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EVALUACIÓN DE METODOLOGÍAS ANALÍTICAS AVANZADAS PARA EL CONTROL DE PLAGUICIDAS Y TOXINAS NATURALES EN AGUAS, ALIMENTOS Y NUTRACÉUTICOS

EVALUATION OF ADVANCED ANALYTICAL METHODOLOGIES FOR THE CONTROL OF PESTICIDES AND NATURAL TOXINS IN WATERS, FOOD AND NUTRACEUTICALS

PROGRAMA DE DOCTORADO EN QUÍMICA

MARÍA DEL MAR APARICIO MURIANA

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Por/By

MARÍA DEL MAR APARICIO MURIANA

DEPARTAMENTO DE QUÍMICA ANALÍTICA

UNIVERSIDAD DE GRANADA

TESIS presentada para aspirar al título de Doctora por la Universidad de Granada

(Mención internacional)

Fdo.: María del Mar Aparicio Muriana

Los directores de la Tesis,

Fdo.: Dr. Francisco Jesús Lara Vargas

Fdo.: Dra. Ana Mª García Campaña

VISADA en Granada el 21 de febrero de 2023

Ana M. García Campaña, Catedrática del Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada y responsable del grupo de investigación "Calidad en Química Analítica, Alimentaria, Ambiental y Clínica" (FQM302),

CERTIFICA:

Que el trabajo que se presenta en esta Tesis Doctoral, con el título de "EVALUACIÓN DE METODOLOGÍAS ANALÍTICAS AVANZADAS PARA EL CONTROL DE PLAGUICIDAS Y TOXINAS NATURALES EN AGUAS, ALIMENTOS Y NUTRACÉUTICOS", ha sido realizado en los laboratorios del citado grupo bajo mi dirección y la del profesor Francisco J. Lara Vargas, en el Departamento de Química Analítica de la Facultad de Ciencias de la Universidad de Granada, y reúne todos los requisitos para poder optar al título de Doctora por la Universidad de Granada con mención de "Doctora Internacional", dentro del programa de Doctorado en Química.

Ana María García Campaña

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Fecha: 2023.02.21 16:12:04 +01'00'

Fdo.: Dra. Ana M. García Campaña

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The doctoral candidate **María del Mar Aparicio Muriana** and the Thesis supervisors Francisco J. Lara Vargas and Ana M. García Campaña,

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Granada, a 21 de febrero de 2023

Los directores de la Tesis / Thesis supervisors:

LARA VARGAS FRANCISCO JESUS - 78036209S
Digitally signed by LARA VARGAS FRANCISCO JESUS - 78036209S
Date: 2023.02.21 18:32:29 +01'00'

Ana María García Campaña
Firmado digitalmente por Ana María García Campaña
Fecha: 2023.02.21 16:12:37 +01'00'

Doctoranda / Doctoral candidate:

APARICIO MURIANA MARIA DEL MAR - 45603289Q
Firmado digitalmente por APARICIO MURIANA MARIA DEL MAR - 45603289Q
Fecha: 2023.02.21 22:16:34 +01'00'

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GOALS OF THE THESIS

The objectives of this Doctoral Thesis are framed in several research projects that have been developed during the last years and that are part of the research lines of the FQM302 group in which this research has been carried out. The main aim has been the development and optimization of new selective and sensitive analysis methods for the determination and control of pesticide residues and contaminants of great interest nowadays in food, food supplements and environmental waters. For this purpose, different sample treatment methods have been proposed depending on the matrices of interest. Likewise, miniaturized analytical separation techniques have been evaluated, such as capillary electrophoresis (CE) or capillary liquid chromatography (CLC), and ultra-high efficiency analytical techniques, such as ultra-high performance liquid chromatography (UHPLC), coupled to different detection techniques such as ultraviolet-visible spectrophotometry (UV-Vis), tandem mass spectrometry (MS/MS) and high-resolution mass spectrometry (HRMS). Also, a new innovative analytical platform, including ion mobility spectrometry (IMS) has been applied, providing of a new dimension for compound identification purposes in the monitoring of contaminants. These techniques have been applied to the control of insecticides, specifically fipronil and its metabolites and cyanotoxins, natural toxins produced by cyanobacteria, proposing alternative analytical methods in their environmental and food control.

The specific objectives of this Doctoral Thesis are the following:

- To evaluate the applicability of CE-UV in its micellar electrokinetic chromatography mode for the determination of fipronil and two of its metabolites

in chicken egg samples using salt-assisted liquid-liquid extraction (SALLE) as sample treatment.

- To evaluate the applicability of CLC-UV for the determination of fipronil and its metabolites in chicken egg samples using SALLE-based sample treatment.

- To develop a multitoxin analysis method based on UHPLC-MS/MS for the determination of cyanotoxins from different families (cyclic peptides, alkaloids, and non-protein amino acids) in reservoir water samples. At the same time, to establish a sample treatment based on solid phase extraction (SPE) using a tandem of two cartridges to achieve simultaneous extraction and preconcentration of analytes with such different physicochemical characteristics.

- To propose an UHPLC-MS/MS method to evaluate the presence of cyanotoxins from different families in samples of commercial algae and cyanobacteria-based dietary supplements, combined with an adequate sample treatment procedure.

- To characterize numerous cyanotoxins in terms of a new parameter specific of the charged molecule, the named collision cross section (CCS), using travelling wave ion mobility spectrometry (TWIMS) and to evaluate the potential of this technique incorporated into LC-HRMS workflows for the qualitative screening of cyanotoxins in algal and cyanobacterial dietary supplements.

OBJETIVOS DE LA TESIS

Los objetivos de esta Tesis Doctoral se enmarcan en distintos Proyectos de Investigación que han sido desarrollados durante los últimos años y que forman parte de las líneas de investigación del grupo FQM302 en el cual se ha desarrollado la Tesis.

El objetivo principal ha sido el desarrollo y optimización de nuevos métodos de análisis, selectivos y sensibles, para la determinación y el control de residuos de plaguicidas y contaminantes de gran interés en la actualidad en alimentos, suplementos alimenticios y aguas medioambientales. Para ello se han propuesto distintos métodos de tratamiento de muestra en función de las matrices de interés. Asimismo, se han evaluado técnicas analíticas de separación miniaturizadas, como son la electroforesis capilar (CE) o la cromatografía líquida capilar (CLC), y técnicas analíticas de ultra alta eficacia como es la cromatografía líquida de ultra alta resolución (UHPLC), acopladas a diferentes técnicas de detección como son la espectrofotometría ultravioleta-visible (UV-Vis), la espectrometría de masas en tándem (MS/MS) y la espectrometría de masas de alta resolución (HRMS). Además, se ha aplicado una reciente e innovadora plataforma analítica, la espectrometría de movilidad iónica (IMS), con el fin de obtener una nueva dimensión en la identificación de compuestos, aplicable en la monitorización de contaminantes. Estas técnicas se han aplicado en el control de insecticidas, concretamente el fipronil y sus metabolitos y de cianotoxinas, toxinas naturales producidas por cianobacterias, proponiendo así métodos analíticos alternativos en su control medioambiental y alimentario.

Los objetivos específicos de esta Tesis Doctoral son los siguientes:

- Evaluar la aplicabilidad de la CE-UV en su modo de cromatografía capilar electrocinética micelar para la determinación de fipronil y dos de sus metabolitos en muestras de huevos de gallina empleando la extracción líquido-líquido asistida por sales (SALLE) como tratamiento de muestra.

- Evaluar la aplicabilidad de la CLC-UV para la determinación de fipronil y sus metabolitos, fipronil sulfuro y fipronil sulfona en muestras de huevos de gallina haciendo uso de un tratamiento de muestra basado en SALLE.

- Desarrollar un método de análisis multitoxina basado en UHPLC-MS/MS para la determinación de cianotoxinas de diferentes familias (péptidos cíclicos, alcaloides y aminoácidos) en muestras de aguas de pantano. Establecer igualmente un tratamiento de muestra basado en la extracción en fase sólida (SPE) en tándem, para lograr la extracción simultánea y preconcentración de analitos con tan distintas características físico-químicas.

- Proponer una metodología analítica basada en UHPLC-MS/MS para evaluar la presencia de cianotoxinas de distintas familias en muestras de suplementos alimenticios comerciales a base de algas y cianobacterias, en combinación con un tratamiento de muestra adecuado.

- Caracterizar numerosas cianotoxinas en términos del parámetro denominado sección transversal de colisión (CCS), intrínseco de la molécula cargada, empleando la espectrometría de movilidad iónica de onda viajera (TWIMS), evaluando el potencial de esta técnica incorporada a los flujos de trabajo de LC-HRMS para la determinación cualitativa de cianotoxinas en muestras de suplementos alimenticios a base de algas y cianobacterias.

SUMMARY

In this Thesis, different analytical strategies using different techniques have been proposed for the determination of residues of fipronil insecticide and its metabolites and cyanotoxins in food products and environmental waters.

Fipronil is an insecticide derived from the phenylpirazole family which is well-known for disturbing the γ-aminobutyric acid (GABA)-gated chloride channels from the cell membranes in the central nervous system. Disruption of the GABA receptors by fipronil prevents the uptake of chloride ions resulting in excess neuronal stimulation and death of the insect. Even though it is known that this insecticide has higher binding affinity for insect receptor complexes, sometimes its effects are negative on other organisms such as vertebrates and humans, thus, it is authorized in the European Union (EU) as a antiparasitic drug for pet animals, but not for food producing animals. In addition, fipronil-sulfone, the primary biological metabolite of fipronil, is reported to be twenty times more active at mammalian chloride channels than at insect ones. In the case of foodstuff, current legislation establishes a maximum residues limit (MRL), which must not be surpassed to guarantee the quality of the product and to permit its distribution and consumption. The approval of fipronil as an agricultural insecticide expired in 2017 and thus, the Commission Regulation (EU) 2019/1792 modified its MRL (as sum of fipronil and fipronil-sulfone) established for foodstuffs, set in the analytical determination limit of 0.005 mg/kg. However bad practices or handling errors sometimes cause that fipronil or its metabolite residues can be found in foodstuffs at levels above this value. If this happens, alerts about their presence in products of animal origin destined to human consumption are notified by the Rapid System of Food and Feed

(RASFF) portal, which provides EU authorities a tool for the exchange of information on the measures taken when a risk is detected in feed or food. This happened with the health alert incident occurred in 2017 in Europe as a result of fipronil contamination of chicken eggs from poultry farms in Netherland and Belgium intended for human consumption.

On the other hand, the presence of not only pesticides, but emerging natural toxins is a matter of interest and awareness. Cyanotoxins are toxic secondary metabolites produced by some genera of cyanobacteria, also known as blue-green algae. Cyanobacteria is a group of oxygenic prokaryotes which habits in a wide variety of freshwater and marine ecosystems and, under certain environmental conditions, such as high levels of nutrients, they reproduce exponentially forming blooms. As a result, cyanotoxins present in water can negatively affect different species and accumulate in animals such as fish and shellfish causing shellfish poisoning through the food chain. In fact, cyanotoxins can be present in different food products such as dietary supplements based on cyanobacteria. Cyanotoxins include potent neurotoxins, hepatotoxins, cytotoxins, compounds with a wide variety of structures and physicochemical properties. However, cyanotoxicity has not been closely studied and therefore the World Health Organization (WHO) has established a drinking water guideline of 1 μg/L only for the most explored cyanotoxin, microcystin-LR.

Considering these concerns, it is obvious that sensitive, selective and effective analytical methods are required for the determination of these compounds in the fields of food and environmental safety to:

a) Monitor the concentration of pesticide residues, particularly the insecticide fipronil and its metabolite fipronil-sulfone, to ensure the quality and safety of food products and to fulfill the current European legislation.

b) Study and evaluate the presence and potential health risks associated with less studied or emerging natural toxic compounds, such as cyanotoxins.

In general, to face with the analytical challenges abovementioned several techniques have been taken into consideration. The proposed methods are based on miniaturized separation techniques, such as capillary electrophoresis (CE) and capillary liquid chromatography (CLC) as they involve low sample volume, low solvent consumption and low waste generation, being recommended as green analytical techniques. Furthermore, the use of high efficiency techniques such as ultra-high performance liquid chromatography (UHPLC) has also been investigated as it offers a lower solvent consumption than in conventional LC methods, higher efficiency and shorter analysis time. Besides UV-Vis detection, mass spectrometry (MS) has been considered in this Thesis due to its advantages such as higher sensitivity and selectivity and its capability to identify unequivocally a large number of compounds. Finally, the coupling of ion mobility spectrometry (IMS) to LC-High Resolution Mass Spectrometry (LC-HRMS) workflow has been assessed as it provides complementary information by means of the collision cross section (CCS) parameter, which can be derived from such a technique. At the same time, suitable sample treatment methods have been investigated to achieve the extraction and preconcentration of the analytes.

It is worthy to mention that this Thesis has sought to obtain novel contributions by exploring alternative methodologies to conventional ones to address these problems. Therefore, it has been developed the first application of CE method for the determination of fipronil and its metabolites in egg samples. Furthermore, it is addressed for the first time the joint determination of cyanotoxins from different families, and therefore, with different characteristics, and the characterization in terms of CCS of a high number of

cyanotoxins employing travelling wave ion mobility (TWIMS) coupled to quadrupole time-of-flight high resolution mass spectrometry (QTOF-HRMS).

The present Thesis has been divided into two different parts. **Part I** presents an introduction including relevant aspects related with the insecticides under study and the experimental work developed. The following chapters are included in this part:

- **Chapter 1** describes a method based on CE in its micellar electrokinetic chromatography mode (MEKC) with ultraviolet detection (UV) as a novel approach for the determination of fipronil and its main metabolite, fipronil-sulfone in chicken egg samples. With this purpose, a sample treatment based on salting out liquid-liquid extraction (SALLE) was developed to efficiently extract the analytes.

- **Chapter 2** involves the employ of CLC coupled to UV as a miniaturized technique for the separation and determination of fipronil and two of its main metabolites, fipronil-sulfone and fipronil-sulfide in chicken egg samples. SALLE was the extraction technique employed to extract the target analytes from the matrix of interest.

On the other hand, in **Part II** an introduction with valuable information about cyanobacteria, cyanotoxins and analytical methods is provided as well as the methods developed in this Thesis involving those analytes. The following chapters can be found in this part:

- **Chapter 3** deals with a comprehensive study of the determination of cyanotoxins from different families in reservoir water samples using hydrophilic interaction liquid chromatography (HILIC) coupled to tandem MS detection (MS/MS). To

achieve the simultaneous extraction of the analytes a novel sample treatment method based on tandem solid phase extraction (SPE) was developed.

- **Chapter 4** consists of the development of a method based on HILIC-MS/MS to determine cyanotoxins from different families in cyanobacteria based dietary supplements. A sample treatment based on solid-liquid extraction (SLE) followed by a tandem-SPE was employed. The method was applied to different dietary supplements samples to study the occurrence of cyanotoxins and evaluate the potential health risk.

- **Chapter 5** presents the research carried out with the supervision of the Professors Dr. Chiara Dall'Asta and Dr. Laura Righetti during the predoctoral research stay at the University of Parma (Parma, Italy). The work consisted of the development of a CCS database as a complementary parameter to characterize 20 cyanotoxins and one marine biotoxin employing travelling wave ion mobility coupled to quadrupole time-of-flight (TWIMS- QTOF) method. The obtained results were discussed, and then, a screening of cyanotoxins was carried out in several dietary supplement samples to evaluate their occurrence.

RESUMEN

En esta Tesis se han propuesto diferentes estrategias analíticas usando diferentes técnicas para la determinación de residuos del insecticida fipronil y sus metabolitos y de cianotoxinas en productos alimenticios y aguas medioambientales.

Fipronil es un insecticida derivado de la familia de los fenilpirazoles, el cual es conocido por perturbar el canal de cloruro regulado por el ácido γ-aminoburírico (GABA) de las membranas celulares en el sistema nervioso central. La perturbación por fipronil de los receptores de GABA impide la captación de iones cloruro, lo que provoca un exceso de estimulación neuronal y la muerte del insecto. Aunque se sabe que este insecticida tiene una mayor afinidad de unión a los complejos receptores de los insectos, a veces sus efectos son negativos en otros organismos como los vertebrados y los seres humanos y por tanto en la Unión Europea (UE) está autorizado como medicamento antiparasitario para animales de compañía, pero no para animales productores de alimentos. Además, se sabe que fipronil-sulfona, el principal metabolito biológico del fipronil, es veinte veces más activo en los canales de cloruro de los mamíferos que en los de los insectos. En el caso de los alimentos, la legislación vigente establece límites máximos de residuos (LMR), que no deben superarse para garantizar la calidad del producto y permitir su distribución y consumo. La aprobación del fipronil como insecticida en agricultura expiró en 2017 y, por tanto, en el reglamento (UE) 2019/1792 se modificó su LMR (como suma de fipronil y fipronil sulfona) establecidos para alimentos, fijado en el límite de determinación analítica, proponiéndose un valor de 0,005 mg/kg. Sin embargo, las malas prácticas o los errores de manipulación hacen que se encuentren residuos de este pesticida o su metabolito principal en alimentos que a veces se supera este valor. Si esto

ocurre, las alertas sobre su presencia en alimentos de origen animal destinados al consumo humano se notifican a través del portal del Sistema de Alerta Rápida para Alimentos y Piensos (RASFF), que proporciona a las autoridades de la UE una herramienta para el intercambio de información sobre las medidas adoptadas cuando se detecta un riesgo en piensos o alimentos. Así ocurrió con el incidente de la alerta sanitaria ocurrido en 2017 en Europa como consecuencia de la contaminación por fipronil de huevos de granjas de los Países Bajos y Bélgica destinados al consumo humano.

Por otro lado, la presencia no sólo de plaguicidas, sino de toxinas naturales emergentes es un tema de interés y concienciación. Las cianotoxinas son metabolitos secundarios tóxicos producidos por algunos géneros de cianobacterias, también conocidas como algas verdeazuladas. Las cianobacterias son un grupo de procariotas oxigénicas que habitan en una gran variedad de ecosistemas marinos y de agua dulce, y en determinadas condiciones, como en medios con altos niveles de nutrientes, se reproducen exponencialmente formando floraciones. Como resultado, las cianotoxinas presentes en el agua pueden afectar negativamente a diferentes especies y acumularse en animales como peces y mariscos, causando intoxicaciones a través de la cadena alimentaria. De hecho, las cianotoxinas pueden estar presentes en diferentes productos alimenticios como son los complementos dietéticos a base de cianobacterias. En las cianotoxinas se incluyen potentes neurotoxinas, hepatotoxinas y citotoxinas, compuestos con una gran variedad de estructuras y propiedades físico-químicas. Sin embargo, la toxicidad de estos compuestos no se ha estudiado aún en profundidad y, por tanto, la Organización Mundial de la Salud (OMS) ha establecido un valor regulado para agua potable de 1 μg/L solo para la cianotoxina más estudiada, la microcistina-LR.

En relación con toda la problemática anterior, es evidente que se requieren métodos analíticos sensibles, selectivos y eficaces para la determinación de estos compuestos en el ámbito de la seguridad alimentaria y medioambiental para:

a) Controlar la concentración de residuos y metabolitos de plaguicidas, en concreto de fipronil y su metabolito, fipronil sulfona, para garantizar la calidad y seguridad de los productos alimentarios y cumplir con la legislación europea vigente.
b) Estudiar y evaluar la presencia y los potenciales riesgos para la salud asociados a compuestos tóxicos naturales, como las cianotoxinas, menos estudiados o emergentes.

En general, para hacer frente a los restos analíticos mencionados, se han considerado distintas técnicas. Los métodos propuestos se basan en técnicas de separación miniaturizadas, como la electroforesis capilar (CE) y la cromatografía líquida capilar (CLC), ya que implican un bajo volumen de muestra y un bajo consumo de disolventes y de generación de residuos, siendo recomendada como una técnica analítica verde. Además, también se ha investigado el uso de técnicas de alta eficiencia, como la cromatografía líquida de ultra alto rendimiento (UHPLC), ya que ofrece un menor consumo de disolvente que los métodos LC convencionales, una mayor eficiencia y un tiempo de análisis más corto. Además de la detección UV-Vis, en esta Tesis se ha considerado la espectrometría de masas (MS) debido a sus ventajas, como una mayor sensibilidad y selectividad y su capacidad para identificar inequívocamente un gran número de compuestos. Por último, se ha evaluado el acoplamiento de la espectrometría de movilidad iónica (IMS) al flujo de trabajo LC acoplado a espectrometría de masas de alta resolución (LC-HRMS), ya que proporciona información complementaria mediante el parámetro denominado sección transversal de colisión (CCS) que puede derivarse de

dicha técnica. Al mismo tiempo, se han investigado métodos de tratamiento de muestra para lograr la extracción y preconcentración de los analitos de manera adecuada.

Cabe destacar que en esta Tesis se ha intentado llevar a cabo aportaciones novedosas explorando metodologías alternativas a las convencionales para abordar estos problemas. Así, se ha desarrollado la primera aplicación de la electroforesis capilar en la determinación de fipronil y sus metabolitos en muestras de huevo. Además, se aborda por primera vez la determinación conjunta de cianotoxinas de diversas familias y por tanto con características físico-químicas muy diferentes y la caracterización en términos de CCS de un gran número de cianotoxinas empleando movilidad iónica de onda viajera (TWIMS) acoplada a espectrometría de masas de alta resolución con analizador de cuadrupolo-tiempo de vuelo (QTOF-HRMS).

Esta Tesis se ha dividido en dos partes diferenciadas. La **Parte I** presenta una introducción que incluye aspectos relevantes relacionados con los insecticidas objeto de estudio y recopila el trabajo experimental desarrollado. En esta parte se incluyen los siguientes capítulos:

- El **Capítulo 1** describe un método basado en CE en su modo de cromatografía electrocinética micelar (MEKC) con detección por batería de diodos (DAD) como un enfoque novedoso para la determinación de fipronil y su principal metabolito, fipronil-sulfona en muestras de huevos de gallina. Con este fin se desarrolló un tratamiento de muestra basado en la extracción líquido-líquido asistida por sales (SALLE) para extraer eficientemente los analitos.
- El **Capítulo 2** trata del empleo de la CLC acoplada a DAD como técnica miniaturizada para la separación y determinación de fipronil y dos de sus principales metabolitos, fipronil-sulfona y fipronil-sulfuro, en muestras de

huevos de gallina. SALLE fue la técnica de extracción empleada para extraer los analitos diana de la matriz de interés.

Por otro lado, en la **Parte II** se ofrece una introducción con información de interés sobre las cianobacterias, cianotoxinas y métodos de análisis, así como los métodos desarrollados en esta Tesis para dichos analitos. En esta parte se encuentran los siguientes capítulos:

- El **Capítulo 3** incluye un estudio exhaustivo de la determinación de cianotoxinas de diferentes familias en muestras de agua de embalses mediante cromatografía líquida de interacción hidrofílica (HILIC) acoplada a detección MS en tándem (MS/MS). Para lograr la extracción simultánea de los analitos se desarrolló un novedoso método de tratamiento de muestra basado en la extracción en fase sólida (SPE) en tándem.
- El **Capítulo 4** consiste en el desarrollo de un método basado en HILIC-MS/MS para la determinación de cianotoxinas de diferentes familias en suplementos dietéticos basados en cianobacterias. Se empleó un tratamiento de muestra basado en extracción sólido-líquido (SLE) seguido de una SPE en tándem. El método se aplicó a diferentes muestras de suplementos dietéticos para estudiar la presencia de cianotoxinas y evaluar el riesgo potencial para la salud.
- El **Capítulo 5** presenta la investigación realizada bajo la supervisión de las profesoras Dra. Laura Righetti y Dra. Chiara Dall'Asta durante la estancia de investigación predoctoral en la Universidad de Parma (Parma, Italia). El trabajo consistió en el desarrollo de una base de datos de CCS como un parámetro complementario para la caracterización de 20 cianotoxinas y una biotoxina marina empleando un método de movilidad iónica de onda viajera acoplado a espectrometría de masas de cuadrupolo-tiempo de vuelo (TWIMS-QTOF-MS). Se

discutieron los resultados obtenidos y posteriormente se llevó a cabo un cribado de cianotoxinas en varias muestras de suplementos dietéticos para evaluar su presencia.

PART I

Determination of residues of fipronil and its metabolites

Introduction

Chapter 1.

A first approach using micellar electrokinetic capillary chromatography for the determination of fipronil and fipronil-sulfone in eggs

Chapter 2.

Simple and efficient method for the determination of fipronil and two main metabolites in eggs by capillary liquid chromatography

INTRODUCTION

DETERMINATION OF RESIDUES OF FIPRONIL AND ITS METABOLITES

1. Pesticides

1.1. Brief history of control pest

Pesticide is a derived from the words "pestis" and "caedere", Latin derivatives that mean "disease, plague" and "to kill". There are different definitions of what a pesticide is. For instance, The World Health Organization (WHO) defines pesticides as "chemical compounds that are used to kill pests, including insects, rodents, fungi and unwanted plants (weeds)" [1], while the Food and Agriculture Organization of the United Nations (FAO) defines pesticide as "any substance or mixture of substances of chemical or biological ingredients intended for repelling, destroying or controlling any pest, or regulating plant growth." [2]

Pest control has a long and storied history. From the beginning of time, pests have been a nuisance to humankind. Not only do they carry and spread disease in people, but they can also destroy food sources, such as crops, and infect and kill animals. From cave dwellers swatting annoying flies and mosquitoes, to the Egyptians having cats to control rodents around their barns, the search for the ideal pest control is very old. Thus, long before the biology of pests was understood, human beings developed many biological, cultural and physical methods to protect crops, animals and themself. Many of these practices eventually proved scientifically valid, though originally derived from empirical evidence [3].

Our knowledge of the earliest forms of pest control, after de development of agriculture approximately 8000 years B.C., is limited to the evidence that has survived to the present day. The first civilization with a recorded proof of pest management were the Sumerians,

located in Mesopotamia in 2500 B.C. [4]. They laid the groundwork for a largely agricultural society, with domesticated livestock, the rotation of crops and people who specialized in field work. In addition, Sumerians introduced the use of chemicals by using sulfur compounds to prevent insect and mites from invading their crops. The Chinese were the next notable civilization to contribute to the development of pest management. They continued and refined the work of the Sumerians by using chemical substances to control pests, and in 1200 B.C., they used insecticides derived from plants as fungicides to protect plant seeds and fumigate plants infested with insect pests [5]. In addition, they learnt how to adjust crop-planting times to avoid pest outbreaks. Greeks and Romans understood the use of fumigants, mosquito nets, granaries on stilts, sticky bands on trees and pesticidal sprays and ointments, although throughout this period and long beyond, such sophisticated practices were accompanied by widespread reliance on offerings to the gods and other superstitions. The Chinese continued to develop their pest-control technology and by 300 A.D., they introduced a new practice using biological controls establishing colonies of predatory (insect-feeding) ants in citrus orchards to control caterpillars and large borer beetles [5,6]. They would tie ropes or bamboo sticks between close branches to ease the movement of ants from one place to another (control pest evolution can be observed in **Figure 1**).

As with most things, the progress made in pest control was greatly disturbed in the Middle Ages. After the Roman Empire, science basically disappeared from Europe, and along with it, much of hygiene and health-related arts while Europeans relied gradually on religious faith rather than biological knowledge. This decline was reversed by the Renaissance era in the 17th century, when the science was brought back to the forefront, interest in biological control was awakened, and various natural pesticides were rediscovered and introduced in Europe.

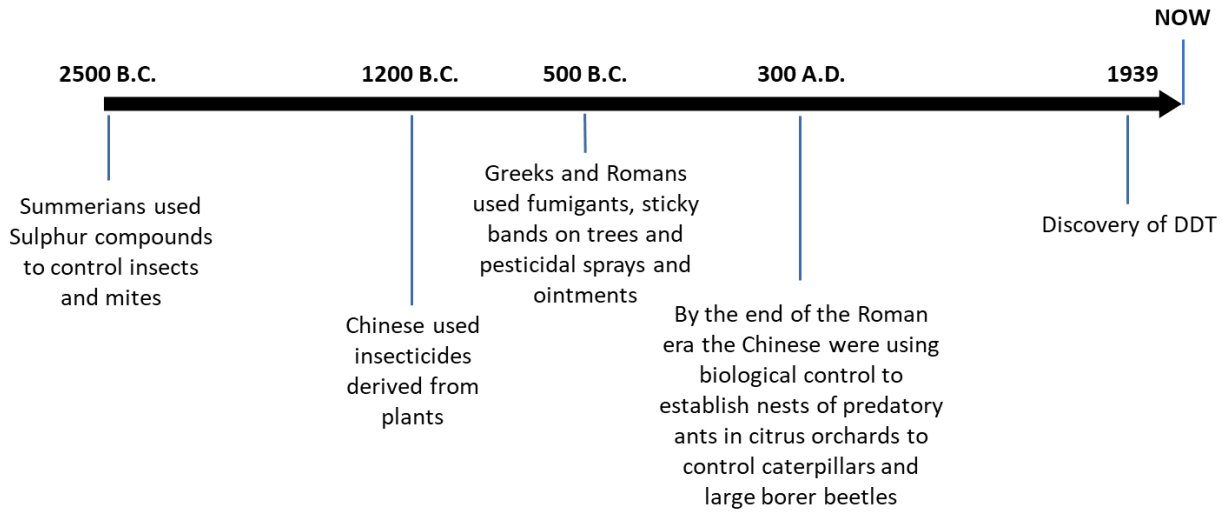

Figure 1. Timeline, drawn to scale, showing the history of the use of pesticides.

By the 19th century there was an interest in developing pest-control techniques, thus five main approaches were well recognized and commonly used. These strategies were based on biological control, chemical control (including inorganic chemicals such as sulfur and copper compounds, or plant-derived alkaloids such as nicotine), mechanical and physical control, cultural and sanitation controls, like crop rotation, and finally, the use of pest-resistant varieties. The progress in pest control continued during the beginning of 20th century, and the Second World War brought a revolution in pest control with highly effective synthetic organic pesticides that replaced inorganic compounds. Since part of the war took place in tropical areas where diseases transmitted by insect, such as malaria or typhus, risked killing the troops, research on pesticides became a priority. It was in 1940s when the growth in synthetic pesticides accelerated, being the discovery of dichloro-diphenyl-trichloroethane, known as DDT, in 1939, the most popular due to its broad-spectrum activity. After the end of the Second World War, DDT continued to be

used for vector control, but it was also widely used in agriculture. This boom in the massive and widespread use of pesticides caused a change in attitude towards pest control, displacing biological control. However, in the middle of the 20th century, resistance to DDT by the housefly was reported and, because of its widespread use, there were reports of harm to non-target plants and animals and problems with its residues [7]. Plants may develop resistance to herbicides over time, and by the end of the 20th century, all agricultural pests in California had developed resistance to DDT. This was noticed because, after the application of the pesticide, the pest population decreased but after a while it rose to levels higher than the previous ones due to the disappearance of the natural enemies. During most of the 1950s, consumers and policy makers were not concerned about the potential health risks of using pesticides; food was cheaper due to the new chemical formulations, and there were no documented cases of people being seriously damaged by the regular use of these pesticides. Parallel to the development of organochlorine compounds such as DDT, it was developed an equally toxic group of insecticides: the organophosphates, as a direct result of research on toxic gases in the nervous system used during the previous wars. These compounds entered the market because their persistence in the environment was lower and there was no accumulation in organisms, which is why they were presented as harmless products for the environment and human health. But as with organochlorines, their intensive use ended up creating resistance in pest and acute toxicity in beneficial fauna. Doubts from entomologists preceded the publication of Silent Spring in 1962 [8], which challenged the idea that chemicals brought benefits with negligible risks. The publication swayed public opinion and led to a reversal in U.S. pesticide policy, a national ban on DDT for agricultural uses, and an environmental movement that led to the creation of the U.S. Environmental Protection Agency (EPA). Research into pesticides continued and by

second part of the 20th century, the glyphosate, one of the world's greatest selling herbicides, was introduced together with the use of genetically modified organisms (GMOs) resistant to its action [9], however, plants developed resistance to glyphosate as well [10]. In these years, research activities concentrated on finding new members of existing pesticide families which have greater selectivity and better environmental and toxicological profiles. Likewise, new insecticide chemistry has allowed better resistance management and improved selectivity. Today the pest management toolbox has expanded to include the use of genetically engineered crops designed to produce their own insecticides, or exhibit resistance to broad spectrum herbicide products or pests. These include herbicide tolerant crops like soybeans, corn, canola and cotton and varieties of corn and cotton resistant to corn borer and bollworm respectively [11].

The use of pesticides has been fundamental in the developing world for agricultural purposes and the reduction of vector-borne diseases. Production of major crops has more than tripled since 1960, thanks largely to pesticides, as in the case of rice [12]. Moreover, pesticides can extend the life of crops and prevent post-harvest losses since threats do not stop once crops leave fields, but in fact, bugs, molds and rodents can cause damage also during storage.

1.2. General aspect and classification of pesticides

Pesticides are a key public health and agricultural input, which help to protect seeds and safeguard crops from undesirable plants, insect, bacteria, fungi and rodents, among others. However, despite their great and undeniable contributions to agriculture, pesticides hold a unique position among environmental contaminants due to their high biological activity and toxicity. They can have a negative environmental impact through contamination of soil and water, especially if used improperly. Furthermore, they are

potentially dangerous to humans, animals or other living non-target organisms since most pesticides do not discriminate between pests and other similar related lifeforms [13]. It is estimated that about from five to twenty thousand people died and more than half a million people get accidentally poisoned every year by pesticides [14,15]. At least half of the intoxicated and 75% of those who die due to pesticide are agricultural workers. The rest is due poisoning by ingestion of contaminated food. Thus, actions must be taken to minimize the hazards of pesticide use, including self-poisonings and toxicity from medium- and long-term exposure. Pesticide is a general term that refers to a wide variety of substances, including insecticides, fungicides, herbicides etc. These substances vary in their chemical and physical properties from one class to another and can therefore be categorized into various groups and studied under their respective groups. According to a classification suggested by Drum [16], pesticides can be mainly categorized according to three criteria: a) the chemical composition, b) the entry mode and c) the pest action and the organism they affect. However, in addition to the above-mentioned classifications, pesticides can also be categorized according to different factors such as source of origin, pesticide formulation, toxicity, biodegradability or the routes of exposure.

1.2.1. Chemical composition

The most widespread and appropriate way of classifying pesticides is based on their chemical composition and description of the active ingredients. This classification is effective in describing chemical and physical properties of particular pesticides. After a pesticide is released to the environmental, its transport, dispersion and transformation is influenced by certain physicochemical properties. Thus, the information on such as properties is key in determining the mode of application, safety measures and

application rates of pesticides. Based on chemical configuration, four main groups can be established, namely organochlorines, organophosphorus, carbamates, and pyrethrin and pyrethroids [17]. Organochlorine pesticides, also known as chlorinated hydrocarbons, were the first synthesized group of pesticides used in agriculture. They are organic compounds with at least five chlorine atoms. Organochlorine pesticides were widely used as insecticides, showing a long-term stability and persistence in the environment. They tend to accumulate in adipose tissue and mainly disrupt the central nervous system causing convulsions, paralysis and death. Their lipophilic nature give rise to a long-term storage in the adipose tissue, followed by a slow release into the circulatory system. As an example, DDT can remain in the body for 50 years [18]. Organophosphate and carbamate are non-polar pesticides that present low water solubility. They are similar in properties and uses, but they are derivates of phosphoric acid and carbamic acid, respectively. They are broad spectrum pesticides that control a great number of pests by affecting the transmission of nerve signals. Organophosphate and carbamate pesticides are biodegradable under natural environment, which cause low environmental pollution. However, they are highly toxic and affect human central nervous system by means of the neurotransmitter acetyl cholinesterase [19]. Examples of organophosphate and carbamate pesticides are malathion and carbaryl, respectively. Finally, synthetic pyrethroids are a group of organic pesticides synthesized by duplicating the structure of natural pyrethrins, which are natural insecticides that affect the dynamics of sodium cation channels in the nerve cell membrane [20]. Synthetic pyrethroids are highly toxic to insect and fish but not to birds and mammals. Most of them are not persistent as they undergo photolysis, which make them the safest insecticides (for instance, cypermethrin is one of the most used synthetic pyrethroid pesticides). However, although these are the four major chemical groups of pesticides,

the chemical-based classification of pesticides is quite complex, and more categories and several subcategories are sometimes defined according to a common chemical characteristic or functional group, such as phenylpirazoles, neonicotinoids, triazines, polar pesticides etc.

1.2.2. Entry mode

There are several ways in which pesticides can come in contact with the target pest. These modes of entry include systemic, contact, stomach toxicants, fumigants and repellents. Systemic pesticides are substances that are absorbed by a certain part of the plant or animal and then they are transferred to untreated parts of the organism, for example, in a plant they move to the roots, leaves or steams through the vascular system of the plant to kill specific pests. The contact or non-systemic pesticides act effectively when they come into physical contact with the target pests. Unlike the previous ones, these pesticides are not absorbed and therefore are not transported, but on the contrary, they act by dermal contact route. Stomach toxicants pesticides are ingested through the mouth and digestive tract, causing the death of the pests by damaging the midgut and stomach poisoning. Fumigants are pesticides that form poisonous vapors which enter the body through the respiratory system causing death by poisoning. Many of them are formed by volatile liquids or substances that are liquids when packaged under high pressure but not when they are released. Finally, repellents are substances that do not kill themselves but are repulsive enough to drive the pests away from the area or product of interest.

1.2.3. Target pest object

Depending on the type of pest they control, pesticides are given specific names to indicate their activities. Most of the category names come from the Latin word *cide*, which means "killer", after the name of the target pest. Various categories of pesticides, their target organism and examples of each one are shown in **Table 1**. However, some pesticides can control more than one single class of pests and can be included in more than one group.

Table 1. Pesticide categories, pesticide example and target pest (modified after Fishel [21]).

Type of pests	Target pests/Function	Pesticides Example
Acaricides	Kill mites that feed on plants and animals	Bifenazate
Attractant	Attracts wide range of pests	Pheromones
Algaecides	Control or kill growth of algae	Copper sulfate
Bactericides	Kill bacteria or acts against bacteria	Copper complexes
Desiccants	Act on plants by drying their tissues	Boric acid
Defoliant	Removes plant foliage	Tribufos
Fungicides	Kill fungi (including blights, mildews, molds, and rusts)	Azoxystrobin
Herbicides	Kill weeds and other plants that grow where they are not wanted	Glyphosate
Insecticides	Kill insects and other arthropods	Aldicarb
Insect growth regulator	Insects	Diflubenzuron
Lampricides	Target larvae of lampreys which are jawless fish latching on vertebrate fish in rivers	Trifluromethyl
Larvicides	Inhibits growth of larvae	Methoprene

Molluscicides	Inhibit or kill mollusks *i.e.*, snails usually disturbing growth of plants	Metaldehyde
Moth balls	Stop any damage to cloths by moth larvae or molds	Dichlorobenzene
Nematicides	Kill nematodes that act as parasites of plants	Aldicarb
Ovicides	Inhibits the growth of eggs of insects and mites	Benzoxazin
Piscicides	Act against fishes	Rotenone
Plant growth regulator	Regulates plant growth	Gibberellic acid
Repellents	Repel pests by its taste or smell, vertebrates and invertebrates	Methiocarb
Rodenticides	Control mice and other rodents	Warfarin
Silvicides	Acts against woody vegetation	Tebuthiuron
Termiticides	Kill termites	Fipronil
Virucides	Act against viruses	Scytovirin

2. Fipronil

2.1. General aspects of fipronil

Fipronil is an insecticide discovered and developed by the French Company Rhône-Poulenc Agro (now Bayer CropScience) between 1985 and 1987 [22], and first introduced to the market in 1993 [23]. It belongs to the phenylpirazole family of pesticides, also known as fiproles, which are characterized by a central pyrazole ring with a phenyl group attached to one of the nitrogen atoms of the pyrazole. Phenylpyrazoles mainly pose herbicide activity, however, fipronil acts as a broad-spectrum systemic insecticide.

When it first appeared, it was considered a "new generation" insecticide since its mode of action, which is explained below, differed from that of the traditional organophosphates and carbamates (both cholinesterase inhibitors) and some pyrethroids (sodium channel activators) [24,25], to which some insects had developed resistance [26]. With this resistance background, fipronil and other insecticides such as neonicotinoids were described as having numerous important features, such as invertebrate specificity, high-persistence, systemic mode of entry and high versatility in application and preparations, which led to the replacement of the traditional insecticides and their quick adoption in both agricultural and urban applications [22].

Fipronil presents low molecular weight, low polarity and high solubility in low polar organic solvent due to its lipophilic nature, therefore, it can be easily bind to lipid matrices and bioaccumulate [27]. Physicochemical characteristics of fipronil are shown in **Table 2**.

Table 2. Physicochemical properties of fipronil [28].

Pesticide type	**Insecticide**
Chemical family	Phenylpyrazoles or fiproles
Chemical name	5-amino-1-[2,6-dichloro-4-(trifluoromethyl)phenyl]-4-[(trifluoromethyl)sulfinyl]-1H-pyrazole-3-carbonitrile
Common name	Fipronil
Trade names	Regent®, Agenta®, Icon®, Frontline®, Termidor®, Combat®, Maxforce®, Adonis®, Choice®, Goliath®, Ascend®, Top Spot®, Chipco-Choice®

Chemical Abstract Service registry number (CAS #)	120068-37-3
Molecular weight	473.2 g/mol
Physical	Powder
Color	White
Odor	Moldy
Melting point	200-201 °C
Density (20 °C)	1.477-1.626 g/mL
Empirical formula	$C_{12}H_4Cl_2F_6N_4OS$
Log $P_{O/W}$	4.5
Solubility in water pH 5 (20 °C)	0.0019 g/L
Solubility in water pH 9 (20 °C)	0.0024 g/L
Solubility in acetone (20 °C)	545 g/L
Solubility in methanol (20 °C)	137 g/L

In the environment, fipronil is degraded into different substances through oxidation, reduction, hydrolysis and photolysis to form four major degradation products: fipronil-sulfone, fipronil-sulfide, fipronil-amide and fipronil-desulfinyl, respectively [29]. Some studies have proven that some fipronil degradation products such as fipronil-sulfone have higher toxicological significance than the original compound [30,31,32,33,34,35,36,37]. Furthermore, it is worthy to mention that fipronil-sulfone is rapidly formed in humans and experimental animals after fipronil exposure and it also persists longer than the parent compound, thus being a useful human biomarker of it [38]. Structures of fipronil and the two degradation products monitored in this Thesis, fipronil-sulfide and fipronil-sulfone, are shown in **Figure 2**.

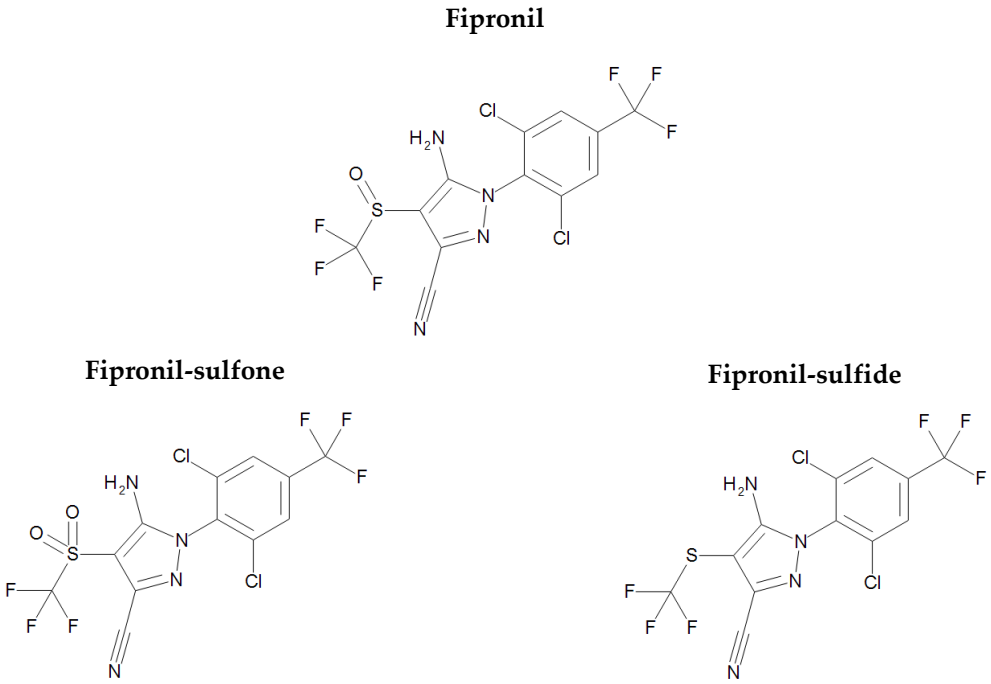

Figure 2. Chemical structures of fipronil, fipronil-sulfone and fipronil-sulfide.

Fipronil and fipronil-sulfone, its major metabolite in mammals and insects, are potent disrupters of the insect central nervous system (CNS) as they interfere in the passage of chloride ions through the γ-aminobutyric acid-(GABA-) regulated chloride channel [39,40], leading to uncontrolled CNS excitation at low doses and paralysis and insect death at high doses [27,29,32]. Although GABA channel plays an important role in nerve transmission in both vertebrate and invertebrate organisms, the binding that fipronil does in the GABA receptor (β3 subunit) found in invertebrates is tighter than in vertebrates and mammals, offering a high degree of selective toxicity toward insects [25,41,42,43,44,45,46,47]. However, its use also exhibits toxic and undesirable side-effects on non-target organisms, such as honeybees, fishes, aquatic invertebrates and land birds [30,48,49]. In fact, fipronil and its metabolites together with neonicotinoid insecticides, have been associated with the colony collapse disorder (CCD), a phenomenon affecting

bees, in which most of the worker bees in a honey colony lose their motor and orientation capacities as a result of an intoxication, leading to a rapid depopulation and consequent death of this pollinators [50,51]. Regarding its toxicity, fipronil is classified by WHO as Class II moderately hazardous pesticide and according to EPA and it belongs to group C, Possible Human Carcinogen [52].

The use of fipronil as pest manager covers different major domains: in agriculture it has been used as plant protection of crops and ornamentals (as soil treatment, seed coating etc.) against herbivorous insects and mites; in urban pest control it has been employed as part of phytosanitary applications to target harmful organisms such as cockroaches, ants, termites, wasps etc. [53] Likewise, fipronil is used in veterinary medical applications as part of ectoparasiticide products where is very effective to kill fleas, mites and all stages of ticks in dogs and cats [22,53,54,55]. The EPA has determined fipronil to be safe for use on pets with no harm to humans who handle these pets. Poisoning by fipronil are mainly due to accidental ingestion or suicidal attempt.

2.2. Fipronil residues and legal framework

Due to its broad-spectrum action and its many applications, fipronil has been widely used throughout the world. Therefore, regulations have been necessary to control its use and to ensure safety for humans, both those who work with it in agricultural or veterinary fields, and those who may ingest it through different food products, as well as for non-target animals and the environment where it is used. Pesticide residues derived from the use of plant protection products (PPPs) on food or feed crops may cause a risk to public health. Therefore, a comprehensive legislative framework has been established in the European Union, which describes rules for: the approval of active substances, the use of PPPs, and the maximum pesticide residue content in foodstuffs.

All issues related to legal limits for pesticide residues in food and feed are covered by Regulation (EC) No 396/2005, which comprises provisions on official controls of pesticides residues in food of plant or animal origin that may result from their use in plant protection [56]. According to the abovementioned Regulation, maximum residue levels (MRLs) are defined as the highest levels of pesticide residues that are permitted in food or animal feed, considering good agricultural practices and the lowest exposure needed to protect vulnerable consumers. Based on risk assessment of the European Food Safety Authority (EFSA) regarding the use of fipronil and taking into account that fipronil-sulfone exhibits higher toxicological significance than fipronil itself, the European Union defined the MRL of fipronil as the sum of fipronil and fipronil-sulfone, altogether expressed as fipronil. The MRL value of fipronil has been progressively decreasing over the last few years to the present day, when its use is prohibited in the European Union. After the Regulation (EC) No 396/2005 were MRLs were set, subsequent Regulation (EC) No 1127/2014 amended Annexes II and III of Regulation (EC) 396/2005 lowered the MRLs for some matrices (such as flowering and head brassicas, swine, bovine, sheep and goat fat and liver, swine kidney, poultry liver and eggs) while for other products it was recommended raising or keeping the existing MRLs [57]. However, the approval period of fipronil expired on 30 September 2017 [58], and although an application was then submitted to renew that approval, the supplementary dossier in support of renewal was not submitted. Since then, fipronil appears as "not approved" by the European Commission, which implies that all use of it as a pesticide in food or feed production is prohibited. Therefore, its MRL is established at 5 μg/kg, considering this value as the limit of analytical determination of a proposed method, in accordance with the current European Regulation (EC) 2019/1792 [59].

2.3. European health alert due to fipronil

Although the use of fipronil is currently not allowed for agricultural purposes or to treat pests in animals intended for human consumption, fipronil residues can still be found in cereals and can be transferred to other livestock products due to its long-lasting residual effects, misuse or presence in imported foodstuffs [60]. Indeed, few years ago fipronil was involved in one of the largest European food health alerts. In July 2017, the Rapid Alert System for Food and Feed (RASFF) was notified about the presence of high residue levels of fipronil in fresh eggs and egg products (720 μg/kg, which was 48 times higher than the MRL which was in force [57,61]). Investigations showed that fipronil was illegally used as a treatment against red mites on poultry Dutch farms [62]. As a result, a great deal of eggs on the market, particularly in the Netherlands but also in other European countries, were traced and withdrawn from the market, as they were not in line with food safety regulations. This incident involved not only European countries but also some areas of Asia suffered economic losses [63].

In view of the above, reliable efficient, selective and sensitive analytical methods are necessary to monitor pesticide residues and to fulfill current legislation requirements in the food safety field.

2.4. Analytical methods for the determination of fipronil in foodstuffs

To date, unambiguous identification of fipronil and its metabolites has been carried out by several techniques including mostly liquid chromatography (LC) and gas chromatography (GC) coupled to ultraviolet-visible (UV-Vis) or mass spectrometry (MS) detection, which will be summarized below. However, different detection approaches such as direct analysis in real time (DART) [64], immunoassays [65,66], Raman [67,68],

electrochemical detection [69] chemiluminescence [70] or colorimetry [71], among others, have also been employed although to a lesser extent. Here is an overview, regarding especially chromatographic methods, of how fipronil and its metabolites have been determined in food samples in the last years.

A critical part in the overall analytical process is often the sample treatment, since different approaches are needed depending on the matrix. As previously commented, fipronil is an insecticide often used for vegetable and fruit samples, but it may also be transferred to other agricultural products or appear in livestock derived products, thus, over the years, fipronil has been determined in several samples. Bulky samples need to be chopped, crushed or grinded to get a homogeneous mixture. Due to the physicochemical properties of fipronil, such as the high affinity for non-polar solvents and its hydrophobicity, a liquid extraction with an organic solvent such as acetonitrile, ethyl acetate, acetone or hexane has been normally employed along with the addition of salts in a QuEChERS or modify QuEChERS procedure. Then, a clean-up step by means of a solid phase extraction (SPE) or dispersive solid phase extraction (d-SPE) with different sorbents was generally required, as it is detailed below. Although these have been the common procedures to determine fipronil in contaminated food samples, lately, sample preparations have included the use of novel approaches such as nano-materials [68,70,72], ionic liquids (ILs) [73] or metal-organic frameworks (MOFs) [74].

2.4.1. Gas chromatography-electron capture detector methods

Regarding analytical techniques, GC coupled to electron capture detector (ECD) has been widely used to determine fipronil and its metabolites from several food matrices. Nonpolar columns composed by 100% dimethylpolysiloxane or (5%-phenyl)-methylpolysiloxane with 30 m length were the most commonly used in GC separations,

in which the elution order is typically fipronil-desulfinyl, fipronil-sulfide, fipronil, fipronil-sulfone and finally fipronil-amide. ECD is a convenient detection system due to the six high electronegative fluorine atoms that are contained in the structure of fipronil and its metabolites. Several matrices contaminated with fipronil have been analyzed by GC-ECD technique, such as sugarcane, maize, edible oils, pollen, honey, cabbage, cotton and grape leaves and berries.

With reference to vegetable samples, fipronil and its metabolites fipronil-sulfide, fipronil-sulfone, fipronil-desulfinyl and fipronil-carboxamide have been determined in sugarcane crop with a modified QuEChERS procedure using ethyl acetate in a liquid extraction step followed by a d-SPE clean-up with $MgSO_4$, primary secondary amine (PSA) and graphitized carbon black (GCB). This method achieved limit of quantification (LOQ) lower than 10 μg/kg for all fipronil derivates, however, baseline separation was not fully achieved between fipronil-sulfone (10.966 min) and fipronil (11.447 min), making quantification difficult [75]. In a later work, the abovementioned method was modified replacing the GCB sorbent with C18 in the d-SPE clean-up, obtaining LOQs of 5 μg/kg [76].

The determination of fipronil in maize grain and maize stem involved two extractions depending on the specific matrix. A mixture of water:acetonitrile or water:methanol was employed as extractant followed by the addition of NaCl and $MgSO_4$ or by a liquid-liquid partitioning with dichloromethane prior aqueous Na_2SO_4 addition. In both cases a SPE with Florisil adsorbent was carried out as the clean-up step obtaining LOQs of 2 μg/kg [77].

Regarding fatty matrixes, such as edible oils, a SPE based on humic acid bonded silica was developed to determine fipronil and metabolites. Humic acid provided

hydrophobic, dipole-dipole, hydrogen bonding and ion exchange interactions with fipronil, achieving high selectivity and low matrix effects (between 0.4% for fipronil-sulfone and 3% for fipronil-sulfide) [78]. On the other hand, edible oils were also treated by means of a novel pine pollen grain based SPE. The outer wall of pollen grain consisted of a biopolymer with carboxylic acids groups joined to unsaturation and ether linkages, which have the potential to be a promising hydrophilic polar sorbent for analytes with polar and electronegative groups, such as fipronil. Pollen grains showed good absorption capacity and stability, achieving LOQs ranging from 0.6 to 1.9 μg/kg [79].

Jimenez et al. did two comparative studies where different sample treatment strategies for pollen samples were examined. The extraction with acetonitrile followed by a clean-up step with C18M or Oasis HLB cartridges was the procedure that provided the best recoveries of fipronil while reducing the co-extraction of other compounds from the matrix. Although ECD is sensitive enough to determine fipronil at trace levels (the LOD obtained was about 0.1 μg/kg), the use of MS spectrometry was recommended for a reliable identification of fipronil [80].A comparison of several types of solvents in LLE (ethyl acetate, dichloromethane, n-hexane, and n-hexane/acetone mixtures) and SPE sorbents (Oasis HLB, Strata-X, Envi-carb and ODS cartridges and Florisil-packed columns) was made to extract fipronil from honey samples. The extraction with n-hexane and the SPE involving the use of Florisil column showed the best performance among all the studied sample treatments. The LOD of fipronil in honey samples was less than 1 μg/kg [81].

GC-ECD methods have been also employed to study dynamics of fipronil residues and their persistence in several vegetable samples. To extract fipronil and its metabolites from Chinese cabbage samples they were soaked in acetone, extracts were evaporated

with the rotary evaporator and then a brine solution was added followed by a liquid-liquid partitioning with dichloromethane. Purification of the extracts was made with a mixed adsorbent column composed by activated carbon, neutral aluminum oxide and Celite545. The minimum detectable quantity of fipronil was 0.2 μg/kg with recoveries between 83.7 and 91.5 %. Results showed that degradation was governed by different processes, and it was faster in vegetable samples than in soil [82]. Another similar persistence study carried out on cabbage samples showed that all compounds were below the detectable limit after 15 days of application [83]. Fate of fipronil was studied on grape leaves and berries, and cotton. Grape leaves and berries were extracted with a mixture of acetonitrile-acetone, saturated with brine solution and partitioned with hexane-ethyl acetate. A column filled with Florisil adsorbent was employed as a clean-up step [84]. On the other hand, cotton lint and seed, were extracted with a mixture of hexane-acetone, diluted with brine solution and partitioned with hexane followed by a clean-up with neutral alumina, Florisil and activated charcoal [85]. Both studies showed that fipronil is highly persistent in soil and it can degrade faster in plants, and temperature and rainfall may reduce the residue content.

2.4.2. Gas chromatography-mass detector methods

Regarding the separation of fipronil and its metabolites, GC methods showed difficulties to achieve a baseline chromatographic separation of fipronil and fipronil-sulfide due to their similar behavior [84]. However, the selectivity of MS makes the identification and integration of the peaks more reliable, mainly at trace level, as different quantitation and confirmation ions can be selected for fipronil and its metabolites. Furthermore, better sensitivity and selectivity are obtained in comparison with other detection systems. The mass spectrometer often operates in electron impact ionization mode (EI) with ionization

energy of 70 eV. Selected ion monitoring (SIM) was the preferred operation mode due to its superior sensitivity.

QuEChERS and modified QuEChERS methods has been broadly utilized to determine fipronil and metabolites in a large variety of vegetable and fruit samples, because of its simplicity, speed, low cost and efficiency. For instance, single-step extraction method based on original QuEChERS, using acetonitrile as extraction solvent along with the addiction of NaCl and $MgSO_4$, was employed to extract fipronil and metabolites from sugarcane juice, jaggery and sugar matrices. No clean-up step was necessary as there were no significant differences between PSA-based clean-up and no clean-up. Recoveries of fipronil varied between 87.5 and 108.5 % and LOQ was set at 5 μg/kg [86]. Modified QuEChERS using ethyl acetate as extraction solvent in place of acetonitrile along with NaCl and different salts such as $MgSO_4$ or Na_2SO_4 have been employed to extract the fipronil and metabolites in vegetables like cabbage, cauliflower, okra and tomato. A mixture of PSA and $MgSO_4$ with and without GCB have been used in the d-SPE clean-up, achieving LOQs of 10 μg/kg with acceptable recoveries [87,88]. Likewise, modified QuEChERS was applied to analyze cauliflower samples, but using acetone as extraction solvent along with $MgSO_4$ and NaCl, and PSA and anhydrous $MgSO_4$ in the d-SPE clean-up step. The mass spectrometer worked in scan and product ion program but in contrast to previous methods, it also worked in multiple reaction monitoring (MRM) mode, achieving lower LOQ of 3 μg/kg [89]. Fipronil has been also extracted from different fresh vegetables with a mixture of ethyl acetate, hexane and acetone. Acidified activated carbon was employed to remove soluble plant pigments and a column packed with Florisil was employed as a final clean-up step before injection into the GC-MS system achieving LOQ of 6.9 μg/kg [90].

QuEChERS and modified QuEChERS were also employed to determine fipronil in animal origin foods, which may be contaminated as a result of irresponsibly insecticide treatments on animals, for example, against cattle ticks. For bovine meat, original QuEChERS method using acetonitrile, $MgSO_4$ and NaCl in the extraction, and C18, PSA and $MgSO_4$ in the d-SPE clean-up, was employed. In the case of bovine fat, hexane and water were also added in the extraction step and only PSA and $MgSO_4$ were used in the d-SPE step. LOQ were about 10μg/kg, but recoveries were higher than 120% with relative standard deviation above 25% for both matrices, showing that the extraction method could improve for meat and fatty matrices [91]. In this sense, the potential transfer of fipronil residues from maize feed to a food of animal origin such as cow milk, another fatty matrix, was assessed. After the extraction with ethyl acetate the mixture was dried, and the fat was removed with isooctane in a liquid/liquid partitioning with acetonitrile. A SPE with an Atoll XC column was carried out to clean the extracts before GC-MS/MS. High sensitivity was achieved with LOQs of 0.025 μg/L for fipronil and desulfinyl, sulfide, sulfone metabolites working on MRM mode. Results suggested a transfer of fipronil from feed to milk under the sulfone metabolite [92].

Aquatic animal derived samples, such as fish liver and crab hepatopancreas, have been analyzed using matrix solid-phase dispersion (MSPD) prior GC-MS. Samples were homogenized with C18 sorbent and then the mixture was extracted using acetonitrile. LOQs for crab hepatopancreas and fish liver were 100 and 250 μg/kg, respectively. Higher LOQs were obtained compared with previous work as there was a dilution factor of 10 and 25 times for hepatopancreas and liver, respectively. Recoveries were acceptable but high enrichment of the signals was found for fish liver due to matrix effect. Thus, matrix matched calibration was needed to improve the accuracy of the results in these complex matrixes [93].

Fipronil and metabolites have also been determined in other animal derived matrices, such as egg, which is a complex matrix rich of proteins and lipids. To extract satisfactorily fipronil and its metabolites from egg samples, acetonitrile was employed to achieve protein precipitation and liquid phase extraction. After the addition of NaCl, hexane was used to remove fats from the mixture. Finally, acetonitrile layer was purified by HLB SPE column. This method could remove protein and lipid matrix interferences, achieving LOQs of 1 and 0.5 μg/kg for fipronil and metabolites, respectively under MRM conditions. Authors observed that fipronil-sulfone was the major metabolite in chicken egg [94].

2.4.3. Liquid chromatography-ultraviolet-visible detector methods

Fipronil and its metabolites are hydrophobic compounds which makes them very suitable for reverse phase LC separation, especially by reversed phase C18 column with water and acetonitrile or methanol as mobile phases. Compared to GC, one of the benefits of LC is that it offers shorter separation times. On the other hand, UV detection is a more accessible detection technique, less expensive and easier to maintain than MS, thus having the potential to be widely used in laboratories around the world. However, UV-Vis detection shows certain weaknesses. For instance, it does not offer much sensitivity when dealing with trace level concentrations due to the low absorbance of some analytes in UV-Vis range, and the presence of interference from multiple absorbing species that are co-extracted with the analytes. Because of this limitation, there are not many methods based on LC coupled with UV-Vis to determine fipronil and its derivates, and many of them are applied to water samples.

Regarding the presence of fipronil in complex animal derived samples such as honey, a comparison between dispersive liquid-liquid microextraction (DLLME) method using

carbon tetrachloride as extractant solvent and acetonitrile as dispersant solvent, and modified QuEChERS method using acetonitrile as extractant solvent was made. In both cases the honey was previously diluted with ultrapure water. With the optimal conditions LOQs were 30 and 600 μg/kg for DLLME and modified QuEChERS, respectively, with acceptable recoveries between 70.7 and 101.1% for both. Despite the better sensitivity offered by DLLME method due to its preconcentration capacity, QuEChERS proved to be more robust when dealing with complex matrix such as honey, although LOQ was quite far from the MRL of 5 μg/kg [95].

Fipronil contamination was studied in turtle eggshells by means of a simple SLE with acetonitrile and low-temperature partitioning achieving LOQ of 8.3 μg/kg. Fipronil is commonly detected between 210 and 220 nm, however, in this work the extracts were analyzed by the HPLC-DAD at two different wavelengths, 230 and 280 nm, because at the lower wavelength the chromatogram showed many co-extracted impurities [96].

On the other hand, chicken eggs were analyzed by means of a novel integrated solid-phase extraction (ISPE) with ionic liquid-thiol-graphene oxide composite (IL-TGO). The ISPE combined pipette-tip solid phase extraction (PT-SPE) and d-SPE. Analytes were adsorbed by IL-TGO through the pipette tip and then eluted through dispersive mode, achieving LOQ of 15.9 μg/kg [73].

During the past decades, numerous advances have been achieved in LC miniaturization, mainly in terms of instrumentation and detection development [97,98]. Miniaturized HPLC systems are based on the use of columns with smaller inner diameter (i.d.) than traditional LC columns. HPLC techniques can be classified according to the i.d. of the chromatographic column used (**Table 3**).

Table 3. Names, i.d. and typical flow rates for HPLC techniques. Adapted from [99].

Name	Column i.d.	Flow rate
Conventional HPLC	3.2-4.6 mm	0.5 – 2.0 mL/min
Microbore HPLC	1.5-3.2 mm	100-500 μL/min
Micro-LC	0.5-1.5 mm	20-100 μL/min
Capillary LC	150-500 μm	1-20 μL/min
Nano-LC	10-150 μm	10-1000 nL/min

The main advantages of the miniaturized techniques is derived from the reduction of column i.d. and the decrease in the flow rate that they employed. The use low flow rate provides the reduction of organic solvent consumption in the mobile phase when compared with traditional LC-based methods, which lowers the economic cost and makes the analysis more environmentally friendly. As a result, more fancy additives, stationary phases or exotic mobile phases could be employed to enhance the method while maintaining a low analysis cost [98]. Similarly, another benefit involves the lower consumption of sample, especially when the available amount is limited, as in the case of natural products, or medical-related analysis. On the other hand, miniaturized techniques are characterized by an increase in sensitivity that they can achieve (for the same injection volume) as a result of a decrease in radial dilution due to small columns. In addition, the low flow rate required to perform the chromatographic separation increases the ionization efficiency when working with electrospray ionization (ESI)-MS detection, as the separated analytes at low flow rates can be efficiently transferred from the liquid phase into gas-phase ions.

However, there are several factors to consider when working with miniaturized techniques since the use of columns with very small i.d. requires an adequate instrumental arrangement selection. For instance, pumps able to produce low flow rates without pulses, proper dimensions of tube connections which minimize dead volumes, and injection valve or detector cell with optimized path length to prevent peak broadening are necessary to reduce the factors that can negatively affect efficiency [100].

Although miniaturized techniques have shown great potential in the analytical field including food analysis [100], to the best of our knowledge, there are no previous references related to the determination of fipronil and its metabolites using capillary liquid chromatography (CLC). Considering the abovementioned, in **Chapter 2**, a novel method based on CLC was proposed for the identification of fipronil, fipronil-sulfone and fipronil-sulfide in egg samples.

2.4.4. Liquid chromatography-mass detector methods

Fipronil and metabolites are mostly determined in negative ESI mode and the transitions usually employed to quantify (Q_{ion}) and identify (I_{ion}) the compounds are 435.0 > 330.1 and 435.0 > 250.0 for fipronil; 387.0 > 351.1 and 387.0 > 282.1 for fipronil-desulfinyl; 419.0 > 262.1 and 419.0 > 383.1 for fipronil-sulfide; 451.0 > 282.1 and 451.0 > 415.1 for fipronil-sulfone; and 453.0 > 303.9 and 453.0 > 347.7 for fipronil-amide [101]. In contrast to most GC separations, fipronil usually eluted before fipronil-sulfide.

As stated above, fipronil and its metabolites together with neonicotinoid insecticides have shown high toxicity to non-target pollinator insects, that can be exposed to the insecticides through pollen and nectar consumption. Their association with the CCD, a phenomenon that causes sudden bee colony death has led to the development of several

methods to determine the presence of fipronil and metabolites in honey and related matrices such as royal jelly or pollen.

Indeed, the first LC-MS/MS method to determine fipronil and metabolites at trace levels was developed in pollen samples with the aim of lowering the LOQs that had been set to date by GC. LOQs of 0.1 μg/kg were achieved for fipronil and metabolites by means of a three-step sample procedure involving a SLE with acetonitrile, a liquid-liquid partitioning with n-hexane saturated with acetonitrile to remove the lipids, and a clean-up step in a glass column filled with Florisil [102].

To analyze raw honey and pollen samples heating was required to reduce the viscosity of the sample, and dilution with a mixture of methanol:water was necessary prior a SPE with Florisil cartridge. No detectable residues of the target pesticides were found in any of the analyzed samples [103]. Fipronil and other pesticides were also determined in honey samples by a multi-residue analytical procedure based on an on-column liquid-liquid extraction (OCLLE), also called supported liquid extraction, which is based on classical LLE principle, but assisted by an inert solid support such as diatomaceous earth. The advantage that OCLLE provided over traditional LLE is the prevention of the formation of emulsion between the organic solvents and the aqueous matrix, ensuring their immiscibility. Fipronil detection capacity was 0.015 μg/kg [104]. In addition, honey and royal jelly have been analyzed using a modified QuEChERS extraction procedure to determine several pesticide residues, including fipronil. Samples were diluted and buffered at pH 9 and extracted with acetonitrile prior to the addition of an extraction kit based on the EN-QuEChERS procedure ($MgSO_4$, NaCl, sodium citrate tribasic dihydrate and sodium citrate dibasic sesquihydrate). Finally, a d-SPE clean-up with PSA, C18 and

$MgSO_4$ was carried out. Fipronil was detected, but not quantified (LOQ of 1 μg/kg) in 86% of the analyzed honey samples [105].

Regarding vegetable samples, a simple and rapid vortex-assisted dispersive liquid-liquid microextraction (VA-DLLME) method was developed for the determination of fipronil and fipronil-sulfone in tomato without further clean-up steps. Chloroform was used as extraction solvent, acetonitrile as dispersive solvent and 1 min vortex was enough to achieve the extraction of the analytes, obtaining LOQ of 0.25 μg/kg with acceptable recoveries [106]. For okra sample, fipronil and metabolites were extracted with ethyl acetate after the homogenization of the sample with water. Then, a clean-up step with PSA was required to reduce the matrix effect to < 25% with LOQ of 1 μg/kg [101].

For cereal, vegetable and fruit samples such as rice, corn, cucumbers, tomatoes, apples and bananas, a simple extraction method based on original QuEChERS followed by a d-SPE clean-up with C18, was employed, achieving LOQs of 10 μg/kg in all matrices [107]. Original QuEChERS extraction method was also employed for soil, corn grain and corn straw samples, followed by a d-SPE clean-up step with C18, C18 and PSA and PSA and GCB, respectively. It was observed that the presence of additives such as formic acid or ammonium acetatae in the acetonitrile:water mobile phase was prejudicial to the peak shape of fipronil and metabolites [108]. Original QuEChERS prior d-SPE clean-up with PSA and $MgSO_4$ was employed for the residue analysis and persistence evaluation of fipronil and its metabolites in cotton. Results showed that fipronil dissipated rapidly in cotton plants and soil mainly though oxidation and photolysis pathways [109]. Modified QuEChERS was employed by other authors to extract fipronil and metabolites from chilli pepper fruits. Samples were extracted with acetonitrile together with NaCl and Na_2SO_4,

and the extract was cleaned-up by a d-SPE with PSA, anhydrous $MgSO_4$, C18 and GCB, achieving LOQs of 10 μg/kg [110]. Peanut kernel, shell, straw and seedling were analyzed by a modified QuEChERS procedure using acidified acetonitrile along with NaCl followed by a d-SPE clean-up with PSA. Although low LOQs of 1 μg/kg were obtained for all compounds, it was noticeable that the retention times of all analytes were less than 0.7 min, which can be explained by the 5 cm column length, and by the isocratic mode with 90% acetonitrile, which resulted in low retention of the analytes in the RP column. Results showed fipronil-sulfone in a higher amount compared to the other metabolites in peanut kernels and soil [111].

Novel materials have been implemented as sorbents in the d-SPE clean-up after original QuEChERS extraction procedure. For instance, magnetic nanoparticles (Fe_3O_4-PSA) along with C18 sorbent used to purify extracts of a large variety of pesticide residues including fipronil from grain samples. The operation efficiency improved compared with conventional method without the magnetic sorbent, mainly because recoveries of the pesticides purified by the magnetic adsorbent focused in the range of 80-100%, while pesticides purified by the non-magnetic adsorbent had a wider recovery distribution, between 40 and 130% [72]. Similarly, the novel use of carbon nanotubes (CNTs) as sorbent along with PSA and C18 in the d-SPE clean-up was carried out to determine fipronil and metabolites in tea and chrysanthemum samples. Compared to GCB sorbent, CNTs presented higher capacity to adsorb pigments and organic molecules, however, matrix match calibration was still required. The use of UPLC Q-Exactive Orbitrap MS provided high selectivity and repeatability with LOQ of 2 μg/kg and recoveries between 86 and 112% [112].

Fipronil and its metabolites were also studied along with neonicotinoids in tea samples by using a modified QuEChERS method. Samples were extracted with acetonitrile, and polyvinylpolypyrrolidone and strong cationic exchange adsorbent were used as dispersive adsorbents to eliminate polyphenols and caffeine from tea extract and minimize matrix interferences. The analysis was carried out using LC coupled with high resolution mass spectrometry (HRMS), obtaining low LOQ values of 0.36 μg/kg [113]. Similarly, the occurrence and temporal variation of fipronil and its metabolites were evaluated in tea samples collected during several years in China, showing that fipronil was the most dominant followed by fipronil-desulfinyl, fipronil-sulfone and fipronil-sulfide [114].

Regarding animal-derived food products, and partly due to the fipronil eggs contamination incident in 2017, fipronil residue has been determined in chicken eggs and related matrices. For instance, chicken egg and muscle were extracted by a combination of sample treatment steps including an extraction with acetonitrile, a salting out with $MgSO_4$ and NaCl, and a SPE purification with C18 cartridge. Signal suppression effect was observed on fipronil, thus, matrix match calibration was used to compensate matrix effect. The detection capability was 0.01 μg/kg with recoveries ranging from 79.7 to 98.0% [115]. Fipronil and metabolites were also determined in egg samples by QuEChERS using acetonitrile to extract the analytes and PSA, C18 and GCB sorbents in the d-SPE purification step. With this mixture of sorbents matrix effects were in the range of -8.6 and 9.3%, and LOQs were below 0.2 μg/kg with acceptable recoveries [116]. Similarly, QuEChERS method was optimized to extract fipronil and its metabolites from chicken egg, muscle and cake. Samples were extracted by means of ultrasonication with acetonitrile, then salted out with NaCl at -20 °C to separate the lipids from the solvent, and purified combining PSA, C18 and anhydrous $MgSO_4$. LOQs were between 0.2 and 5

ng/kg. Results showed that fipronil-sulfone metabolite was detected in most of the 214 samples, and it was the main residue in egg and cake [117]. In other multi-residue study, MSPD involving Na_2SO_4 and sea sand was developed to disrupt the egg matrix and facilitate the extraction, which was performed in a glass column with a mixture of cyclohexane and ethyl acetate. Then, a small-scale gel permeation chromatography was employed to remove high molecular lipids and a SPE with pure C18 sorbent in combination with acidified MeOH elution was used to remove remaining low molecular lipids [118]. In a recent work an in-syringe-assisted fast pesticides extraction (FaPEx) has been developed and applied for the determination of fipronil and its metabolites in chicken egg samples. This sample preparation strategy comprised two steps. First, the sample was placed into a syringe and a mixture of acetonitrile and NaCl and $MgSO_4$ was added for the liquid-liquid partitioning. Then, the extractant from the first step was directly transferred into a FaPEx syringe-type SPE kit containing PSA, C18 and anhydrous $MgSO_4$. The FaPEx method required less amount of sorbents and time. The LOQs ranged between 0.1-0.25 μg/kg for target analytes with recoveries between 88.7 and 110.9% [119].

Due to its hydrophobicity, fipronil and its metabolites can persist in aquatic environments, and thus, aquatic organisms can be exposed to their residues. In that sense, PRiME pass-through cleanup was satisfactorily employed to greatly reduced the ion suppression matrix effect observed when analyzing different seafood samples such as crab, shrimp, fish and shellfish. Samples were extracted with acetonitrile prior the PRiME cleanup procedure. However, an isotope internal standard was applied as the matrix effect still could not be fully ignored. The PRiME pass-through cleanup method exhibited LOQ ranging between 2 and 0.4 μg/kg with satisfactory recoveries (81.4–114%) [120].

Even if the use of fipronil is prohibited in agricultural products, it can still be found in food of unknown origin. Thus, fipronil and its metabolites were determined in dietary samples by means of a cold-induced aqueous two-phase system (CI-ATPS) involving the extraction with water and acetonitrile, a freezing step for phase separation, and a d-SPE clean-up with C18, PSA and NanoCarb. The analysis was carried out by LC-HRMS in target single ion monitoring (tSIM) which allowed to reduce the LOQs in the range of 0.01 and 0.03 μg/kg. The CI-ATPS approach did not need to utilize salts or additives to induce phase separation, and it could effectively precipitate and remove lipids by freezing [121].

2.4.5. Chiral separation methods

Fipronil is a chiral insecticide as it possesses an asymmetric center of sulfur atom in its structure. Thus, it has a pair of enantiomers that have been identified as S-fipronil and R-fipronil. As a result, chiral methods have been proposed to determine and study its enantioselective properties such as degradation, acute toxicity, bioaccumulation etc.

For instance, an HPLC method using a (R,R) Whelk-O 1 column was developed for cabbage samples [122]. Analytes extraction was carried out with acetonitrile and NaCl, and purified through a glass chromatography column filled with Na_2SO_4, neutral aluminum oxide and activated carbon. Two detectors in series were employed for the chiral separation of fipronil; a circular dichroism (CD) detector performing at 234 nm, and a UV detector performing at 225 nm. The first eluted enantiomer was S-(+)-fipronil and the second was R-(-)-fipronil. LOQ were 50 and 60 μg/kg for S- and R-fipronil, respectively. Results suggested that the R-enantiomer degraded faster ($t_{1/2}$ = 2.52 days) than the S-enantiomer ($t_{1/2}$ = 3.99 days), resulting in a relative enrichment of the S-residue, which is positive since it possesses higher insecticide activity and lower mammalian

toxicity [123,124]. It was also observed that fipronil-sulfone was the main metabolite after 240 h of fipronil application.

On the other hand, the enantioselective acute toxicity, bioaccumulation and degradation of fipronil in earthworms were examined. The extraction employed acetonitrile and a liquid-liquid partition with n-hexane to remove lipids followed by a SPE procedure with C18 cartridges. Analyses were carried out using a (R,R) Whelk-01 chiral column in a HPLC instrument with DAD detection at 225 nm and chiral recognition of R- and S-enantiomers by CD detector. Results demonstrated that R-fipronil was approximately 2 times more toxic than the S-enantiomer, although toxicity of the racemate and both enantiomers was low to earthworms. It was also observed a preferential degradation of the less toxic S-enantiomer [125].

The enantioselective bioaccumulation, transformation and elimination of fipronil and its metabolites have been also studied in *Anodonta woodiana* mussel samples. The extraction was carried out with ethyl acetate followed by an SPE with Florisil cartridge, and the chiral analysis was conducted using a BGB-172 column in a GC-ECD instrument. In contrast to what was observed with earthworms, in *Anodonta woodiana* mussels S-fipronil was more toxic than the R-enantiomer and the racemic mixture. Fipronil-sulfone and fipronil-sulfide were more than ten times more toxic than racemic fipronil, being fipronil-sulfone the most abundant during the bioaccumulation [126].

2.4.6. Capillary electrophoresis methods

As already mentioned, LC and GC coupled to different detection systems have been the preferred techniques for the determination of fipronil and its metabolites in food matrices. To the best of our knowledge, the capillary electrophoresis (CE) technique has

not yet been reported for the determination of these insecticides, neither in food matrices nor in any other matrices. Briefly, CE is a separation technique consisted of a fused silica capillary filled with an electrolyte solution, two electrolyte buffer reservoirs, a high-voltage power supply and a detector (**Figure 3**).

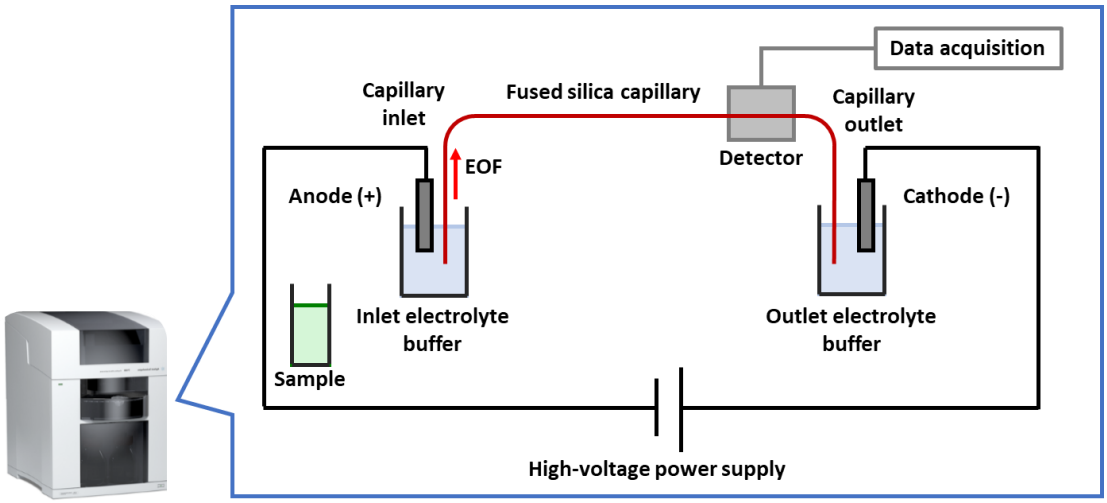

Figure 3. Scheme of capillary electrophoresis separation in normal mode.

When high voltage is applied through the capillary ends, the sample molecules, which have previously been introduced into the capillary inlet, are separated by their electrophoretic mobility. When working in normal mode, i.e., with the detection near the cathodic end of the capillary, all ions, positive or negative, and neutral molecules are pulled through the capillary in the same direction by electroosmotic flow (EOF). Positive ions migrate faster toward the capillary outlet since the EOF and the ion movement are in the same direction, then neutral molecules migrate only by the EOF, and finally, negative ions, as their electrophoretic mobilities are against the EOF. This CE mode is known as capillary zone electrophoresis (CZE) However, separation and selectivity can be controlled by modifying several factors such as pH or ionic strength, or by the use of additives in the electrolyte solution.

It is well-known that CE has been integrated in analytical laboratories within many fields, especially in pharmaceutical and biochemistry sciences, although its use has also been extended to environmental and food analysis [127,128,129]. Among all the techniques discussed above, CE is an interesting and green alternative due to its short analysis time, high separation efficiency and resolving power, and low solvent and sample consumption. Furthermore, CE eliminates the need for organic solvents and extends the experimental working pH range above that of most silica-bonded stationary phases used in RPLC. Additionally, CE instruments and their maintenance are more affordable than HPLC or GC ones, fact that could make CE widespread in routine laboratories from different economic backgrounds. The major limitation of CE is the lack of sensitivity, mainly using UV-Vis detection, because of the low sample volumes injected and the short optical path length of the capillary detection window. This drawback can be overcome by applying sample preconcentration strategies, by extending the optical path length using extended light path capillaries or "bubble" cell capillaries, or by using more sensitive detection techniques, such as laser induced fluorescence, or MS detection. Considering the advantages associated to this technique and the absence of previous information related to the determination of fipronil, in **Chapter 1** the feasibility of CE and related electromigration techniques such as micellar electrokinetic capillary chromatography (MEKC) has been assessed for the determination of fipronil and its primary degradation products in egg samples.

MEKC combines the separation mechanisms of chromatography, i.e., degree of affinity of the analyte for a mobile phase and for a stationary phase, with the electrophoretic and electroosmotic movements of solutes and solutions, extending its functionality not only to charged but also not neutral analytes. In addition, it increases the selectivity of the CZE mode separation by selectively interacting the solutes with one of the components

of the BGE, thereby altering the electrophoretic mobility of one or more of the sample components.

The separation is carried out in an electrolyte solution containing a "stationary phase", usually a surfactant at a concentration above the critical micellar concentration (CMC), so that micelles are formed and the neutral compounds are distributed in equilibrium inside and outside the micelles. Among all surfactants, some of the most commonly used are sodium dodecyl sulphate (SDS) and ammonium perfluorooctanoate (APFO). With both surfactants, the spherical micelles have the hydrophobic side inwards and the hydroph8ilic side outwards (negatively charged surface), however, although they are attracted to the positive pole (anode) of the electrophoretic system, the micelle or the micelle- neutral analyte assembly migrates towards the cathode due to the effect of the EOF, which at neutral or basic pH is greater than the electrophoretic mobility. The neutral molecules interact with the micelles to different extents depending on their distribution coefficients in the micelles, which depend on the hydrophobicity of these analytes. The most apolar compounds have high affinity for the interior of the micelles, while polar compounds are poorly soluble in the apolar interior of the micelle, and therefore remain free in the solution. **Figure 4** schematizes the mechanisms of MEKC with anionic micelles.

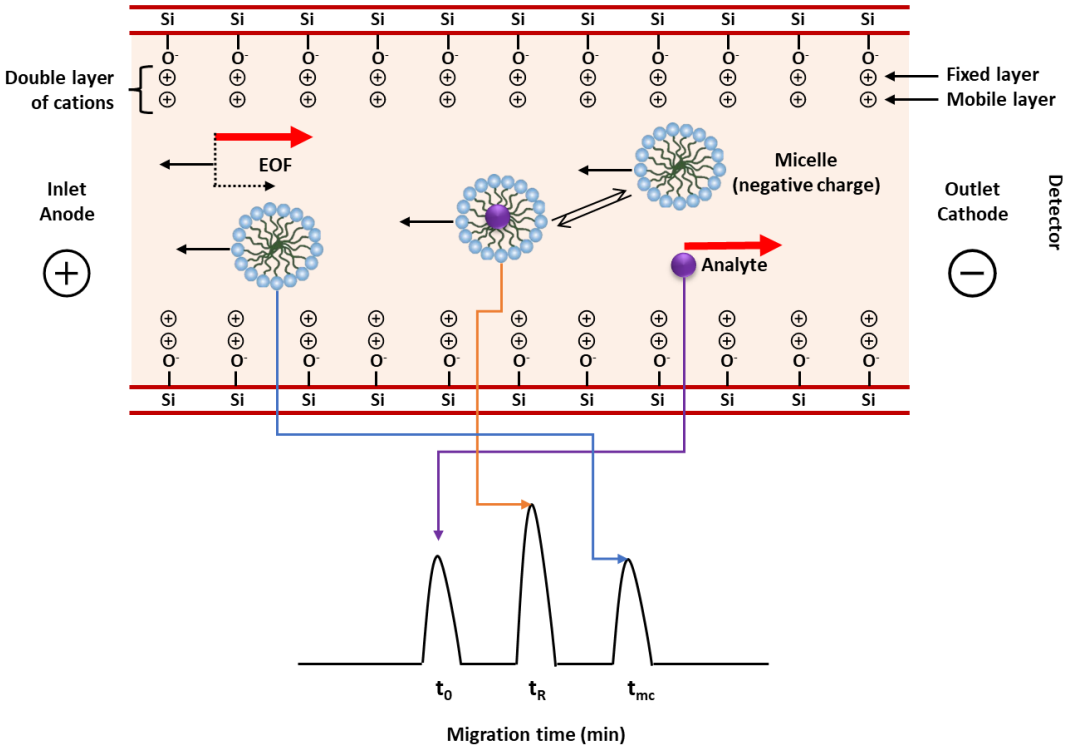

Figure 4. Scheme of a MEKC separation using negative micelles and normal polarity.

The time it takes for an analyte to elute from the capillary depends on its distribution coefficient in the micelles. If it is fully incorporated in the micelle, it will migrate at the same rate as the micelle (t_{mc}), whereas if the analyte is totally free in BGE it will migrate at the rate of the EOF (t_0). The stronger the solute-micelle interaction, the longer the migration time (t_R), as the micelles hold the solute against the EOF current.

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CHAPTER 1

A FIRST APPROACH USING MICELLAR ELECTROKINETIC CAPILLARY CHROMATOGRAPHY FOR THE DETERMINATION OF FIPRONIL AND FIPRONIL-SULFONE IN EGGS

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1. Summary

Fipronil is a broad-spectrum insecticide from the phenylpyrazole family which is currently not approved in the European Union in food. In 2017 fipronil was involved in a European health alert due to its presence in fresh hen eggs because of an illicit use in poultry farms. This issue highlighted the importance of developing reliable analytical methods to determine fipronil and its main metabolites in this matrix. In this work, it has been reported the first approach to the study of fipronil and two metabolites, fipronil-sulfone and fipronil-sulfide by capillary electrophoresis (CE). Micellar electrokinetic chromatography (MEKC) mode was employed using as background electrolyte a solution of 50 mM ammonium perfluorooctanoate pH 9.0 with 10% (v/v) methanol. The proposed method was combined with a simple sample treatment based on salting-out assisted liquid-liquid extraction (SALLE) using acetonitrile as extraction solvent and ammonium sulfate as salt. The SALLE-MEKC-UV method allowed the simultaneous quantification of fipronil and fipronil-sulfone. Validation parameters yielded satisfactory results, with precision, expressed as relative standard deviation, below 14% and recoveries from 83 to 88%. Limits of detection were 90 μg/kg for fipronil and 150 μg/kg for fipronil-sulfone, so in terms of sensitivity further studies with sample treatment allowing extra preconcentration or more sensitive detection, such as mass spectrometry (MS), would be need.

2. Experimental

2.1. Materials and reagents

Analytical standards of fipronil, fipronil-sulfide and fipronil-sulfone (≥99%) were supplied by Sigma-Aldrich (Darmstadt, Germany) and were used without further purification. All reagents used in this study were of analytical grade and the solvents were of HPLC grade. Acetonitrile (MeCN), methanol (MeOH) and ethyl acetate were obtained from VWR (Center Valley, PA, USA).

Perfluorooctanoic acid (PFOA, 96%) was obtained from Sigma Aldrich (St. Louis, MO, USA). Ammonia solution (NH_3, 30% v/v) and sodium hydroxide (NaOH) were purchased from Panreac-Química (Madrid, Spain). A solution of 50 mM ammonium perfluorooctanoate (APFO) at pH 9.0 was prepared from PFOA, dissolving the necessary amount of this acid in ultrapure water and adjusting the pH to 9 with a 5 M NH_3, 30%. Sodium dodecyl sulfate (SDS, ≥98.5%) was purchased from Sigma-Aldrich (Darmstadt, Germany). Salts used in the sample treatment procedure were acquired from different suppliers: ammonium sulfate ($(NH_4)_2SO_4$, >99.5%) was obtained from VWR (Center Valley, PA, USA), sodium chloride (NaCl, >99%) and magnesium sulfate anhydrous ($MgSO_4$, 96%) were obtained from Panreac (Barcelona, Spain). Sodium tetraborate decahydrate (>99.5%) was supplied from Sigma-Aldrich (Darmstadt, Germany). Perchloric acid ($HClO_4$, 72%) and sodium dihydrogen phosphate monohydrate ($NaH_2PO_4 \cdot H_2O$, ≥98) were from Panreac (Barcelona, Spain). Cyclodextrins (CDs): α-CD, β-CD, hydroxypropyl-β-CD and γ-CD (>98%) were from Sigma-Aldrich (Darmstadt, Germany). Ultrapure water (18.2 MΩ·cm) (Milli-Q plus system, Milllipore, Bedford, MA, USA) was employed throughout the work.

Polytetrafluoroethylene (PTFE) syringe filters (0.2 μm × 13 mm) (VWR, Center Valley, PA, USA) were also used.

2.2. Preparation of standard solutions

Individual standard stock solution (1000 mg/L) of fipronil, fipronil-sulfide and fipronil-sulfone were prepared in MeOH. The standards were accurately weighed and dissolved in 4-mL amber glass vial and stored in dark under refrigeration at -20 °C until further use. When necessary, sonication was applied to ensure the complete dissolution of the analytes. The intermediate stock solutions (100 mg/L) were obtained by dilution of the standard stock solutions with MeOH. Working standard solutions containing all analytes were prepared by mixing and diluting the intermediate stock standard solutions in 20 mM SDS.

2.3. Instrumentation

The analyses were carried out with an Agilent 7100 CE instrument (Agilent Technologies, Waldbronn, Germany) equipped with a diode array detector (DAD). The software provided with the HP Chemstation (version B.02.01) was used for instrument control and data acquisition. Separations were carried out in polyimide-coated fused-silica capillaries of 48.5 cm total length (40 cm effective length, 50 μm inner diameter) from Polymicro Technologies (Phoenix, AZ, USA).

A pH meter (Crison model pH 2000; Barcelona, Spain), a Vortex-Genie 2 (Scientific Industries; Bohemia, NY, USA), an ultrasonic bath (USS-300 model; VWR, West Chester, PA, USA), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA), an evaporator with nitrogen (System EVA-EC from VLM GmbH; Bielfeld, Germany) and a centrifuge (Universal 320R model; Hettich, Leipzig, Germany) were used.

2.4. MEKC procedure

Before its first use, the capillary indicated in section 2.3., was conditioned by means of the following rinse sequence: 10 min with deionized water, 10 min with 0.1 M NaOH, again 10 min with deionized water and finally 50 s with the running buffer. In all cases, temperature was set at 25 °C and a pressure of 1 bar was applied. The background electrolyte (BGE) was a solution of 50 mM APFO pH 9.0 (prepared as explained in section 2.1. Material and reagents), containing a 10% (v/v) of MeOH as organic modifier.

In order to obtain a good repeatability between days, at the beginning of the working day the capillary was conditioned by flushing with 5 M NH_3 solution for 10 min and deionized water for 10 min, and then with BGE for 50 s. Longer conditioning times with BGE resulted in deficient reproducibility of the migration time. Thus 50 s was the time selected as it allows a satisfactory reproducibility, and it is enough time to fill the capillary volume. Moreover, the capillary was rinsed between runs with 5 M NH_3 solution for 1 min, deionized water for 1 min and BGE for 50 s. Furthermore, to guarantee a satisfactory repeatability it is essential to change the BGE vial after six injections. At the end of the working day, the capillary was washed consecutively with 0.1 M NaOH, deionized water and MeOH for 5 min each, followed by air for 1 min and stored dried with isopropanol:water (1:1, v:v) vials in both extremes to avoid dust particles to obstruct it. In all instances, temperature was set at 25 °C and a pressure of 1 bar was applied.

Samples were injected hydrodynamically at 50 mbar for 20 s. Separation was performed in MEKC mode, using the BGE previously described, and a voltage of 25 kV with an initial ramp time of 0.3 min. The temperature of the capillary cassette was set a 25 °C. UV-detection was performed a 220 nm wavelength with a bandwidth of 4 nm for all analytes. The data acquisition rate was set at 20 Hz. PTFE syringe filters (0.2 μm × 13 mm) were used to filter the sample extracts before injection into the CE system.

2.5. SALLE procedure

Organic hen eggs were purchased at the local supermarket. Eggs were cracked, mixed and stirred until homogenization. Four mL of MeCN were added to 2.0 g of homogenized sample for protein precipitation and analyte extraction. The mixture was stirred in a mechanical shaker for 4 min followed by centrifugation at 9000 rpm at 4 °C for 10 min in order to separate the resulting supernatant from the precipitated proteins. Then, the MeCN organic phase was separated from the aqueous portion of the supernatant by adding 0.8 g of ammonium sulfate, inducing phase separation by salting-out effect. The mixture was vortexed for 1 min and centrifuged at 9000 rpm and 4 °C for 5 min. Three layers were perfectly distinguishable in the tube: some salt in the bottom, a clear aqueous phase in the middle and a yellowish organic phase in the top. Then, 3.4 mL of the organic phase were isolated and transferred to a 4-mL glass vial. The organic extract was evaporated under a gentle N_2 stream at 30 °C. The residue was re-dissolved in 500 μL of the sample injection medium (20 mM SDS). The resulting solution was injected hydrodynamically in the CE system and analyzed by the optimized MEKC-UV procedure, previous filtration through a 0.2 μm PTFE filter.

3. Results and discussion

3.1. Optimization of the CE method

No previous references were found in bibliography about the determination fipronil and its metabolites by CE, so different strategies were checked, including capillary zone electrophoresis (CZE), non-aqueous capillary electrophoresis (NACE) and MEKC with different micellar media. Although the wavelength of 280 nm has been widely used to determine fipronil and metabolites using UV detection systems, the wavelength used in

this work was 220 nm, also employed in some studies [1,2,3], because greater sensitivity was achieved.

Initially, both basic and acidic BGEs were considered for CZE to separate a mixture of 10 mg/L in MeOH of fipronil, fipronil-sulfide and fipronil-sulfone. In this CE mode, separation takes place because the different components of the sample mixture migrate as discrete bands with different velocities. The velocity of each component depends on its electrophoretic mobility and the electroosmotic flow (EOF) inside the capillary. Thus, on the one hand, 50 mM sodium tetraborate buffer at pH 9.2 was used with the aim of negatively charge the analytes by deprotonation of the amino group (**Figure 1A**). On the other hand, 50 mM phosphate buffer at pH 2.0 adjusted with 0.1 M NaOH was intended to make the analytes positively charged by protonation of the amino group (**Figure 1B**). Applied voltage was 25 kV in both cases. However, neither of the two mentioned BGEs was satisfactory since no peaks were observed in either analysis, which indicated that these analytes are neutral in a wide range of pH, so CZE was discarded.

Then, NACE, which is a variation of CZE that employs electrolytes in organic solvents as BGE, was evaluated. NACE provides additional mechanisms and interactions in relation to aqueous solvents, which might lead to interesting changes in separation or selectivity. In this sense, the tested BGE was MeOH:MeCN (1:2, v/v) with 20 mM $HClO_4$ as BGE. These conditions have been previously used to determine weakly basic pesticides with satisfactory results [4]. However, no signals were detected for fipronil, fipronil-sulfide and fipronil-sulfone when similar separation conditions were applied, so NACE was also discarded.

MEKC was also evaluated using 20 mM sodium tetraborate as BGE with SDS. The SDS concentration tested ranged between 60-120 mM, which is above its critical micelle concentration (CMC) of 8 mM. The SDS micelle aggregates have polar negatively

charged surfaces and are naturally attracted to the positively charged anode. However, the alkaline pH of the sodium tetraborate BGE, which was around 9 led to a strong EOF towards the cathode, so a positive voltage of 25 kV was applied. No signals were observed when water was the injection solvent, probably due to poor solubility of analytes in water (**Figure 1C**). Therefore, a solution composed by 20 mM sodium tetraborate with 60 mM SDS was tested as injection solvent. In this case, and regardless the SDS concentration in the BGE, a single peak was observed as a result of comigration of the three analytes (**Figure 1D** and **Figure 1E**). Subsequently, the addition of CDs in an acidic SDS BGE was evaluated to enhance the separation of the analytes [5]. In this case, negative voltage (-25 kV) was applied as the EOF is minimized at acidic pH. However, no effect on resolution was observed although different types of CDs were tested such as α-CD, β-CD (**Figure 1F**), hydroxypropyl-β-CD and γ-CD. Analytes seemed to have low affinity for CDs, just interacting with the SDS micelles.

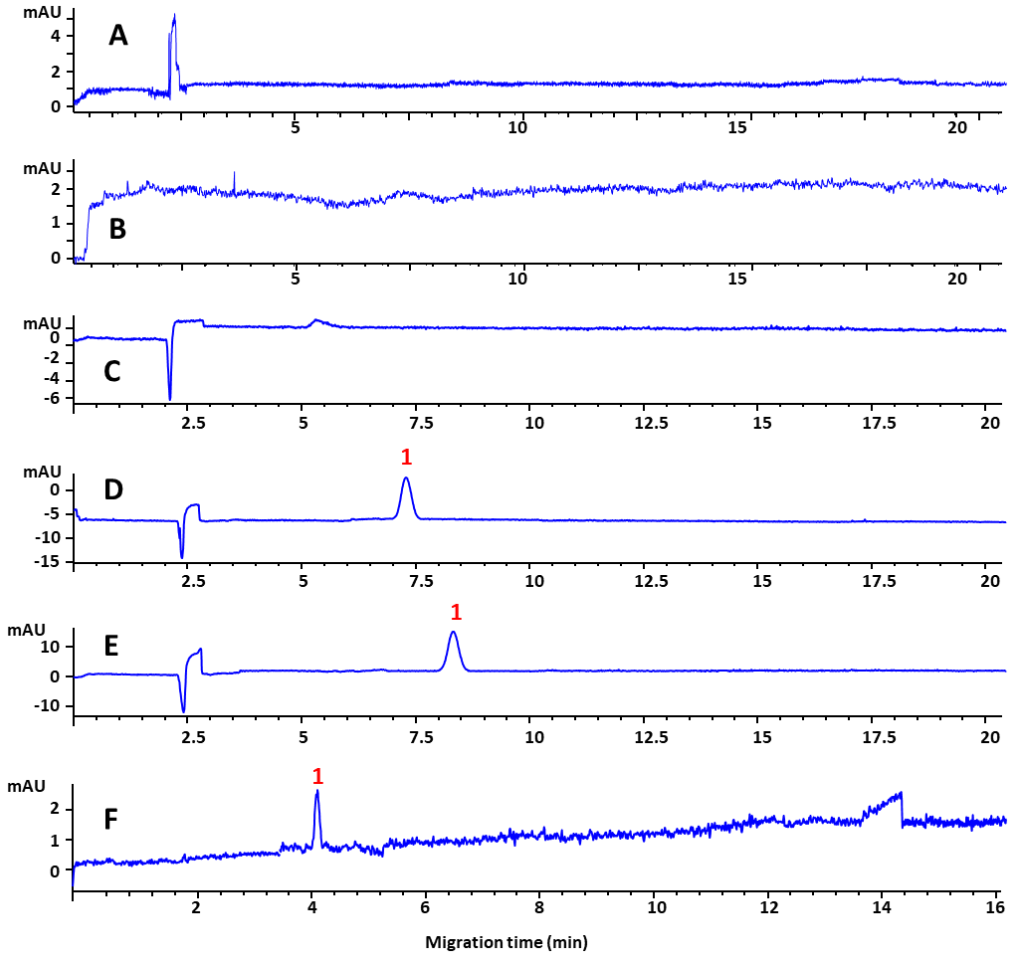

Figure 1. Different MEKC conditions for the separation of a standard mixture of fipronil, fipronil-sulfide and fipronil-sulfone. A) CZE mode: BGE 50 mM sodium tetraborate buffer, pH 9.2. Injection solvent H_2O; B) CZE mode: BGE 50 mM phosphate buffer, pH 2.0. Injection solvent H_2O; C) MEKC mode: BGE 20 mM sodium tetraborate and 60 mM SDS. Injection solvent H_2O; D) MEKC mode: BGE 20 mM sodium tetraborate and 60 mM SDS. Injection solvent 20 mM sodium tetraborate and 60 mM SDS; E) MEKC mode: BGE 20 mM sodium tetraborate and 120 mM SDS. Injection solvent: 20 mM sodium tetraborate and 60 mM SDS; F) MEKC mode: BGE 50 mM phosphoric acid, 50 mM SDS and 15 mM β-CD. Injection solvent 50 mM phosphoric and 50 mM SDS. Peak 1: Coelution of fipronil, fipronil-sulfide and fipronil-sulfone (100 μg/L of each analyte). Other experimental conditions: separation voltage, 25 kV (A, B, C, D, E), -25 kV (F); temperature, 25 °C; hydrodynamic injection, 10 s at 50 mbar.

Finally, the use of an alternative pseudo-stationary phase, such as APFO, a (semi)volatile surfactant, was checked. In comparison to SDS, APFO shows some advantages such as high compatibility with MS detection, which would be positive for further studies. In

addition, APFO, which CMC is 20 mM, is not only an anionic micellar agent but also a BGE due to its buffer capacity. An alkaline pH (9.0) provided by adjusting with 5 M NH_3 solution was required to dissolve PFOA in water, obtaining the APFO solution. With this alkaline buffer there was a strong EOF towards the cathode, thus, a positive electric field was assessed even though AFPO micelles are negatively charged. A solution of 50 mM APFO was tested for both, BGE and injection solvent, and a positive voltage of 25 kV was applied. Using these conditions, three baseline separated broad peaks were observed for the first time (**Figure 2A**). However, narrower peaks were obtained when the micellar medium composition of the injection solvent was 50 mM SDS solution instead of 50 mM APFO solution (**Figure 2B**). Then, using 50 mM SDS solution as injection solvent, the BGE APFO concentration was increased up to 70 mM. The best signals were obtained using a concentration of 50 mM since resolution between fipronil-sulfide and fipronil strongly decreased when the APFO concentration increased up to 70 mM (**Figure 2C**). Nevertheless, a broad peak was obtained for fipronil-sulfone so the effect of adding an organic solvent to the BGE was investigated.

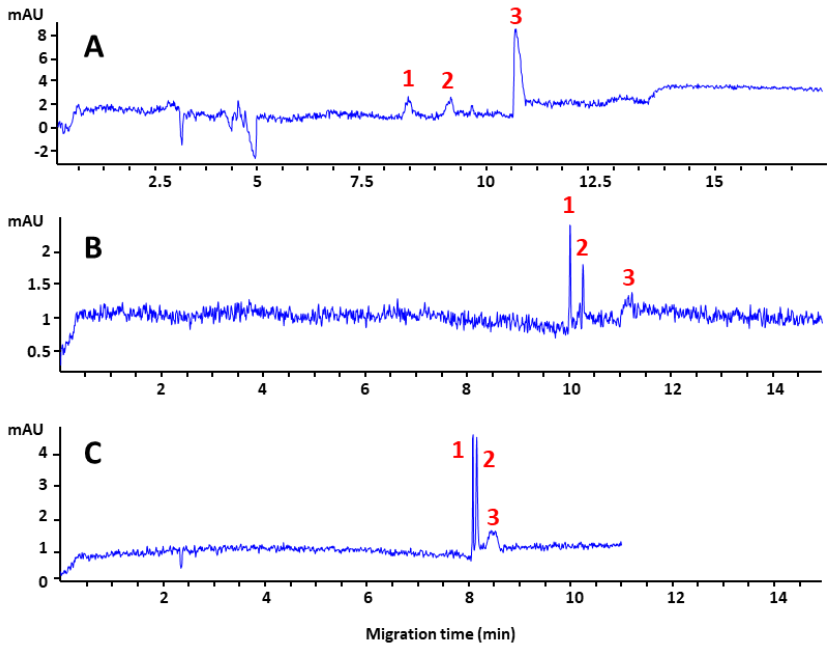

Figure 2. MEKC mode separation using A) BGE 50 mM APFO, injection solvent 50 mM APFO; B) BGE 50 mM APFO, injection solvent 50 mM SDS; C) 70 mM APFO, injection solvent 50 mM SDS. Peaks: 1: Fipronil-sulfide; 2: Fipronil; 3: Fipronil-sulfone (10 µg/L of each analyte). Other experimental conditions: separation voltage, 25 kV; temperature, 25 °C; hydrodynamic injection, 10 s at 50 mbar.

Organic BGE modifiers can produce variations in the mobility of the analytes as in the viscosity and polarity of the solution, resulting in changes in selectivity, migration time and peak shape. In order to improve the peak shape of fipronil-sulfone, different percentages of MeOH (0, 5, 10 and 20% v/v) were added to 50 mM APFO (**Figure 3**). The resolution and efficiency of the fipronil-sulfone peak significantly improved with the increase of the MeOH concentration in the BGE. A 10% (v/v) of MeOH in a 50 mM APFO BGE was the optimum value because higher values involved the micelles breakdown, deteriorating the separation.

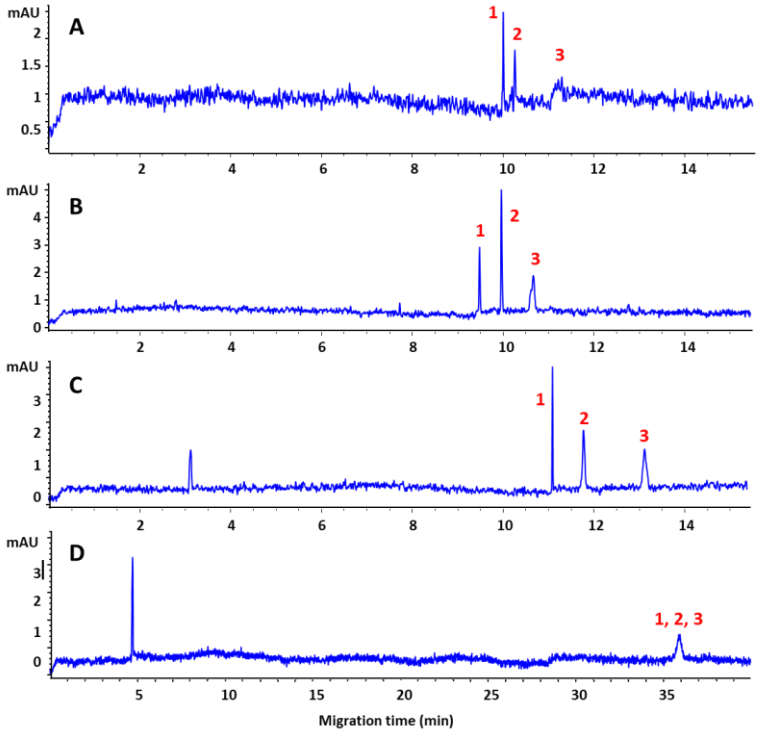

Figure 3. Influence of MeOH as organic modifier in the 50 mM APFO BGE. A) 0% MeOH; B) 5% MeOH; C) 10% MeOH; D) 20% MeOH. Peaks: 1: Fipronil-sulfide; 2: Fipronil; 3: Fipronil-sulfone (10 μg/L of each analyte). Other experimental conditions: separation voltage, 25 kV; temperature, 25 °C; hydrodynamic injection, 10 s at 50 mbar.

Once the CE mode was selected, the rest of variables were optimized. Using the above mentioned BGE, capillary temperature was studied in the range of 20-30 °C. An increase in the peak intensity, resolution and efficiency were observed when the temperature was set a 25 °C, so this value was selected. The separation voltage was evaluated in the range of 20-30 kV. The best results were obtained using a voltage of 25 kV, as a good compromise between peak resolution, analysis time, and electrophoretic current (32 μA).

Once these parameters had been optimized, sample injection medium was re-evaluated in order to check if stacking preconcentration was possible. An aqueous solution of 10% MeOH (v/v), the selected BGE (50 mM APFO with 10% (v/v) MeOH) and a 50 mM SDS

solution were tested. Satisfactory separation was achieved only with the SDS solution, so its concentration was evaluated between 20 and 50 mM. The best results were obtained using a concentration of 20 mM, since peak area and peak efficiency improved. Finally, the injection volume was studied through the hydrodynamic injection time, in the interval from 12 to 30 s. Obviously, an increase in the injection time and consequently, in the sample volume, produced an increase of the sensitivity, however, resolution and peak efficiency were adversely affected. Accordingly, 20 s and 50 mbar (equivalent to a 2% of the capillary volume) were selected as a compromise between peak shape, efficiency and run time. In **Figure 4** it can be observed an electropherogram with the final optimized conditions.

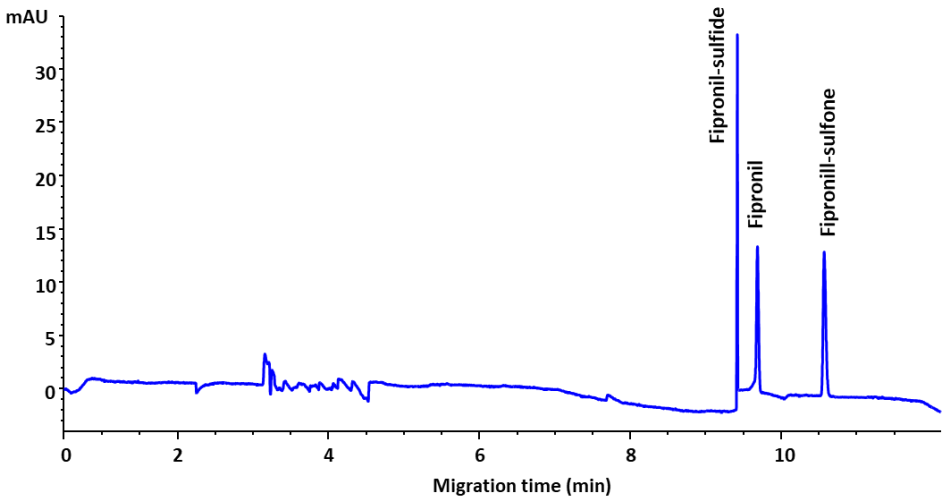

Figure 4. Electropherogram showing fipronil-sulfide, fipronil and fipronil-sulfone peaks with the optimized electrophoretic conditions (10 μg/L of each analyte). Experimental conditions: BGE, 50 mM APFO with 10% (v/v) MeOH; injection solvent, 20 mM SDS; separation voltage, 25 kV; temperature, 25 °C; hydrodynamic injection, 20 s at 50 mbar..

3.2. Optimization of the sample treatment

Reported extraction methods for the analysis of fipronil in egg samples prior to their analysis by chromatography techniques are mostly based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe) using MeCN or other organic solvents such as ethyl

acetate or acetone as extraction solvents, and a mix of different salts as salting-out reagents. To separate lipids from the solvent, a salting-out step at -20 ° C or a partitioning with hexane can be done. Finally, those steps are commonly followed by a dispersive solid phase extraction (d-SPE) clean-up by combining sorbents such as primary secondary amine (PSA) and C18 [6].

In contrast with these time-consuming procedures, a simple and fast extraction method based on salting-out assisted liquid-liquid extraction (SALLE) was considered in this work. The influence of several variables (nature and volume of the extraction solvent, nature of salt and mechanical shaking time) on the extraction efficiency was evaluated by means of recovery studies. Recoveries were calculated by comparing peak areas of samples spiked before sample treatment with peak areas of samples spiked after sample treatment. The study was carried out in triplicate (n=3) using a homogenized pool of hen eggs. An amount of 2 g was used throughout the sample treatment optimization, which was previously spiked with the target analytes at 1 mg/kg.

3.2.1. Nature of the extraction solvent

In order to select the extraction solvent, 4 mL of two water-miscible organic solvents such as MeOH and MeCN, and also ethyl acetate, which is slightly soluble in water, were examined. After mixing with a mechanical shaker, the samples were centrifuged at 4 °C and 9000 rpm for 10 min. With MeOH no phase-separation was observed, while ethyl acetate created a three-phase gelatinous mass when it was mixed with the egg, thus both solvents were discarded. The addition of MeCN led to egg protein precipitation and phase-separation, thus, it was selected as the extraction solvent.

3.2.2. Nature and amount of salt

The supernatant was isolated, and three different salts were checked as salting-out agents: NaCl, $MgSO_4$ and $(NH_4)_2SO_4$. In the case of NaCl, very low recoveries were achieved in comparison to those obtained with sulfate salts. Ammonium sulphate provided the best results considering reproducibility and recoveries. An amount of 0.8 g of $(NH_4)_2SO_4$ was enough to induce phase separation by salting-out with recoveries higher than 80%.

3.2.3. Volume of the extraction solvent

Then, the influence of the volume of MeCN on the extraction recoveries was studied between 2 and 4 mL, showing that 4 mL of MeCN was the optimum value, with recoveries higher than 80% for fipronil and fipronil-sulfone (**Figure 5A**). Although MeCN has higher boiling point, and it is more difficult to evaporate than the other tested solvents, the advantages of using it are its ability to induce protein precipitation and its suitable polarity to extract the analytes. At these conditions, the electropherogram of a blank egg sample showed an endogenous interferent peak at fipronil-sulfide migration time, so fipronil-sulfide could not be quantified by using this extraction. More extensive sample treatment involving further clean-up steps would be needed to determine fipronil-sulfide using the proposed MEKC-UV method. However, this fact does not preclude the use of the proposed method for legislative purposes as the EU sets the MRL of fipronil as the sum of fipronil and fipronil-sulfone.

3.2.4. Mechanical stirring time

Finally, the mechanical stirring time was evaluated between 2 and 5 min, achieving higher extraction recoveries when the time increased up to 4 min (**Figure 5B**), so this value was selected as the optimum, with recoveries higher that 83% for both analytes.

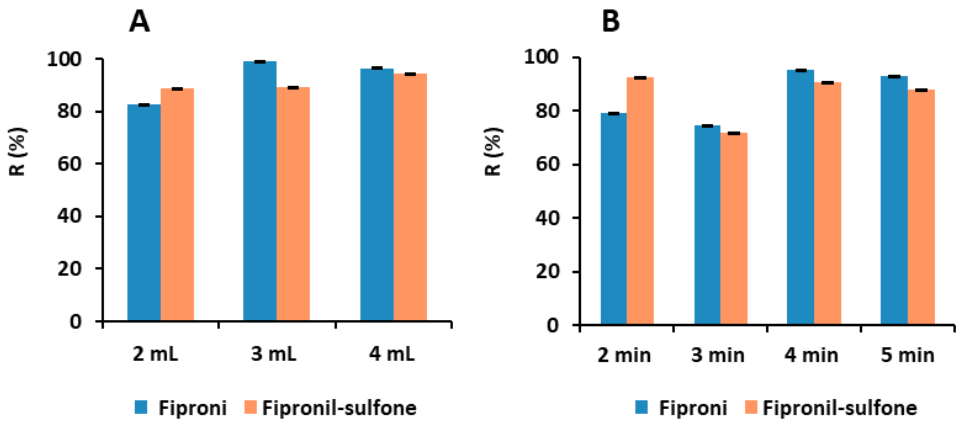

Figure 5. Influence of: A) volume of extraction solvent and B) time of mechanical agitation on the extraction efficiency of the SALLE sample treatment. Error bars represent the standard error (n=6).

3.3. Method characterization

The proposed SALLE-MEKC-UV method for the determination of fipronil and fipronil-sulfone in eggs was characterized in terms of linearity, limits of determination (LODs), limits of quantification (LOQs), precision (repeatability and intermediate precision) and recovery.

3.3.1. Calibration curves and analytical performance characteristics

The proposed method was evaluated by procedural calibration curves, using ecological hen eggs free of analytes. The calibration curves were established by spiking the egg samples with fipronil and fipronil-sulfone at five different concentration levels (300, 800, 1500, 2200 and 3000 μg/kg for fipronil and 500, 1500, 2500, 3500 and 5000 μg/kg for fipronil-sulfone). After equilibration for fifteen minutes, each level was extracted in duplicate, and each extract was injected in triplicate (n=6). Considering that the reproducibility of the migration times (as well as the peak areas) depends on the EOF and usually it may vary from capillary to capillary, batch to batch or with slight changes in the APFO BGE composition, calibration curves were obtained by considering the

migration time-corrected peak areas (calculated by dividing the analyte area by its migration time). **Figure 6** shows an electropherogram of a blank egg sample and an egg sample spiked with fipronil and fipronil-sulfone at a concentration of 800 and 2200 µg/kg respectively. It can be observed the fipronil-sulfide interference before the fipronil peak. A blank sample was also analyzed to ensure that samples were free of these compounds.

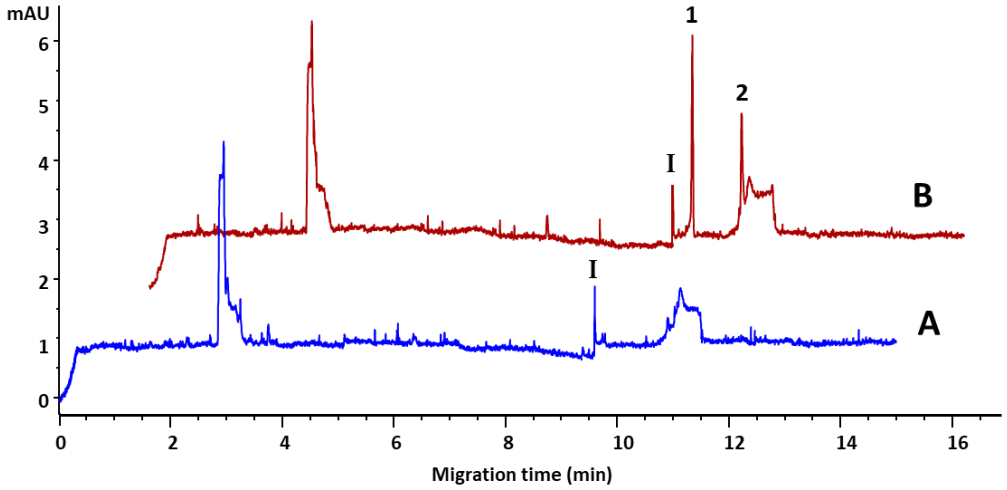

Figure 6. A) Blank egg sample. B) Egg sample spiked with fipronil at 0.8 mg/kg and fipronil-sulfone at 2.2 mg/kg, both submitted to the optimized SALLE extraction procedure and MEKC analysis. Peaks: I: interferent at fipronil-sulfide migration time, 1: fipronil, 2: fipronil-sulfone.

The coefficients of determination (R^2) for both analytes were 0.993 for fipronil and 0.991 for fipronil-sulfone, indicating that a good linearity was obtained over the studied concentration range. The LODs and LOQs were calculated as the minimum analyte concentration yielding 3 and 10 times the signal-to-noise (S/N) ratio, respectively. LODs were 90 µg/kg for fipronil and 150 µg/kg for fipronil-sulfone while LOQs were 300 µg/kg for fipronil and 500 µg/kg for fipronil-sulfone.

3.3.2. Precision studies

Precision was studied in terms of repeatability (intra-day) and intermediate precision (inter-day) by the application of the proposed MEKC-UV methodology to hen egg samples spiked at two concentration levels: 800 μg/kg and 2200 μg/kg for fipronil and 2200 μg/kg and 3500 μg/kg for fipronil-sulfone. Repeatability was evaluated by extracting two spiked egg samples at each studied concentration levels and the obtained extracts were then injected in triplicate (n=6) on the same day, under the same experimental conditions. Similarly, intermediate precision was assayed by extracting two spiked egg samples per concentration level for three consecutive days. Each extract was injected in triplicate (n=18). The results of both intra and inter-day precision, which were expressed as the relative standard deviations (% RSD) of the migration time-corrected peak areas, are shown in **Table 1**. Acceptable values, lower than 5% for intra-day and 13.4% for inter-day precision, were obtained in all cases.

3.3.3. Recovery study

For recovery studies, samples were spiked at two concentration levels previously used for precision study. Each concentration level was extracted in duplicate, and each extract was injected in triplicate (n=6). Blank samples were analyzed with the proposed method and no analytes were detected. Recoveries were calculated by comparing the migration time-corrected peak areas of the egg samples spiked before the sample treatment with those spiked after the sample treatment. As **Table 1** shows, recoveries were higher than 80% with RSD lower than 10% for both analytes and both level concentrations.

Table 1. Precision and recovery studies of the SALLE-MEKC-UV method for spiked egg samples. The two concentration levels established (L1 and L2) were set to 800 μg/kg and 2200 μg/kg for fipronil and 2200 μg/kg and 3500 μg/kg for fipronil-sulfone.

			Fipronil	Fipronil-sulfone
Repeatability (n=6)	**L1**	**RSD (%)**	4.9	3.9
	L2	**RSD (%)**	2.6	4.4
Intermediate precisión (n=18)	**L1**	**RSD (%)**	10.7	6.1
	L2	**RSD (%)**	13.3	6.6
Recoveries (n=6)	**L1**	**Recoveries (%)**	83.3	87.2
		RSD (%)	9.3	3.6
	L2	**Recoveries (%)**	86.2	84.0
		RSD (%)	6.7	3.8

4. Concluding remarks

In the present study, we have proposed, to the best of our knowledge, the first methodology for the separation of fipronil and two metabolites, fipronil-sulfide and fipronil-sulfone by using CE coupled with diode array spectrophotometry detection. Among the different separation modes tested, just MEKC provided satisfactory results. In addition, injection solvent was found to have a significant effect on peak resolution. This inexpensive and fast electrophoretic method was combined with a low-cost and simple sample treatment based on SALLE, which makes the proposed analytical method ideal for routine laboratories. The sensitivity achieved might be enough for some real cases, although lower limits of detection would be desirable for regulatory purposes. This drawback could be solved using mass spectrometry as the developed method is fully compatible with this detection. Moreover, the selectivity achieved by a mass spectrometer could allow the quantitation of fipronil sulfide in egg samples.

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CHAPTER 2

SIMPLE AND EFFICIENT METHOD FOR THE DETERMINATION OF FIPRONIL AND TWO MAIN METABOLITES IN EGGS BY CAPILLARY LIQUID CHROMATOGRAPHY

This work was published as:

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1. Summary

The miniaturized technique capillary liquid chromatography (CLC) coupled to UV-diode array detection has been proposed for the first time to determine fipronil and two metabolites, fipronil-sulfide and fipronil-sulfone in hen egg samples. Under optimum conditions, analytes eluted in less than 12 minutes. Despite the complexity of egg samples, a simple and fast sample preparation method based on salting-out assisted liquid-liquid extraction (SALLE) was developed using acetonitrile as extraction solvent and ammonium sulfate as salting out reagent. To obtain satisfactory extraction efficiencies for the target compounds, several parameters affecting the procedure were optimized, including the nature and amount of the extraction solvent and the type and amount of salting-out reagent, among others. Validation parameters of the proposed SALLE-CLC-UV method yielded satisfactory results with repeatability and intermediate precision, expressed as relative standard deviation (RSD), below 9 and 11%, respectively and recoveries above 80%. Good linearity was obtained (R^2>0.990) with LODs below 5 μg/kg.

The benefits of miniaturized techniques such as CLC come from the smaller column inner diameter and lower flow rate they employ. This results in less consumption of organic solvent in the mobile phase, making the analysis more eco-friendly and lowering the economic cost. Similarly, less sample consumption is required, making it suitable when the amount of sample is limited, such as in natural products or medical related analysis. In addition, low flow rates may increase sensitivity due to the reduction of chromatography dilution derived from smaller columns, and due to the increase in ionization efficiency in electrospray ionization (ESI)-MS.

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However, certain considerations must be taken when using these techniques, such as the use of appropriate instrumentation including pumps, tubing etc. Besides, the small diameter of the columns used in CLC can make them susceptible to clogging, leading to decreased performance or even column failure, which usually shorten the lifetime of the CLC columns.

2. Experimental

2.1. Material and reagents

Analytical standards of fipronil, fipronil-sulfide, and fipronil-sulfone (≥99%) were supplied by Sigma–Aldrich (Darmstadt, Germany) and were used without further purification. Individual standard stock solutions (1000 mg/L) were dissolved in methanol (MeOH). The standards were accurately weighed and dissolved in 4-mL amber glass vial and stored in the dark under refrigeration at –20 °C until further use. When necessary, sonication was applied to ensure the complete dissolution of the analytes. The intermediate stock solutions (20 mg/L) were obtained by dilution of the standard stock solutions with MeOH.

All reagents used in this study were of analytical grade and the solvents were of HPLC grade. Acetonitrile (MeCN), MeOH and ethyl acetate were obtained from VWR (Center Valley, PA, USA). Trifluoroacetic acid (TFA) was obtained from Merck (Darmstadt, Germany). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Salts used in the SALLE procedure for aqueous and organic-phase partitions were acquired from different suppliers: ammonium sulfate ($(NH_4)_2SO_4$, >99.5%) was obtained from VWR (Center Valley, PA, USA), sodium chloride (NaCl, >99%), anhydrous magnesium sulfate ($MgSO_4$, >96%), anhydrous sodium sulfate (Na_2SO_4, >99%) and anhydrous calcium chloride ($CaCl_2$, >96%) were obtained from Panreac (Barcelona, Spain), potassium chloride (KCl, >99.5%) and ammonium chloride (NH_4Cl, >99%) were obtained from Merck (Darmstadt, Germany).

Polytetrafluoroethylene (PTFE) hydrophilic syringe filters (0.2 μm × 13 mm) (VWR, Center Valley, PA, USA) were used to filter the sample extracts before injection into the capillary LC system.

2.2. Instrumentation

Chromatographic separations were carried out on a 1200 Series Capillary LC System from Agilent Technologies (Waldbronn, Germany) that consisted of an online degasser, capillary pump (maximum flow rate 20 μL/min), 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 Luna C18 (150 × 0.3 mm, 5 μm particle size) capillary column acquired from Phenomenex (Torrance, CA, USA) was employed.

A Vortex-Genie 2 (Scientific Industries, Bohemia, NY, USA), an ultrasonic bath (USS-300 model; VWR, West Chester, PA, USA), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA), a nitrogen dryer (System EVA-EC from VLM GmbH, Bielfeld, Germany) and a centrifuge (Universal 320 model from Hettich, Leipzig, Germany) were also used.

2.3. Chromatographic conditions

Chromatographic separation was performed at 50 °C on a Luna C18 capillary analytical column (150 x 0.3 mm, 5 μm), using a mobile phase consisted of water as eluent A and a mixture of MeOH:MeCN (3:1, v/v) as eluent B and supplied at a flow rate of 7 μL/min. Separation of fipronil and metabolites was carried out employing isocratic elution with a 70% (v/v) of eluent B and water. Afterwards, mobile phase composition was set at 100% of eluent B to clean the column and then it was back to 70% of eluent B and maintained to guarantee column equilibration and achieve a reproducible and stable separation.

Injection volume was set at 8 µL (full loop injection) and injection solvent was 45% aqueous MeOH. Wavelength was monitored at 280 nm, as it was a maximum of absorption (**Figure 1**).

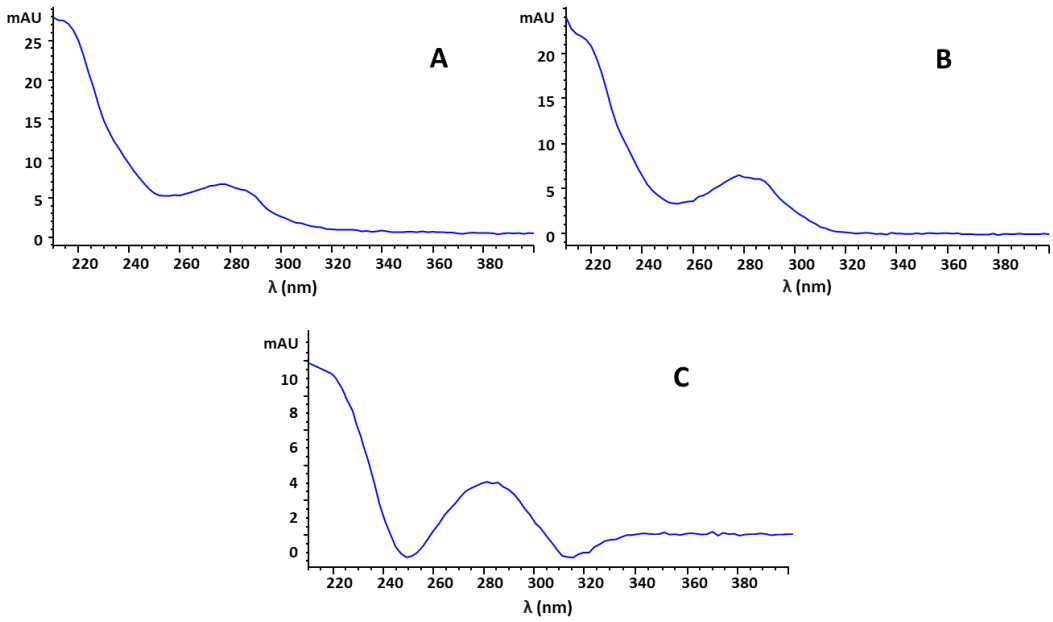

Figure 1. UV absorption spectrum of A) Fipronil; B) Fipronil-sulfide; C) Fipronil-sulfone.

2.4. SALLE procedure

Organic hen eggs were purchased at the local supermarket. Eggs were cracked and mixed until homogenization. Initially, 1 g of homogenize egg was placed in a 15-mL conical tube and stirred with 1.5 mL of water in order to dilute and increase the sample volume. Afterwards, 4 mL of MeCN were added for both protein precipitation and analytes extraction. The mixture was stirred in a mechanical shaker for 3 min followed by centrifugation at 9000 rpm and 4 ˚C for 10 min to separate the egg precipitated proteins from the liquid supernatant. Then, the supernatant was transferred to a 15-mL conical tube and 1 g of $(NH_4)_2SO_4$ was added to induce the separation of the MeCN organic phase from the aqueous portion by salting-out effect. The mixture was vortexed 1 min and centrifuged at 9000 rpm and 4 ˚C for 5 min. Two layers were perfectly

distinguishable in the tube: a clear aqueous phase in the bottom and a yellowish organic phase on the top. Then, the upper organic phase was collected with a syringe, transferred to a 4-mL glass vial and dried under a nitrogen stream at 30 ˚C. The residue was re-dissolved in 500 μL of 45% aqueous MeOH using vortex agitation. Finally, 8 μL of the final extract were injected into the CLC system previous filtration through a 0.2 μm PTFE filter.

3. Results and discussion

3.1. Optimization of chromatographic separation

Initially, separation of fipronil, fipronil-sulfide and fipronil-sulfone was tested in two capillary columns: a Zorbax XDB-C18 and a Luna C18 using MeOH (eluent B) and H_2O (eluent A) as mobile phase at a flow rate of 10 μL/min and 40 ˚C. Gradient program was established as follows: mobile phase composition was kept at 45% (v/v) of eluent B from 0 to 1 min, increasing MeOH content to 70% (v/v) until 30 min. This MeOH concentration was kept for 1 min and after that the initial conditions were re-established by a 1 min linear gradient and then maintained for 10 min. An injection volume of 8 μL (full loop) was considered and 45% aqueous MeOH was initially selected as injection solvent. Standard solutions (100 μg/L of each analyte) were analyzed under these conditions and analytical signals were monitored at 280 nm. Preliminary studies showed similar results in terms of retention time (around 30 min) as both columns were reversed phase capillary columns with the same length (150 mm) and particle size (5 μm). However, peak shape and resolution were slightly preferable for Luna column, thus it was selected for further optimization of chromatographic variables.

In order to achieve an optimum separation, parameters such as the influence of the mobile phase composition, gradient program, mobile phase flow rate, sample injection volume, column temperature and injection solvent were studied.

First of all, the effect on the separation of different organic solvents in the mobile phase (eluent B), such as MeOH, MeCN, and a mixture of both at different ratios were evaluated. When MeCN was used as eluent B only two peaks appeared because fipronil-sulfide and fipronil-sulfone coeluted. When MeOH was employed, the chromatogram showed the three target compounds baseline resolved. An improvement in sensitivity and peak shape was observed when using a mixture of MeOH:MeCN, so different ratios of both solvents were tested, being a mixture of MeOH:MeCN (3:1, v/v) selected as the optimum composition of organic mobile phase (**Figure 2**). The aqueous phase composition (eluent A) was water without additives, as the analytes are very nonpolar compounds and they do not ionize easily. Thus, the final composition consisted of water as eluent A and MeOH:MeCN (3:1, v/v) as eluent B.

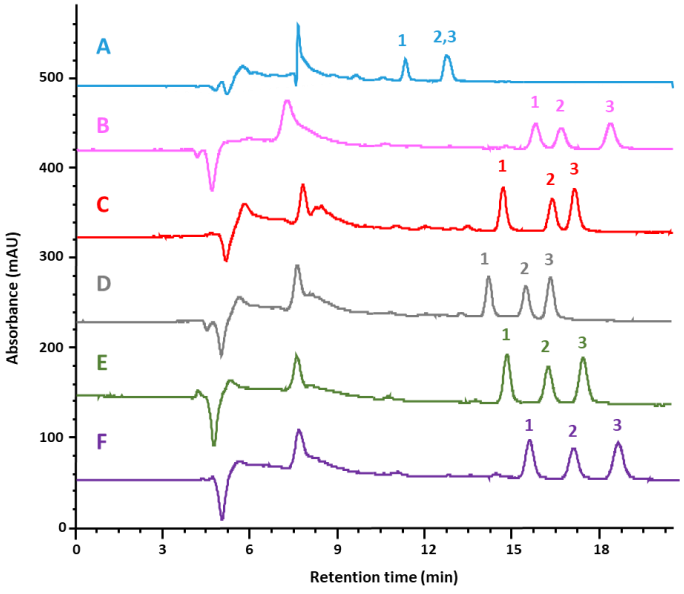

Figure 2. Influence of eluent B composition on the analyte separation and peak resolutions. A) MeCN; B) MeOH; C) MeOH:MeCN 1:1; D) MeOH:MeCN 2:1; E) MeOH:MeCN 3:1; F) MeOH:MeCN 4:1. Peaks: 1: Fipronil; 2: Fipronil-sulfide; 3: Fipronil-sulfone (100 µg/L of each analyte).

Then, the effect of column temperature was evaluated from 40 to 55 ˚C. It was observed that higher temperatures reduced the analysis time because the analytes solubility in the mobile phase increased, but the resolution between peaks got worse (**Figure 3**). A temperature of 50 ˚C was selected since the three peaks were baseline resolved in 18 min.

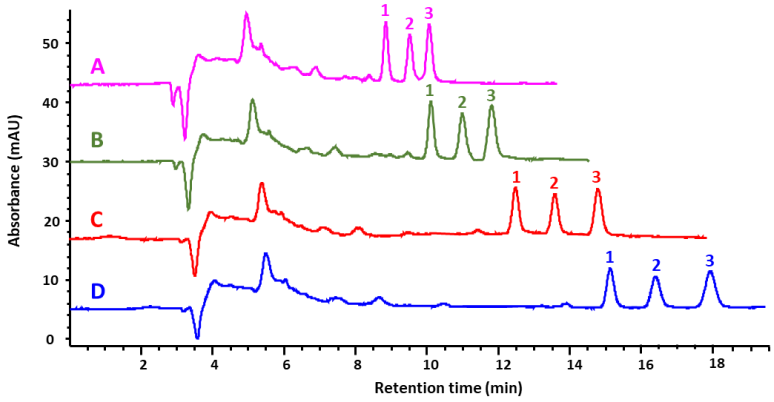

Figure 3. Influence of temperature on the analyte separation and peak resolutions. A) 55 ˚C; B) 50 ˚C; C) 45 ˚C; D) 40 ˚C. Peaks: 1: Fipronil, 2: Fipronil-sulfide, 3: Fipronil-sulfone (100 µg/L of each analyte).

Injection volume was then studied between 1 and 8 µL. As expected, the higher injection volume the higher intensity and peak area, so 8 µL was maintained as optimum value as it did not result in any decrease in efficiency or resolution. Flow rate was evaluated from 4 to 12 µL/min and a flow rate of 7 µL/min was established as optimum because higher flow rates involved a decrease in peak resolution between fipronil-sulfide and fipronil-sulfone. Considering the optimized separation parameters, mobile phase composition through the separation was re-optimized to shorten the analyte elution time. An isocratic elution was established: content of eluent B was set to 70% (v/v) and this composition was maintained for 13 min until the three analytes eluted. After that, the content of B was increased to 100% in 1 min and maintained for 10 min to ensure the cleaning of the column when working with egg samples. Then, mobile composition of B was changed in 1 min for reaching initial conditions and they were stated for a period of

20 min for ensuring column equilibration, obtaining a stable and reproducible separation. After that, analyte elution time was reduced to 12 min. Finally, different injection media were evaluated including MeOH, MeCN and its mixtures with H_2O at different ratios, and MeOH:MeCN (3:1, v/v). When using 100% organic solvent as injection medium no peaks appeared. Finally, 45% of aqueous MeOH was selected as the optimum injection solvent composition. In conclusion, under optimum conditions the three analytes were fully separated in 12 min (**Figure 4**).

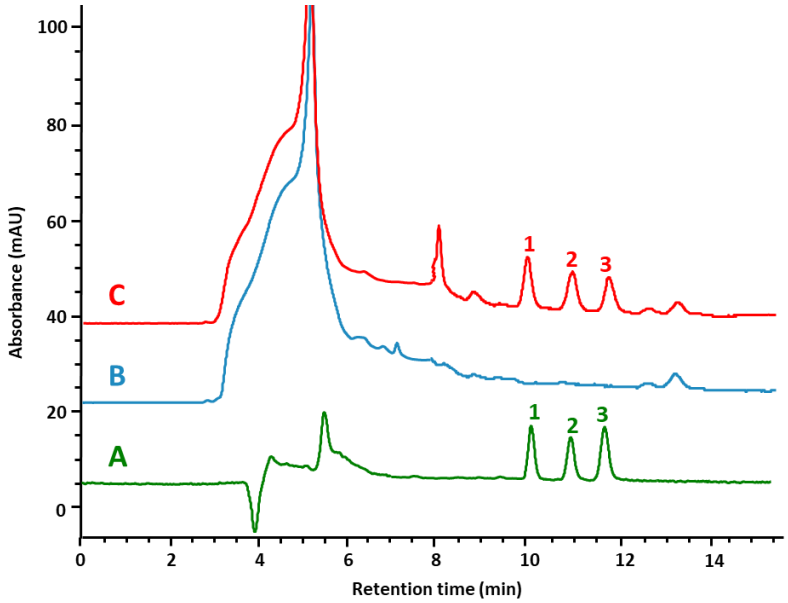

Figure 4. Chromatograms of: A) a standard mixture obtained with the optimized separation conditions (100 µg/L of each analyte); B) Blank egg sample submitted to the optimized SALLE-CLC-UV method; C) Egg sample spiked at 80 µg/kg of each analyte and submitted to the optimized SALLE-CLC-UV method. Peaks: 1: Fipronil; 2: Fipronil-sulfide; 3: Fipronil-sulfone.

3.2. Optimization of the sample treatment

The applicability of the proposed CLC-UV method was evaluated for the analysis of fipronil, fipronil-sulfone and fipronil-sulfide in hen egg samples. SALLE was chosen as sample treatment as it is a cheap, relatively fast and simple process that involves low

organic solvent volumes, making it a suitable extraction procedure for routine laboratories.

In **Chapter 1** it is detailed the optimization of a SALLE procedure for the determination of fipronil and fipronil-sulfone in egg samples and their subsequent analysis by capillary electrophoresis (CE). In the present **Chapter 2**, the analyzed samples (egg) and the proposed sample treatment (SALLE) are the same as in **Chapter 1**. However, in this case, it was possible to perform also the determination and quantification of fipronil-sulfide. It was supposed that fipronil-sulfide behavior should be similar to that of the parent compound and the sulfone-derivate, however, extraction conditions have been also checked in this chapter to select the optimum values considering the three analytes.

Therefore, to start the study, 1 g of homogenized hen egg sample spiked at 100 μg/kg was placed in a 15 mL-centrifuge tube and MeCN was used as extraction solvent as in **Chapter 1**. The influence on the extraction efficiency of the MeCN volume, salt nature, amount of salt, and vortex agitation time were checked by means of recovery studies taking into account fipronil-sulfide. Recoveries were calculated by comparing the peak area of the analytes in two samples, spiked before and after the sample treatment, respectively. All the experiments were carried out with two experimental replicates and analyzed in duplicate (n=4).

3.2.1. Volume of the extraction solvent

The volume of MeCN was checked in the range of 2 to 5 mL. As it is shown in **Figure 5A**, the influence of the volume of MeCN is crucial, as the average recoveries were lower than 50 % for the three analytes when 2 mL were used, and they increased for the above 80% when 4 mL of MeCN were employed. The extraction efficiency did not improve above this value, so 4 mL was maintained as optimum.

3.2.2. Nature of extraction medium

Afterwards, the use of an acidic medium that facilitates or increases egg protein precipitation was evaluated comparing the addition of 1.5 mL of TFA (0.01 M and 0.02 M) with water. It was observed that the presence of TFA did not improve the extraction efficiency; in fact, recoveries were slightly lower for fipronil metabolites in comparison with those obtained with MeCN (**Figure 5B**). Nevertheless, MeCN has the ability itself to induce protein precipitation as well as analyte extraction, so an acidic medium was not required. In this sense, a volume of 1.5 mL of water was maintained to dilute the sample and facilitate its mixture with MeCN.

3.2.3. Nature and amount of salt

Next, 0.5 g of three different chloride salts ($CaCl_2$, KCl, and NH_4Cl) and one sulfate salt (Na_2SO_4) were tested in addition to the salts studies in the SALLE development of **Chapter 1** (NaCl, $MgSO_4$ and $(NH_4)_2SO_4$). With Na_2SO_4, phase separation was not achieved. When NaCl and KCl were used, the extraction efficiencies were very low so they were discarded. In **Figure 5C** the extraction efficiency expressed as recovery (%) was represented for $CaCl_2$, NH_4Cl, $MgSO_4$ and $(NH_4)_2SO_4$. The best results in terms of reproducibility and recoveries were obtained again when $(NH_4)_2SO_4$ was employed, so it was maintained as optimum salting-out reagents. Subsequently, the amount of salt was studied in the range between 0.5 and 2 g. No significant differences were observed so 1 g was selected as slightly better results were obtained (**Figure 5D**).

3.2.4. Mechanical stirring time

Finally, the agitation time was studied in the range of 2-4 min. No significant differences were observed between 3 and 4 min, so 3 min was selected as vortex agitation time.

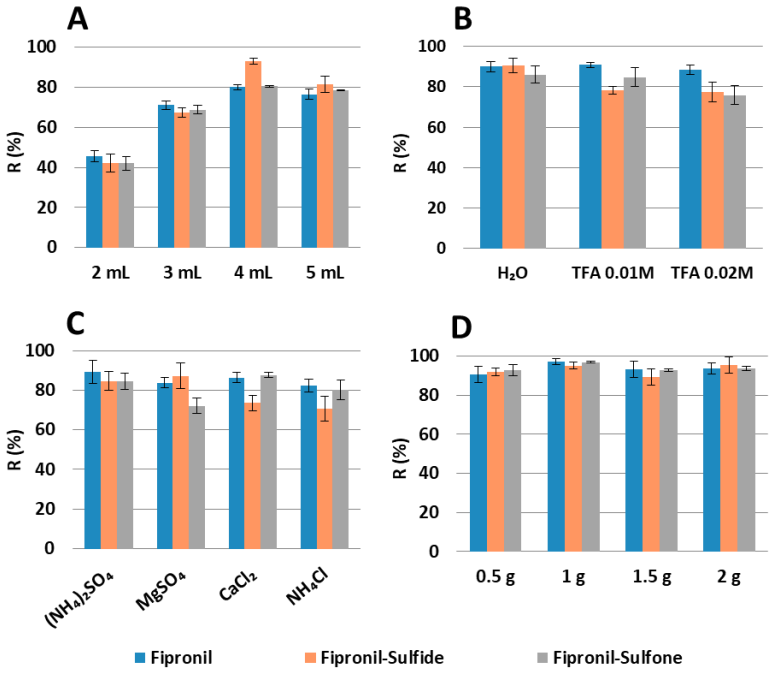

Figure 5. Influence on the extraction efficiency of some parameters of the sample treatment. A) Volume of extraction solvent; B) Nature of the extraction medium; C) Nature of the salt; D) Amount of salt. Error bars represent the standard error (n=4).

3.3. Method characterization

In order to test the applicability of the method for the determination of fipronil, fipronil-sulfide and fipronil-sulfone, hen egg samples were treated by the optimized SALLE procedure and analyzed by CLC-UV. The proposed method was evaluated in terms of linearity, LODs, LOQs, repeatability, intermediate precision and recovery.

3.3.1. Calibration curves and analytical performance characteristics

Procedural calibration curves were established in blank hen egg samples and spiked at six different concentration levels: 20, 40, 60, 80, 100 and 120 μg/kg for each analyte. Two spiked hen egg samples per level were treated following the SALLE procedure and each sample was analyzed in duplicate (n=4) according to the proposed CLC-UV method. A

blank hen egg sample was analyzed as described before and no matrix interferences were found as no interfering peaks were coeluting with the analytes. Peak area of each compound was considered as the analytical response. The determination coefficients (R^2) were higher than 0.990 in all cases, indicating that a good linearity was obtained over the studied concentration range. LODs and LOQs of the method were calculated as the minimum analyte concentration corresponding with a signal–to–noise ratio equal to three and ten, respectively. LODs were 4.2, 4.7 and 4.9 μg/kg while LOQs were 14.0, 15.6 and 16.3 μg/kg for fipronil, fipronil-sulfide and fipronil-sulfone, respectively.

3.3.2. Precision studies

Precision studies were carried out in order to evaluate the repeatability (intra-day precision) and the intermediate precision (inter-day precision) of the proposed SALLE-CLC-UV method. Repeatability was evaluated over two concentration levels (20 and 80 μg/kg) by extracting two spiked egg samples per level and injecting in triplicate (n=6). Similarly, intermediate precision was assayed at two concentration levels (20 and 80 μg/kg) by extracting one spiked egg samples in triplicate per level and per day for three consecutive days (n=9). Results expressed as peak area RSDs (%) are shown in **Table 1**. In all cases RSDs were lower than 9% and 11% for repeatability and intermediate precision, respectively.

3.3.3. Recovery study

Recovery assays were accomplished in order to demonstrate the efficiency of the extraction procedure. Two egg samples were spiked at two concentration levels (20 and 80 μg/kg). Samples were treated by the whole extraction procedure and analyzed in triplicate by the proposed CLC-UV method (n=6). Moreover, a blank of egg sample was analyzed to ensure no matrix interferences were present. Recoveries were obtained by

comparing the signal of the egg samples spiked before the sample treatment with the signal from a blank sample spiked after the sample treatment and prior to the analysis. As can be observed in **Table 1**, recoveries (R) higher than 80% were obtained for the three analytes, demonstrating the convenience of using the proposed SALLE procedure as cheap and easy sample treatment for fipronil, fipronil-sulfide and fipronil-sulfone extraction from egg samples in combination with CLC-UV method.

Table 1. Precision and recovery studies of the SALLE-CLC-UV method for spiked egg samples. The two concentration levels established were set to 20 μg/kg (L1) and 80 μg/kg (L2) for the three analytes.

			Fipronil	Fipronil-sulfide	Fipronil-sulfone
Repeatability (n=6)	**L1**	**RSD (%)**	8.7	7.2	8.5
	L2	**RSD (%)**	1.9	1.4	1.2
Intermediate precision (n=9)	**L1**	**RSD (%)**	7.5	10.1	7.6
	L2	**RSD (%)**	2.4	2.7	2.5
Recoveries (n=6)	**L1**	**Recoveries (%)**	85.6	80.5	79.2
		RSD (%)	8.6	16.0	6.4
	L2	**Recoveries (%)**	84.5	77.5	83.2
		RSD (%)	3.4	2.1	4.1

In comparison of previously reported LC-based methods using UV detection (see

Table 2), the proposed CLC-UV method achieves LODs at the level of the MRL proposed by the EU legislation and LOQs comparable or even lower to those obtained for other LC-UV methods which determine only fipronil in egg or honey samples with more complex sample treatments. In contrast with methods based on HPLC-MS/MS or GC-MS, which use QuEChERS or SPE pretreatment [1], the proposed method is simpler, low cost, faster and greener, even if LODs inherent of UV detection are slightly higher in comparison to those obtained with MS detection.

Table 2. Comparison of reported LC-based methods using UV detection and the proposed CLC-UV method for the analysis of fipronil and metabolites in food samples.

Sample	Analytes	Extraction	Technique	LOQ (μg/kg)	R (%)	Ref.
Honey	Fipronil	DLLME and QuEChERS	LC-UV	30 (DLLME) 600 (QuEChERS)	70.7 – 101.1 (DLLME) 76.3 – 87.7 (QuEChERS)	[2]
Cabbage	Fipronil	LPE - SPE	LC-UV	50	78.8 – 90.9	[3]
Egg	Fipronil	ISPE-IL-TGO	LC-UV	15.9	83 – 97.7	[4]
Egg	Fipronil, fipronil-sulfide and fipronil-sulfone	SALLE	CLC-UV	14-16.3	79.5 – 85.6	This article

4. Concluding remarks

In the present work, CLC has been applied for the first time to determine fipronil and two of its main metabolites, fipronil-sulfide and fipronil-sulfone in egg samples. As a miniaturized technique, CLC involves lower sample and hazardous organic solvent consumption compared to conventional HPLC, which supposes a great advantage as it reduces the total cost and waste generation. A rapid and easy sample treatment based on SALLE without further clean-up steps has been developed to extract the analytes from egg samples. The proposed SALLE-CLC-UV method has been characterized for the three analytes, which were baseline separated in less than 12 min, with LODs at the level of the MRL proposed by the EU legislation and LOQs comparable or even lower to those obtained for other LC-UV methods which determine only fipronil in egg or honey samples with more complex sample treatments. Satisfactory validation parameters, such as precision, recoveries and linearity demonstrate that the method could be applied in

routine analysis laboratories, mainly with screening purposes of fipronil and metabolites. Therefore, it is a feasible alternative to traditional HPLC methods for quantification of these residues in eggs, or as a screening method before MS/MS detection.

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PART II

Determination of cyanotoxins

Introduction

Chapter 3.

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INTRODUCTION

DETERMINATION OF CYANOTOXINS

1. Cyanobacteria

1.1 General aspects

Cyanobacteria are a structurally and morphologically diverse group of oxygenic photosynthetic prokaryotes that convert carbon dioxide into biomass using sunlight as an energy source. It is believed to be one of the oldest life forms on Earth. They appeared around 3 billion years ago [1], and their photosynthetic action initiated one of the most decisive events in the evolution of the Planet Earth which has led to life as it is known today: the change from a primitive reducing atmosphere to an oxidizing one [2]. Cyanobacteria are also known as blue-green algae (BGA), however they are not algae, as algae only refers to photosynthetic eukaryotes. Besides, many cyanobacteria are not blue-green because the distinctive cyan tone of cyanobacteria pigment phycocyanin is often concealed by the abundant green chlorophyll and by other pigments, leading to a wide variety of colors [3].

Cyanobacteria are widespread all over the world since they can tolerate and even thrive under extreme conditions such as arid and dry environments, high UV radiation, low light, lack of nutrients and high salt concentrations [4]. Indeed, they have been found in the Antarctic or in habitats with temperatures up to 70 °C [5]. As a result, cyanobacteria are ubiquitous not only in many aquatic ecosystems such as lakes, ponds, rivers, brackish waters, marine environments and hydrothermal vents, but also in deserts, bare rocks, rain forests, subsurface soils, etc. These microscopic microorganisms exist as individual cells or filaments, however they can be visible when forming of colonies, such as crusts

or blooms. Most cyanobacteria are an innocuous source of natural products with applications in several fields and industries [6]. However, an important proportion of cyanobacteria are known to be harmful producers of cyanotoxins, which are toxic secondary metabolites that can impact on ecosystem, animal and human health [7,8].

1.2 Beneficial health effects and potential of cyanobacteria in industry

The intake of cyanobacteria dates back to Aztec civilization, where they were employed as an endurance-booster [9]. However, it was in early 1950s when a large-scale production of cyanobacteria and microalgae was proposed [10]. Cyanobacteria present many distinct features that have fostered their use on an industrial scale. For instance, they can grow faster and have higher harvest potential compared with traditional food crops; they do not compete with resources used for traditional land-based feedstock or food because they can be cultivated on non-productive areas such as aquatic environments, thus providing the extra benefit of entire-year cultivation; and finally, they can generate oxygen as a byproduct of their photosynthetic activity. The first works related with the potential use of BGA focused on their photosynthetic, therapeutic, antibiotic and toxicological properties, as well as in their use as agricultural product. At present, BGA production provides biomass for a wide range of applications [11].

1.2.1 BGA as dietary supplements

One of the areas in which the use of BGA is well established is in the nutraceutical field [12,13], where BGA based dietary supplements have become well known around the world because of health awareness increase and disease prevention. They are considered

a great source of protein, complex sugars, amino acids, active enzymes, pigments, essential fatty acids, vitamins and minerals [14]. Moreover, BGA contain bioactive components which can be beneficial for improving blood lipid profiles and for preventing inflammation and oxidative stress [15], which makes them suitable as natural and sustainable high-value alternatives to medicinal products without any prescription. There are a large number of BGA dietary supplements available in the market in the form of pills, capsules, and powders, and most of them derive from two filamentous genera of cyanobacteria, which are *Arthrosphira*, including *Arthrospira platensis*, *A. fusiformis*, and *A. maxima*; and *Aphanizomenon flos-aquae*. In addition, several BGA dietary supplements also include the microalga *Chlorella pyrenoidosa* [16]. *Arthrosphira* (commonly known as Spirulina), is usually produced under cultured conditions in open tank systems. Because of its nutritious potential and health benefits, and because it can be easily harvested and processed, the food manufacturers use spirulina as the preferred cyanobacterial strain to prepare dietary supplements [17,18]. *Aphanizomenon flos-aquae* (also referred to as Klamath) is naturally present in freshwater sources and it is harvested in open environment lakes, being the most important the Upper Klamath Lake in Oregon (USA). The green microalgae *Chlorella* is harvested in artificial ponds, so contamination with other toxin producer organisms is not common, however, in the final product it is frequently mixed with other BGA increasing the risk of toxin contamination.

1.2.2 Other industrial applications

One of the promising applications of BGA is as a source of energy. The traditional use of fossil fuels as an energy resource has caused pollution concerns through the whole

society. In this sense, new ways to explore natural environment-friendly energy resources are being seeking, and biorefinery, including production of biofuels and bio-based chemicals, is one of them. Cyanobacteria have caught attention as an alternative carbon source to produce biofuels due to their few growth requirements with high grow rates, and high photosynthesis efficiency [19,20]. Thus, a vast commercial scale mass cultivation of BGA could be possible in the near future to develop the production of biofuels as well as other valuable co-products.

Cyanobacteria can also be used as a natural biofertilizers in a variety of agricultural conditions. The current agricultural practices, and the excessive and widespread use of chemical fertilizers, contribute to greenhouse gas emission, soil degradation and eutrophication [21]. In this sense, BGA research has gained interest in recent decades as a sustainable agricultural-production enhancer which can perform comparably to traditional fertilizers [22,23]. It has been observed that BGA biomass reduce soil salinity and soil exhaustion, and can also reduce weed growth and decomposition [24].

Furthermore, cyanobacteria have the potential to be used for bioremediation of various contaminants such as heavy metals, crude oil, pesticides, aromatic pollutants and xenobiotics [25,26]. BGA have some advantages over other microorganisms due to their photoautotrophic activity, i.e., the use of light energy and inorganic carbon to produce organic materials, their ability to fix atmospheric N_2 and their well extreme-environment adaptation, which make them self-sufficient in strongly polluted environments [23].

Cyanobacteria generate a large number of biomolecules that are used as dyeing pigments in food and cosmetic industry and as fluorescent substances in clinical or research centers [27]. Likewise, cyanobacteria secondary metabolites are bioactive

substances that can be employed in cosmetic and medicinal field through biotechnological applications as it has been stated to possess antifungal, antimicrobial, anti-inflammatory, and anticancer properties among others [28,29,30]. In addition, other co-products extracted from BGA can be used in a wide variety of applications including biopolymers and biodegradable plastics [31].

1.3 Cyanobacterial blooms

Cyanobacteria have developed cellular mechanisms that allow them to grow rapidly and adapt easily to environmental changes, facilitating the production of dense populations. Under certain combination of environmental conditions and physicochemical factors, a phenomenon known as Harmful Algal Blooms (HABs) occurs [32,33]. HABs are the rapid and excessive growth of algae or cyanobacteria forming dense blooms that can cause negative impact to living organisms and local ecology. The HBAs are particularly hazardous when they are formed by toxin-producing cyanobacteria [34]. In first instance, cyanobacteria blooms can adversely affect water quality [35]. When HABs appear, water turbidity increases, and submerged vegetation is smothered by the high density of BGA which prevents the light from reaching them. Not being able to photosynthesize, submerged vegetation dies and is decomposed by microbes which, in doing so, consume the oxygen in the water. Oxygen depletion by the microbial degradation of senescent blooms generate hypoxia and anoxia that can be deadly for fish and some invertebrates [36]. This trend is of great concern, as it generates negative impact on the biodiversity and activity of aquatic food webs. Moreover, it threatens the use of waters for drinking,

bathing, fishing and other recreational purposes due to the production of odor, taste and toxic compounds.

Although determining the causal factors for HAB events are complex [37], it is known that pH, light, CO_2 enrichment or salinity influence the occurrence, frequency, intensity and duration of cyanobacterial blooms across the globe [32]. Among all these elements, it has been observed that changes in climate and anthropogenic nutrient inputs are the most significant ones contributing global eutrophication and expanding HABs [38,39,40,41].

1.3.1 Nutrients

Among all the potential environmental factors promoting cyanobacterial blooms, the anthropogenic nutrient pollution has been the one receiving most attention. Studies show that the increase of certain anthropogenic activities, such as intensive agriculture, have intensified the inputs of nitrogen and phosphorus into the aquatic ecosystems [42]. The excessive increase of these nutrients, which are essential for cyanobacteria growth, is called eutrophication, and it can affect estuaries, rivers, lakes and reservoirs, promoting HABs [43], a worldwide environmental issue that has been recognized since the 1960s [44].

1.3.2 Global warming

The use of fossil fuels and the resulting increase in the amount of CO_2 in the atmosphere has caused the earth's surface temperature to rise by about one degree in the 20^{th} century,

most markedly in the last 40 years [45]. It has been observed that the communities of aquatic microorganisms, including cyanobacteria and other phytoplankton, are strongly influenced by the increase in temperature, resulting in a competitive advantage for cyanobacteria [46,47,48]. On the one hand, the growth rates of many of them are intimately, but differentially, linked to temperature. As temperatures exceed 20 °C, the growth rates of freshwater eukaryotic phytoplankton usually become stable or decline while several cyanobacteria strains reach their maximum. In addition to direct effects, temperature also modifies physical characteristics of aquatic environments in such a way that may benefit cyanobacteria dominance. One of these indirect temperature effects is the reduction of water density and viscosity, which favors buoyancy-regulating toxins and prevent sedimentation in stratified waters, i.e., waters where distinct layers occupy the vertical water column, while increase sinking loss of eukaryotic competitors [46,49,50]. As a result, BGA tend to be the most abundant phytoplankton in eutrophic freshwater environments during the warmest periods of the years. However, these bloom-enhancing effects vary among species as some taxa of cyanobacteria are more responsive to an increase in nutrients while other benefit more from an increase in temperature [51].

2. Cyanobacterial derived toxins: Cyanotoxins

Many genera of cyanobacteria are known to be harmful producers of cyanotoxins, a diverse array of toxic secondary metabolites that can impact on ecosystem, animal and human health [35,52,53,54,55,56,57]. Toxin production by BGA blooms fluctuates and it is difficult to predict from species composition and abundance as cyanotoxin contents

vary broadly among different strains of the same specie [58,59]. However, cyanobacterial blooms usually contain both toxic and non-toxic strains from the same species and therefore, changes in strain composition of the cyanobacterial bloom can lead to variations in the toxin composition [59]. In addition, some cyanotoxins can be produced by different types of cyanobacteria genera as well as one genus can produce different types of cyanotoxins.

2.1 Cyanotoxin classification

Cyanotoxins are a large group of compounds that differ in their structure and toxicological properties [52]. They can be classified into different groups according to their chemical structure and mechanisms of action, being the following the largest and most relevant ones.

2.1.1 Cyclic peptides

Cyclic peptides are water soluble and stable molecules which inhibit protein phosphatase causing mainly severe hepatotoxicity but also damage in organs such as heart, brain or reproductive system [55,56,60,61,62]. The most significant cyclic peptides are microcystins and nodularins.

Microcystins (MCs) are cyclic heptapeptides with various unusual amino acids, including the distinguishing 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4(E),6(E)-dienoic acid (ADDA). They form the major and the most studied family of cyanotoxins. As it can be observed in **Figure 1**, MCs are cyclic compounds enclosing 7

amino acids, being the ADDA often associated with the toxicity of the molecule [63,64]. While five amino acids remain almost always unchanged, the X and Z amino acids refer to variable protein amino acids residues in positions 2 and 4, where different combinations lead to more than 240 microcystins variants [65,66]. Thus, a universal nomenclature system based on the term microcystin-XZ was established [67]. For instance, the most relevant and studied microcystin, from which the regulatory guidelines for cyanotoxins in water have been established [68], contains a leucine (initial L) in position 2 and arginine (initial R) in position 4, thus being identified as MC-LR. Microcystins studied in this Thesis are shown in **Figure 3**. The name of the family comes from *Microcystis*, the first cyanobacteria genus related with their synthesis, although they are produced by several genera such as *Anabaena*, *Nostoc, Aphanizomenon* or *Planktothrix* (*Oscillatoria*) [61].

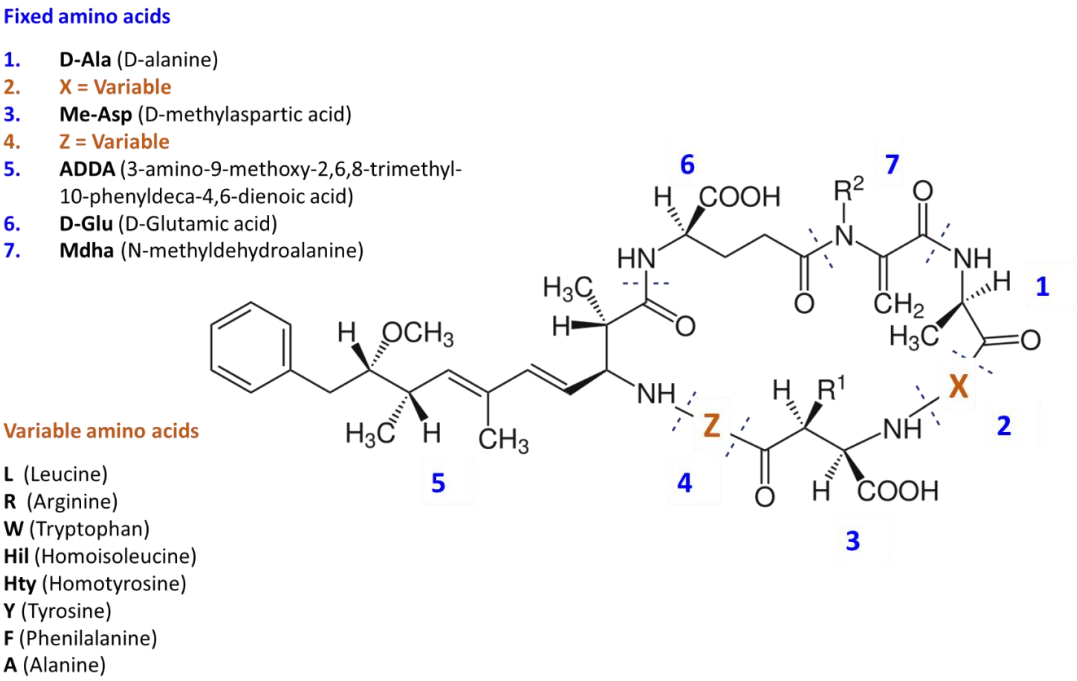


Figure 1. General structure of Microcystins.

Nodularins (NODs) are cyanotoxins only associated with *Nodularia spumigena* specie from *Nodularia* genus. NODs are cyclic pentapeptides structurally related to MCs, including the ADDA moiety but with only one variable amino acid. Currently, ten variants have been identified [69] and the one with arginine as the variable amino acid (NOD-R) is the most common one.

In addition to MCs and NODs, other common cyclic peptides derived from BGA found in the environment are anabaenopeptins (APs) [70]. They contain six amino acid residues and show phosphatase, carboxypeptidase and protease inhibitory activity. APs have been detected in a wide range of cyanobacteria genera, such as *Aphanizomenon*, *Anabaena*, *Nostoc*, *Microcystis*, *Planktothrix*, *Lyngbya*, and *Brasilonema*, in freshwater, marine and terrestrial environments [71]. Although they have been poorly explored, recent investigations suggested that they could be more abundant in freshwater than other more studied cyclic peptides such as the well-known MCs [72,73,74].

2.1.2 Alkaloids

Anatoxin-a (ANA), whose structure can be observed in **Figure 3**, and its structural variants, are water-soluble alkaloids associated with different genera of cyanobacteria such as *Anabaena*, *Aphanizomenon*, *Planktothrix* (*Oscillatoria*) etc. ANA shows rapid neurotoxicity in mammals and birds, and once in the organism, it produces paralysis by binding on acetylcholine receptors without being degraded by acetylcholinesterase [75]. Due to its acute toxicity, ANA has been involved in several animal poisonings, remarkably the death of dogs [76,77,78,79] and in some human poisoning [80].

Saxitoxins (SAXs) are water-soluble tricyclic guanidine alkaloids with many structural variants [81]. They are some of the most potent natural neurotoxins and they can be produced by several species of cyanobacteria such as A*nabaena, Aphanizomenon, Lyngbya, Cylindrospermopsis* or *Planktothrix*, and dinoflagellates [82,83]. Also known as paralytic shellfish poisons, SAXs act blocking the sodium ion channels in nerve axon membrane and preventing the transmission of action potentials, which lead to rapid paralysis [84].

Cylindrospermopsin (CYN) was named according to *Cylindrospermopsis raciborskii*, the first cyanobacteria specie related to its biosynthesis, although several other genera, such as *Aphanizomenon, Umezakia*, *Lyngbya*, *Raphidiopsis or Anabaena* are also producers [85]. CYN is a highly water-soluble tricyclic alkaloid enclosing a guanidine entity along with an uracil moiety, potentially responsible for its toxicity [86]. CYN mainly affects the liver through the irreversible inhibition of protein synthesis leading to cell death [87]. The most notable case of human intoxication by CYN occurred in 1979 in Australia and it is often referred to as the Palm Island mystery disease [88].

2.1.3 Cyanobacterial non-protein amino acids

The cyanotoxin non-proteinogenic amino acid β-N-methylamino-l-alanine (BMAA) is a small, hydrophilic and polar molecule naturally produced by a diverse number of aquatic cyanobacteria, marine diatoms and dinoflagellates [89,90]. It possesses neurotoxic activity affecting motor neurons by activation of glutamate receptors [91]. Moreover, BMAA could cause intraneural protein misfolding, which is the trigger of neurodegeneration [92]. Indeed, it has been associated with human neurodegenerative diseases, such as amyotrophic lateral sclerosis [92], Alzheimer's [93], and Parkinson's

diseases [94], however, the neurodegenerative disease relationship is still under investigation [95]. In the environment BMAA has been detected along with its structural isomers 2,4-diaminobutyric acid (2,4-DAB, mostly referred to as DAB) and N-(2-aminoethyl) glycine (AEG) [96,97] (chemical structures are shown in (**Figure 3**), and in some cases both isomers have been found more frequently and at higher concentrations than BMAA [98,99].

2.2 Routes of exposure to cyanotoxins

There are many routes by which humans can be exposed to cyanotoxins [100]. Among them, the major ones include chronic and accidental ingestion of contaminated drinking water; consumption of contaminated vegetables and fruits irrigated with contaminated water; consumption of contaminated aquatic organisms (fish, shellfish, etc.); consumption of cyanobacteria dietary supplements; dermal contact through recreational activities; and inhalation of aerosols generated by HBAs.

2.2.1 Drinking water

The direct consumption of contaminated drinking water affected by cyanotoxins resulting from HABs is a common and the easiest route of exposure of cyanotoxins. If the water is obtained from a surface water source when a HABs was occurring, it may contain toxins released during cyanobacteria cell decomposition. This is considered a global problem in drinking water resources because the ingestion of small quantities of contaminated water, or the prolonged chronic exposure to contaminated water, are linked with high level of risk to human health [53,100 ,101,102,103].

2.2.2 Food chain

When surface waters contaminated with cyanotoxins are used to irrigate or water the crops, they come into contact with the toxins, entering the food chain and affecting both the quality and quantity of the harvest and [104,105,106]. Moreover, cyanobacteria may be present in areas used for livestock grazing or may directly feed livestock [107,108], increasing the likelihood of bioaccumulation and human exposure. In addition, the bioaccumulation process can occur in a wide range of aquatic animals used for human consumption that have lived in contaminated environments [109,110,111].

2.2.3 Dietary supplements

Cyanobacteria based dietary supplements are widely sold in many countries due to their beneficial health effects [112]. Since these products are natural, it is assumed that they are safe, and therefore BGA may be taken at high doses and over a long period of time without medical supervision. In fact, the consumption of food supplements based on BGA biomass is considered safe and is approved by food regulatory bodies such as the European Food Safety Agency (EFSA) or the Food and Drug Administration (FDA) [113]. However, the increase in BGA derivate products consumption has an associated risk as many of the existing BGA species are cyanotoxin producers, which makes important to clarify the specific species used for human consumption. As stated previously, most of the algae dietary supplements derive from *Spirulina* and *Aphanizomenon flos-aquae*. To date, *Spirulina* is considered to be non-toxin producing, while it has indeed been observed that Klamath is able to produce toxins itself [114]. In addition, Klamath is generally harvested in natural lakes, where other toxin producing species coexist, which

may increase the probability of contamination of BGA dietary supplements. As a result, several studies have demonstrated the presence of cyanotoxins in *Aphanizomenon flos-aquae* based dietary supplements [16,114,115,116,117].

2.2.4 Dermal contact and inhalation

Cyanotoxin dermal contact occurs during various recreational activities, such as swimming o water sports, when the waters experience HABs. A wide range of symptoms associated with this recreational exposure to BGA have been observed, like desquamation, skin rashes or eye irritation [118]. Besides dermal contact, during intense HABs, harmful aerosols can be created dispersing algae and cyanotoxins by air, threating air quality and causing respiratory disorders through inhalation [119,120]. In **Figure 2** it can be observed the origin of toxic cyanobacterial blooms and human exposure.

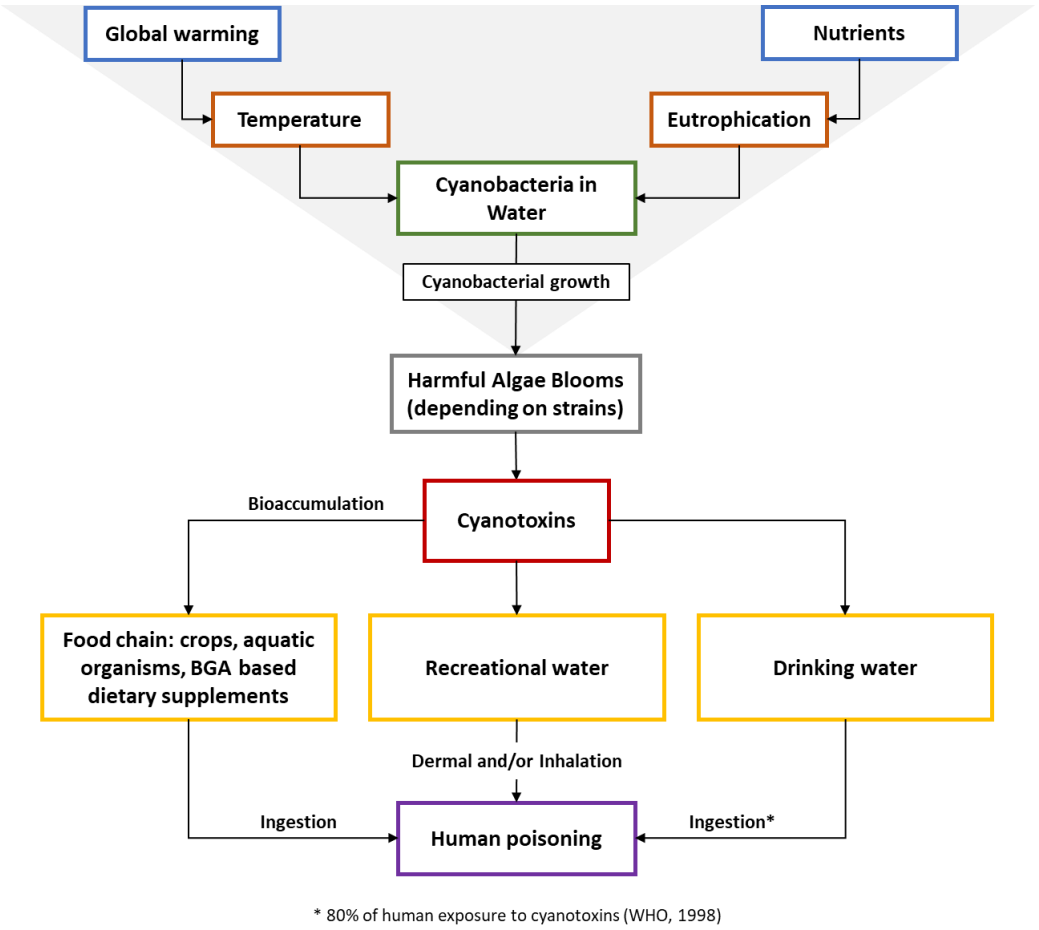


Figure 2. Origin of toxic cyanobacterial blooms and human exposure [52].

2.3 Toxicity

Cyanotoxicity studies are mainly focused on the biggest and greatest studied family of cyanotoxins, the cyclic peptide MCs. They focused specifically on a single congener, being most of the available toxicological data based on MC-LR. While legislation on the presence of MCs in waters for different purposes is clear, toxicological analyses are not compulsory for food supplements. Considering the information provided by the toxicological studies, tolerable daily intake (TDI) and guidance values have been

proposed by some institutions to prevent cyanotoxin contamination. The World Health Organization (WHO) established a TDI of 0.04 μg/kg body weight (bw) for chronic exposure to MC-LR and recommends a safe limit of 1 μg/L in drinking water [121]. This guideline value normally represents the concentration of a constituent that does not result in any significant risk to health over a lifetime of consumption. The WHO guideline value is stated as being "provisional" because it covers only MC-LR congener due to the absence of toxicological studies for other cyanobacterial toxins. According to these provisional guidance levels, infants (5 kg), children (20 kg) and adults (60 kg) could tolerate a maximum daily intake of 0.2, 0.8 and 2.4 μg MCs, respectively [122]. Similarly, the Oregon Health Authority (OHA) established a regulatory limit of 1 μg/g for microcystins in BGA-containing products [123] and set provisional guideline values for ANA, CYN, MCs and SAX for drinking and recreational waters use for humans and recreational waters use for dogs (**Table 1**) [124].

Table 1. Summary of Oregon's tolerable daily intakes and guideline values for four cyanotoxins for use in acute or short-term exposures [124].

Guideline value	Anatoxin-a	Cylindrospermopsin	Microcystins	Saxitoxin
Human TDI (μg/kg-day)	0.1	0.03	0.05	0.05
Dog TDI (μg/kg-day)	None—used human TDI	None—used human TDI	None—used human TDI	0.005
Drinking Water (μg/L)	3.0	1.0	1.0	1.0
Recreational Water (μg/L)	20.0	6.0	10.0	10.0
Dog-specific (μg/L)	0.4	0.1	0.2	0.02

Considering the lack of toxicological information regarding toxins beyond microcystins [70], and the fact that possible hazards of the consumption of BGA-related products have not been clearly established, safety measures would be necessary for the consumption of these kind of products, especially for some part of the population such as pregnant women or nursing mothers.

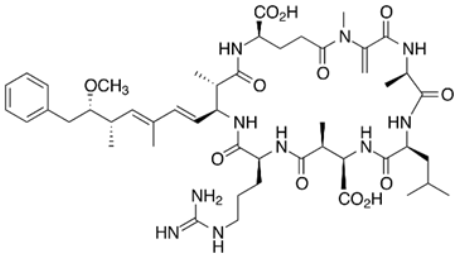
In **Chapter 5**, the okadaic acid (OA), a toxin that is not classified as cyanotoxin, but as a marine biotoxin, is included along with cyanotoxins. Therefore, this group of toxins will be introduced briefly below. Together, cyanotoxins and marine toxins are part of a larger group of toxins called phycotoxins, which are defined as complex secondary metabolite chemicals produced by eukaryotic and prokaryotic algae.

3. Marine biotoxins

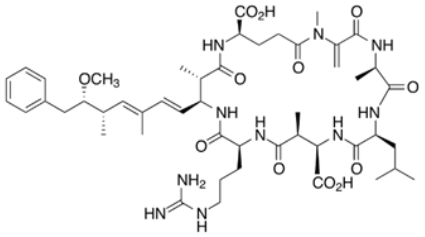
Marine biotoxins, also known as shellfish toxins, are bioactive compounds mainly synthetized by algae or phytoplankton. These toxins can be accumulated in the hepatopancreas of filter-feeding shellfish, such as oyster, mussels or clams, thus may be a health risk to humans through the food chain [125]. The ingestion of marine biotoxins can generate problems ranging from less severe such as headaches, vomiting and diarrhea, to more dangerous such as neurological problems and even death. The OA and its dinophysistoxin analogues constitute the DSP group of toxins, as they cause Diarrhetic Shellfish Poisoning [126]. The OA-group toxins are temperature-stable lipophilic polyether compounds, usually produced by dinoflagellates belonging to *Dinophysis spp.* and *Prorocentrum spp*. genera. As was the case of cyanotoxins, the amount

of marine biotoxins-producing organisms varies over periods. Similarly, explosive growth may occur as a result of changes in weather, temperature, salinity and nutrients amount [127], which can lead to multi-species contamination and human poisoning events [128]. The regulation established that live bivalve mollusks must not contain amount of marine biotoxins including OA, dinophysistoxins and pectenotoxins together exceeding the limit of 160 μg equivalents[1]/kg [129].

Chemical structure, molecular formula and family of every toxin studied in this Thesis are shown in **Figure 3.**

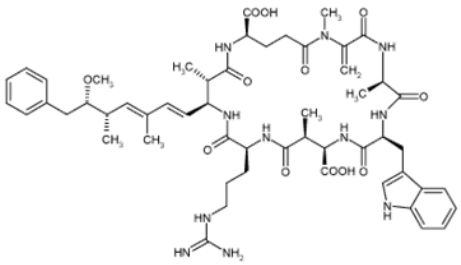


Microcystin-Leucine-Arginine (MC-LR)
$C_{49}H_{74}N_{10}O_{12}$
Cyclic peptides

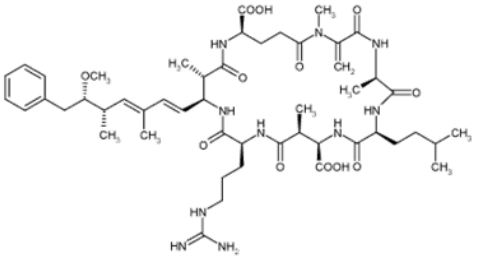


Microcystin-Arginine-Arginine (MC-RR)
$C_{49}H_{75}N_{13}O_{12}$
Cyclic peptides

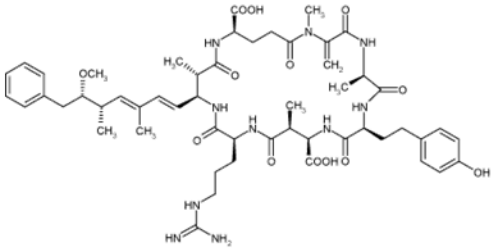
[1] Equivalents: the amount of toxins expressed as the amount of okadaic acid that gives the same toxic response followed intraperitoneal administration to mice. [https://efsa.onlinelibrary.wiley.com/doi/pdf/10.2903/j.efsa.2008.589]



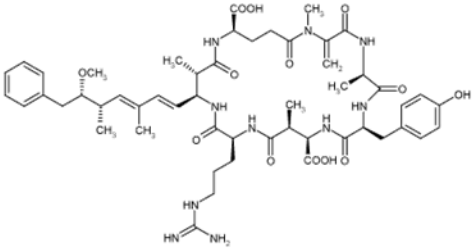
Microcystin-Tryptophan-Arginine (MC-WR)
$C_{54}H_{73}N_{11}O_{12}$
Cyclic peptides



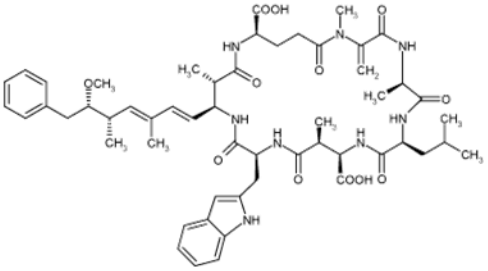
Microcystin-Homoisoleucine-Arginine (MC-HilR)
$C_{50}H_{76}N_{10}O_{12}$
Cyclic peptides



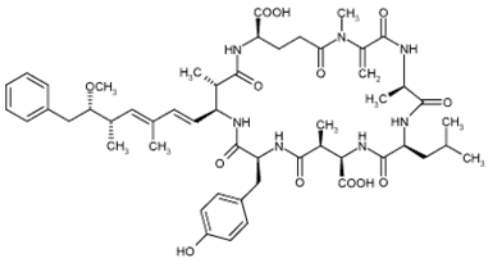
Microcystin-Homotyrosine-Arginine (MC-HtyR)
$C_{53}H_{74}N_{10}O_{13}$
Cyclic peptides



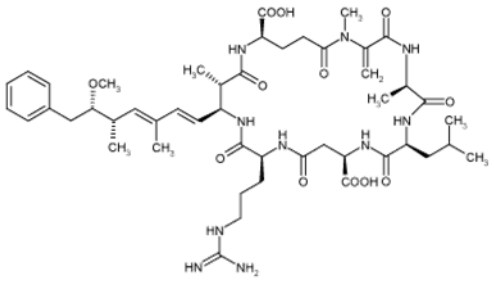
Microcystin-Tyrosine-Arginine (MC-YR)
$C_{52}H_{72}N_{10}O_{13}$
Cyclic peptides



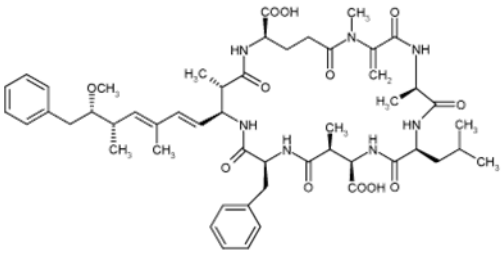
Microcystin-Leucine-Tryptophan (MC-LW)
$C_{54}H_{72}N_8O_{12}$
Cyclic peptides



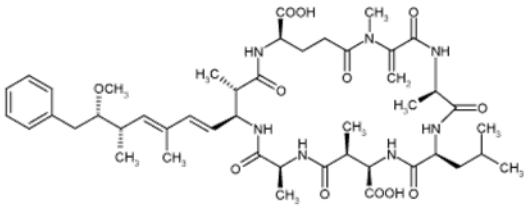
Microcystin-Leucine-Tyrosine (MC-LY)
$C_{52}H_{71}N_7O_{13}$
Cyclic peptides



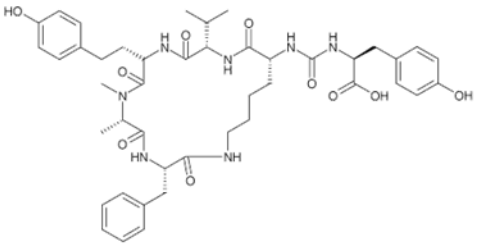
[D-Asp3]Microcystin-Leucine-Arginine ([D-Asp3]-MC-LR)
$C_{48}H_{72}N_{10}O_{12}$
Cyclic peptides



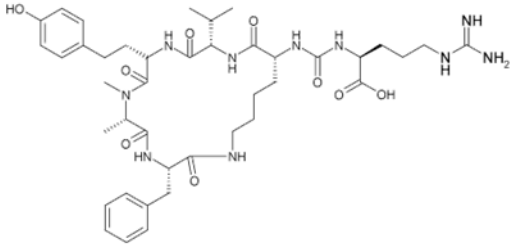
Microcystin-Leucine-Phenilalanine (MC-LF)
$C_{52}H_{71}N_7O_{12}$
Cyclic peptides



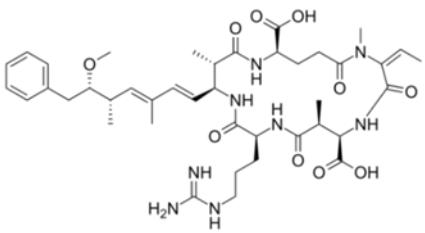
Microcystin-Leucine-Alanine (MC-LA)
$C_{46}H_{67}N_7O_{12}$
Cyclic peptides



Anabaenopeptin A (APa)
$C_{44}H_{57}N_7O_{10}$
Cyclic peptides



Anabaenopeptin B (APb)
$C_{41}H_{60}N_{10}O_9$
Cyclic peptides



Nodularin (NOD)
$C_{41}H_{60}N_8O_{10}$
Cyclic peptides

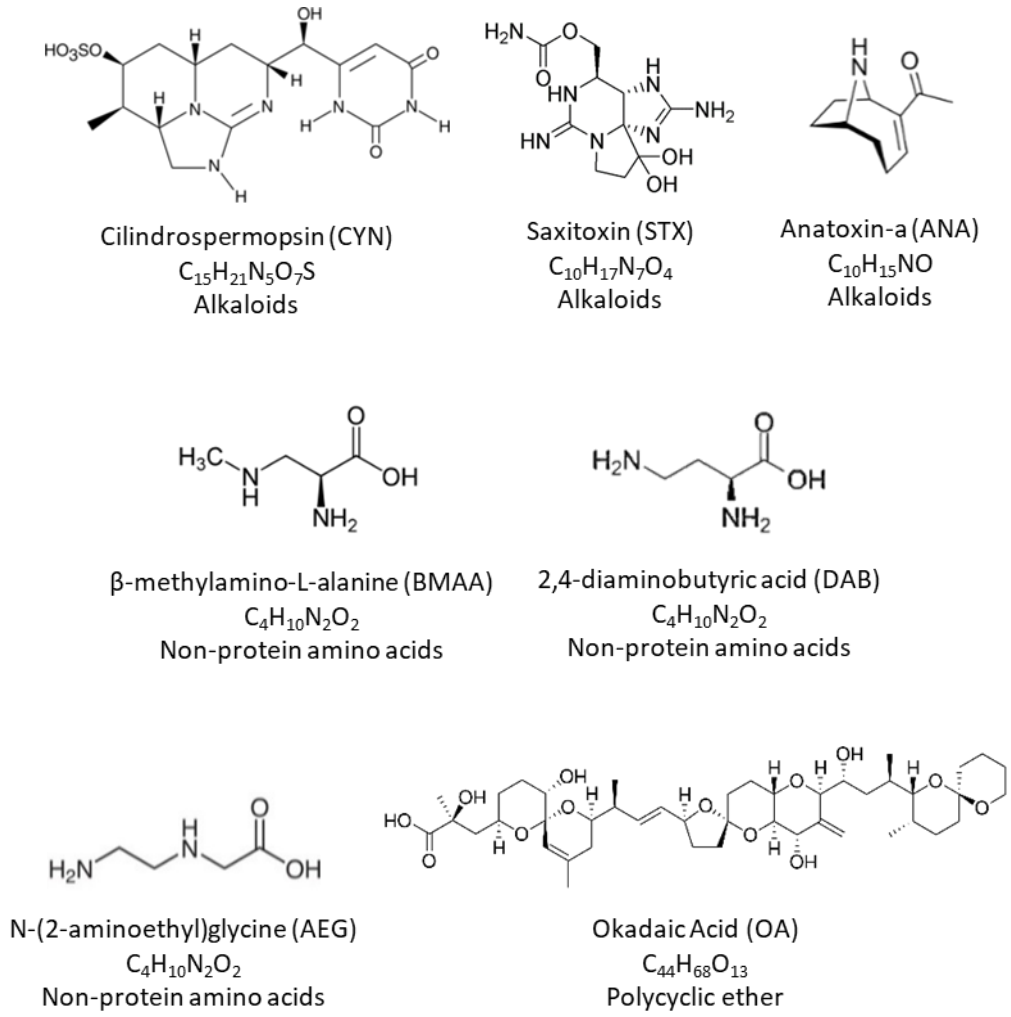


Figure 3. Structure, name, molecular formula and family of the target toxins.

4. Analytical methods for the determination of cyanotoxins

Since MCs were firstly detected, several methods have been developed to analyze environmental samples [130]. In recent years, the presence of cyanotoxins threatening humans and wildlife health, and the problems related with these toxins have called for awareness in many countries around the world. Therefore, the number of studied

cyanotoxins and analytical methods to detect them have increased. Cyanotoxins monitoring in potential contamination avenues such as drinking and recreational waters or BGA derived dietary supplements has become crucial to adequately control their presence and prevent or reduce the risks associated with their contamination.

Detection approaches employed differ in terms of accuracy, sensitivity and specificity. The two distinctive analytical methods most widely used to determine cyanotoxins are liquid chromatography (LC) combined with different detectors, and enzyme-linked immunosorbent assay (ELISA). However, other methods such as mouse bioassay, protein phosphatase inhibition assay, capillary electrophoresis, gas chromatography and biosensors have also been reported [131,132,133,134]. Among all of them, LC coupled with mass spectrometry (LC-MS) has been the most popular for identification and quantitation purposes, as it allows to quantify several analytes with enough sensitivity to fulfill low provisional limits and it offers the reliability and selectivity necessary for large-scale studies [135,136,137]. Moreover, LC methods must achieve a satisfactorily baseline separation of BMAA and its naturally occurring isomers DAB [138] and AEG. In this respect, it has been observed a lack of consensus over the widespread presence of BMAA in aquatic ecosystems [139,140,141].

Although numerous methods have been reported for the determination of a single toxin family, the development of reliable, sensitive and selective analytical approaches able to cover a wide range of cyanotoxins is essential, since they normally co-exist in any environment. However, due to their variability in chemical structures and physicochemical properties, this task is highly challenging [52]. For instance, biological methods are usually just toxin specific, and chemical methods, such as LC-MS, also show

limitations as quantifying several cyanotoxins might require a trade-off among each toxin for both extraction and separation. In addition, reliable quantification of cyanotoxins in complex samples has proven to be difficult due to matrix interferences [142,143].

As a consequence, special emphasis is being put on the development of multitoxin analytical approaches, which is one of the main challenges to be faced when developing analytical methods nowadays. Multitoxin methods are able to encompass the extraction and determination of toxins from different families, reducing the time and resource consumption as well as the amount of required sample.

Several LC-MS methods have been developed for the determination of cyanotoxins from different classes. Some of them use C18 reversed-phase (RP) columns [144,145] or RP biphenyl columns [136] to separate toxins from cyclic peptide family, such as MCs and NOD, and toxins from alkaloid group, mainly ANA and CYN. Similarly, RP columns functionalized with hydrophilic groups such as HSS T3 [146,147,148], Hypersil Gold C18 [74,149], Hypersil Gold aQ C18 [150], Atlantis T3 [151] or YMC ODS AQ [152], have also been extensively employed to determine cyclic peptide and alkaloid cyanotoxins. When more polar cyanotoxins, like BMAA and its isomers, are analyzed together with other toxins, a derivatization step is sometimes introduced, allowing the chromatographic separation and fluorescence detection (FD) with a RP C18 column [153,154,155]. In this sense, hydrophilic interaction liquid chromatography (HILIC) appeared as an alternative to the abovementioned derivatization methods for the determination of the highly polar BMAA [143,156,157].

HILIC is an ultra-high-performance liquid chromatography (UHPLC) mode suitable for the separation of polar, hydrophilic and amphiphilic compounds that are too polar to be retained in RP-LC, whereas do not have enough charge to be electrostatic retained in ion-exchange chromatography [158]. Thus, HILIC have been successfully applied to solve many separation difficulties regarding neutral and charged compounds and several biomolecules [159,160,161,162]. HILIC employs polar stationary phases, traditionally used in normal-phase LC, based on bare silica or silica gels modified with polar functional groups. The mobile phase is similar to those employed in RP-LC mode, including a polar organic solvent miscible with water and a small amount of water [163]. The separation can be accomplished using isocratic mode with a high proportion of organic solvent or using gradient mode, starting with high proportion of organic solvent and gradually increasing the proportion of aqueous solvent to favor the elution of the most polar compounds.

Therefore, taking advantage of the potential of HILIC, in some works the chromatographic method was developed separately for lipophilic and hydrophilic toxins using both RP-C18 and HILIC columns [164,165,166,167,168,169]. Likewise, in a previous work, a method using a TSKgel Amide instead of a HILIC column was employed to retain ANA, CYN and SAX and a method using a RP-C18 column was applied to retain MCs and NOD [16]. In other study, all the target cyanotoxins were determined with the same Hypersil Gold C18 column but employing two different chromatographic conditions, and thus in two different runs, one for MCs and ANA and other for CYN, SAX and BMAA [170].

Despite the advances in determination of cyanotoxins in multitude of matrices, there are still considerable data gaps on analytical methods [171], including the lack of all relevant toxin standards [172], the need to validate methodologies beyond water matrices, the need to standardize cyanotoxins analytical methods to be used in monitoring programs [173], the need of new methods able to cover simultaneous identification of several cyanotoxins and the need to improve robustness and detection limits of the methods.

Regarding the sample treatment, in the case of multitoxin analysis it is essential to consider their diverse physicochemical properties, from the highly polar NPAs to the more lipophilic MCs. These disparities complicate the extraction and purification or preconcentration procedure adding difficulty when a combined simultaneous extraction and analysis is desired. Thus, workflows to extract multiple toxin classes usually endeavor to either group similar species together or to assume compromises in terms of recoveries. In the case of complex matrices such as BGA derived dietary supplements, some multitoxin studies cover the presence of diverse cyanotoxin families, especially MCs, CYN, SAX, ANA, and some of them also include BMAA. However, as was the case for chromatographic separation, they apply different extraction procedures depending on the family. In such cases, sample treatment approaches are generally based on solid-liquid extraction (SLE) followed by a purification step using solid-phase extraction (SPE). Due to their similar structure, MCs and NOD have been commonly extracted using mixtures of methanol:water from 70 to 90%, sometimes with acid. The mixture containing 75% MeOH is the more frequently employed for both hydrophobic and hydrophilic MCs congeners [134]. For the extraction of more polar compounds, such as alkaloids or NPAs, there is less uniformity in the extraction solvents used. Toxins

belonging to these groups have been extracted from dietary supplement samples using pure methanol with and without acid, water, and acidified mixtures of water and acetonitrile [16,170,174,175].

When working with water samples, sample treatment is mainly based on SPE [176]. However, when working with more complex matrices, such as BGA dietary supplements, a SPE is usually required as a preconcentration and clean-up step after the SLE procedure. Usually, cation exchange SPE cartridges are used to purify low molecular mass basic compounds such as NPA isomers in matrices like water [143,154] or dietary supplements [170]. In contrast, MCs and NOD, as moderately polar organic compounds, are usually retained on hydrophobic cartridges such as reversed phase or hydrophilic-lipophilic balance (HLB) cartridges [177,178]. On the other hand, supelclean ENVI-carb cartridges and polygraphitized carbon (PGC) cartridges are more effective for the retention of CYN and ANA in some situations due to its high-water solubility [164,179]. As a result, achieving a multiclass target extraction is not an easy task. In this regard, the use of a dual cartridge assembly of above-mentioned cartridges has been previously investigated with the aim of covering different cyanotoxin families or improving matrix interference clean-up [143,144,146,147,151,180]. However, the literature often describes one SPE procedure for each family of cyanotoxins or separate elutions of the cartridges turning the extraction into more time-consuming and tedious [145,146,181].

In addition to the technical challenges associated with an accurate extraction, detection, quantification and interpretation of the results of multi-cyanotoxin analysis, some of them such as MCs and BMAA can be found in their free form or bound to proteins [122],

in which case it is important to use a sample treatment fitted for the intended purpose, and it is necessary to indicate whether the free, bounded or total fraction has been determined.

Taking into account all these considerations and the intrinsic limitations of a possible sample treatment covering the three groups of cyanotoxins, in **Chapter 3** it is described the development of an analytical method for the simultaneous extraction, preconcentration and determination of the free fraction of cyanotoxins belonging to cyclic peptide family (MC-LR, MC-RR and NOD), alkaloid family (ANA, CYN), and NPA family (BMAA, DAB and AEG) from reservoir water samples. The method consisted of a tandem-SPE sample treatment followed by a hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry (HILIC-MS/MS). Based on the progress achieved in such work, an analytical method for comprehensive determination of some of these multiclass cyanotoxins from BGA and microalgae dietary supplements was proposed in **Chapter 4**. Likewise, it was intended to evaluate the feasibility, challenges and pitfalls that arise in the field of multi-toxin analysis when they are carried out in such complex matrices.

Although the use of UHPLC coupled to low resolution mass spectrometry (LRMS) detection systems has been one of the most employed techniques to determine cyanotoxins in food matrices due to the high sensitivity offered in target analysis, it shows as well some limitations due to the acquisition mode, such as the time-consuming compound-depending optimization of the acquisition parameters [182], or the incapacity to conduct retrospective data analysis [183]. Thus, current target methods rely on defined compounds and are commonly unsuitable to detect and identify emerging toxins. The

use of high-resolution mass spectrometry (HRMS) provides a powerful alternative to LRMS as it allows the settlement of some of these handicaps. The main improvement of HRMS-based approaches is the acquisition of high-resolution full scan mass spectrometry data which aids a retrospective data analysis of non-target compounds without re-injecting the samples [184]. In addition, HRMS allows the combination of target and non-target analysis, the so-called suspect screening, which enables the identification at high levels of confidence of novel, uncommon and unknown compounds based on the exact mass to charge ratio (m/z), isotope pattern, common adducts and secondary fragmentation, and the semi-quantification even without reference standards [185,186,187,188], providing significant improvements in screening of complex mixtures [189,190]. In this line, the hyphenation of ion mobility spectrometry (IMS) with HRMS and its introduction in traditional UHPLC-MS workflows is becoming a powerful technique that enhances the quality, quantity and specificity of the information that can be obtained [191].

5. Ion mobility spectrometry

IMS has emerged as a powerful tool for the separation, identification and structural characterization of molecules. Although it is not a recent technique, its coupling to MS in the 1970s pushed its development over the years and brought the technique from the domain of specialists to more commonplace laboratories [192]. IMS is electrophoretic separation technique that allows the separation of ionized compounds generated by a source, by driving them through a gas chamber, generally filled with nitrogen or helium, using an electric field [193,194]. Once in the ion mobility drift cell, analytes are separated

by their size, charge and geometric conformation. The time that it takes for the ions to move through the gas-filled chamber under a specific electric field is known as drift time. The drift time of an ion is dependent from the ion mobility system employed, so this value cannot be compared among different instruments. It is preferable to report the collision cross section (CCS, Ω), which is defined as the orientationally averaged collision area from the interaction between an individual ion and the neutral gas molecule. The CCS is considered a molecular descriptor because it is an intrinsic property of each molecule, its value is directly linked to the chemical structure and three-dimensional conformation of the charged analyte and carrier gas used in the IMS experiment [195], and it is independent from the matrix. Therefore, CCS can be used as an additional separation dimension alongside with the traditional parameters, such as retention time and accurate *m*/*z*, to improve screening capacity, selectivity and sensitivity, as it was already demonstrated for different substances including food contaminants [196,197,198,199,200,201]. In this sense, the incorporation of CCS as an identification parameter in routine analysis requires the use of reliable CCS databases that provide these experimental values for as many molecules as possible. However, several compounds, such as cyanotoxins, remain uncharacterized in terms of CCS, thus further efforts are needed to overcome the lack of appropriate CCS databases.

There is a wide variety of IMS-MS instruments based on different technologies and thus with diverse advantages and disadvantages [202]. CCS values can be obtained directly from drift time values by applying the Mason-Schamp equation in the case of drift tube ion mobility (DTIMS) [203]. However, when using other IMS technologies such as travelling wave ion mobility spectrometry (TWIMS) or trapped ion mobility

spectrometry (TIMS), CCS measurements are obtained through calibration with a specific mixture of compounds with known CCS [204,205].

With this background, the main goal of **Chapter 5** was to generate the first TWIMS-derived CCS database for phycotoxins, starting from 21 compounds of different chemical nature (20 cyanotoxins and 1 marine biotoxin), aiming to implement the use of IMS in routine phycotoxin workflows and to extend the current IMS knowledge about natural toxins. To establish the potential of the CCS as a molecular descriptor, CCS values were measured through different experimental conditions. Likewise, the strengths and the challenges that arose from the developed library were discussed. Finally, as a proof of concept, the database was applied to the qualitative screening of BGA dietary supplements of different composition.

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CHAPTER 3

MULTICLASS CYANOTOXIN ANALYSIS IN RESERVOIR WATERS BY TANDEM SOLID-PHASE EXTRACTION FOLLOWED BY ZWITTERIONIC HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY

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1. Summary

The presence of cyanobacteria and cyanotoxins in all water bodies, including ocean water and fresh water sources, represents a risk for human health as eutrophication and climate change are enhancing their level of proliferation. For risk assessment and studies on occurrence, the development of reliable and sensitive analytical approaches able to cover a wide range of cyanotoxins is essential. This work describes the development of an Hydrophilic Interaction Liquid Chromatography (HILIC) multiclass method coupled with tandem mass spectrometry (MS/MS) detection for the simultaneous analysis of eight cyanotoxins in reservoir water samples belonging to three different classes, according to their chemical structure: cyclic peptides (microcystin-LR, microcystin-RR and nodularin), alkaloids (cylindrospermopsin, anatoxin-a) and three non-protein amino acids isomers such as β-methylamino-L-alanine, 2,4-diaminobutyric acid and N-(2-aminoethyl)glycine. A Zwitterionic HILIC column (SeQuant ZIC-HILIC, Millipore) was employed to achieve the chromatographic separation in less than 12 min. Previously, a novel sample treatment based on a tandem solid-phase extraction (SPE) system using mixed cation exchange (MCX) and Strata-X cartridges was investigated with the aim of extracting and preconcentrating this chemically diverse group of cyanotoxins. The Strata-X cartridge, which was configured first in the line of sample flow, retained the low polar compounds and the MCX cartridge, which was at the bottom of the dual system, retained mainly the non-protein amino acids. The optimization procedure highlighted the importance of sample ion content for the recoveries of some analytes such as the isomers β-N-methylamino-L-alanine and 2-4-diaminobutyric acid. Method validation was carried out in terms of linearity, limit of detection (LOD) and quantification (LOQ), recoveries, matrix effect and precision in terms of repeatability and intermediate precision. This work represents the first analytical method for the simultaneous analysis of these multiclass cyanotoxins in reservoir water samples,

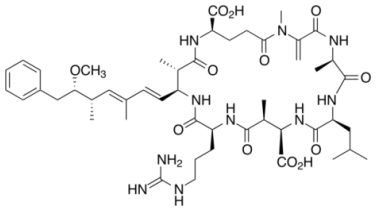
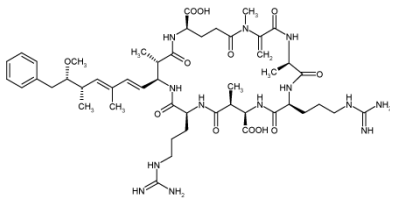
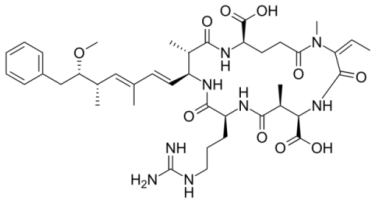
achieving LOQs in the very low range of $7 \cdot 10^{-3}$ – 0.1 μg/L. Despite high recoveries obtained at the LOQ concentration levels (101.0-70.9%), relative standard deviations lower than 17.5% were achieved.

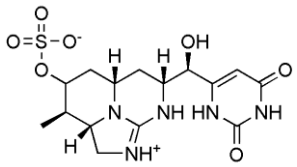
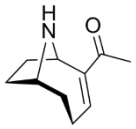
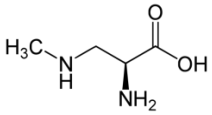
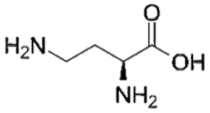
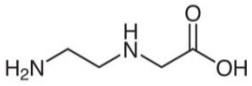
2. Experimental

2.1. Material and reagents

Microcystins (MCs), such as microcystin-leucine-arginine (MC-LR ≥ 99%) and microcystin-arginine-arginine (MC-RR ≥ 99%), nodularin (NOD ≥ 95%), cylindrospermopsin (CYN ≥ 95%) and anatoxin-a (ANA ≥ 98%), were supplied by Enzo Life Sciences, Inc. (Lausen, Switzerland). Isomers β-N-methylamino-L-alanine hydrochloride (BMAA ≥ 97%), 2-4-diaminobutyric acid dyhydrochloride (DAB ≥ 95%) and N-(2-aminoethyl)glycine (AEG ≥ 98%) were supplied by Sigma Aldrich (Darmstadt, Germany). The toxicity, structure and physicochemical properties of the studied cyanotoxins are shown in **Table 1**.

Table 1. Toxicity, structure and physicochemical properties of target cyanotoxins.

Cyanotoxin	Toxin group	Toxicity	pKa[a]	pKa[a]	log P[a]	Chemical structure
Microcystin-leucine-arginine (MC-LR)	Cyclic peptides	Hepatotoxin	2.2	12.4	2.4	
Microcystin-arginine-arginine (MC-RR)	Cyclic peptides	Hepatotoxin	3.0	13.7	-0.22	
Nodularin (NOD)	Cyclic peptides	Hepatotoxin	3.4	10.8	1.5	

Cylindrospermopsin (CYN)	Alkaloids	Cytotoxin	-1.5	10.5	-0.82	
Anatoxin-a (ANA)	Alkaloids	Neurotoxin		9.6	0.8	
β-methylamino-L-alanine (BMAA)	Non-protein amino acids	Neurotoxin	2.1	6.6	-0.1	
2,4-diaminobutyric acid (DAB)	Non-protein amino acids	Neurotoxin	2.6	8.4	-4.0	
N-(2-aminoethyl)glycine (AEG)	Non-protein amino acids	Neurotoxin	2.2	9.8	-3.8	

[a]Advanced Chemistry Development software V11.02 (ACD/Labs, Toronto, Ontario, Canada)

Stock standard solutions were prepared by adding 1 mL of the desired solvent directly into the vial of toxin supplied by the manufacturer and gently swirling the vial to dissolve the toxin. The obtained solutions were: 50 μg/mL MC-LR in methanol (MeOH), 25 μg/mL MC-RR in 80% aqueous MeOH, 50 μg/mL NOD in 50% aqueous MeOH, 25 μg/mL CYN in MeOH, 1000 μg/mL ANA in water. Stock solutions of 1000 μg/mL for the three standard isomer molecules (BMAA, DAB and AEG) were prepared by dissolving the desired amount of analyte in water. All of them were stored in the dark at −20 ˚C. Intermediate standard solutions of each compound at 2.5 μg/mL were prepared by dilution of the stock solutions with the corresponding solvent for each toxin. These solutions were used to prepare the working solutions that consisted of a mixture of all cyanotoxins in concentration levels according to the experiment in 50% aqueous MeOH. These solutions were stored at 4 ˚C and equilibrated to room temperature before use.

Unless otherwise specified, analytical grade reagents and HPLC grade solvents were used in this work. Acetonitrile (MeCN) and MeOH were purchased from VWR (Radnor, PA, USA). Ammonia solution ($NH_3 \cdot H_2O$) (30% assay) was purchased from Merck (Darmstadt, Germany). Formic acid (FA) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water (Milli-Q plus system, Millipore, Bedford, MA, USA) was used throughout the work.

Oasis WCX cartridges (60 mg), Oasis MCX cartridges of different sorbent mass (60 mg and 150 mg), and Oasis HLB cartridges (60 mg) from Waters (Milford, MA, USA); Strata-X cartridges (200 mg) and Strata WCX cartridges (100 mg) supplied by Phenomenex (Torrance, CA, USA) and WCX cartridges (60 mg) from Supelco Inc. (Bellefonte, PA, USA) were tested for cyanotoxin extraction from water samples. SPE tube adapters from Supelco Inc. (Bellefonte, PA, USA) were employed.

A large variety of syringe filters were evaluated: CLARIFY polytetrafluoroethylene (PTFE) hydrophilic filter (0.2 μm × 13 mm), CLARYFY PTFE hydrophobic (0.2 μm × 13 mm), CLARIFY cellulose acetate (CA) filter (0.2 μm × 13 mm) and CLARIFY glass microfiber (GF) filter (1 μm × 13 mm) from Phenomenex (Torrance, CA, USA); Nylon filter (0.2 μm × 25 mm) and PTFE filter (0.2 μm × 13 mm) from VWR International (West Chester, PA, USA); CA filter (0.2 μm × 13 mm), GForde filter (0.7 μm × 13 mm) and polyvinylidene fluoride (PVDF) filter (0.2 μm × 13 mm) from Thermo Fisher Scientific (Whatman, Maidstone, UK).

2.2. Instrumentation

Chromatographic separation of cyanotoxins was performed on an Agilent 1290 Infinity II System (Agilent Technologies; Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler (with 20 μL injection loop) and a column thermostat. The LC system was coupled to an API 3200 triple quadrupole (QqQ) mass spectrometer (AB Sciex; Darmstadt, Germany) equipped with a Turbo V

electrospray ionization source. Instrumental data were collected by the Analyst® Software (version 1.5) using the Scheduled MRM™ Algorithm (AB SCIEX).

Separation was achieved using a SeQuant ZIC-HILIC column (2.1 × 250 mm, 3.5 μm particle diameter, EMD Millipore; Billerica, MA). A Zorbax RRHD (Rapid Resolution High Definition) Eclipse Plus C18 (2.1 × 50 mm, 1.8 μm) column from Agilent Technologies (Waldbronn, Germany), a Luna Omega C18 Polar (2.1 × 100 mm, 1.6 μm) column, a Kinetex Biphenyl (2.1 x 50 mm, 1.7 μm) column and a Kinetex HILIC (2.1 × 100 mm, 1.7 μm) column, all supplied by Phenomenex (Torrance, CA, USA), were also tested.

An analytical balance with 0.0001 g resolution (Sartorius; Goettingen, Germany), a multi-tube vortexer (model BV1010 from Benchmark Scientific; Sayreville, USA), a vortex-2 Genie (Scientific Industries; Bohemia, NY, USA), an Universal 320R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany), a pH-meter (Crison model pH 2000; Barcelona, Spain), a nitrogen dryer EVA-EC System (VLM GmbH; Bielefeld, Germany) and a Visiprep solid-phase extraction unit from Supelco (Bellefonte, PA, USA) were used for sample treatment.

2.3. Sample preparation

Water samples collected in March 2021 from different freshwater swamps named "El Portillo" (Castril), "Canales" (Güéjar Sierra), "Bermejales" (Arenas del Rey) and "Cubillas" (Albolote), all located in Granada (Andalucía, Spain), were considered in this study. All samples were collected in amber glass bottles. After sampling procedure, the pH and the conductivity were measured. Conductivity ranged between 115 and 555 μS/cm, while pH was around 8.0 for all of them. The content of sulfates, chlorides, nitrates, fluorides and phosphates determined by Ion-Exchange Chromatography is shown in **Table 2**. Water samples were filtered through a 0.22 μm

cellulose acetate membrane filter to remove suspended particles and they were kept at 4 ˚C until analysis.

Table 2. Content of anions* and conductivity of water samples from different reservoirs.

	El Portillo	Canales	Bermejales	Cubillas
Sulfates (mg/L)	3.7	22.3	124.2	153.4
Nitrates (mg/L)	1.0	2.5	2.1	8.8
Chlorides (mg/L)	2.3	6.7	5.6	27.0
Fluorides (mg/L)	n.d.	n.d.	n.d.	0.2
Phosphates (mg/L)	n.d.	n.d.	n.d.	n.d
Conductivity (μS/cm)	191.7	115.4	367.0	555.0

n.d.: non detectable; *by Ion-Exchange Chromatography

2.4. SPE procedure

A sample treatment was developed for cyanotoxin extraction and preconcentration using an assembly of two cartridges, a Strata-X (200 mg, 6 mL) and Oasis® MCX (150 mg, 6 mL), connected in series, which enabled the simultaneous extraction and preconcentration despite variations in their physicochemical properties. The SPE was carried out using a 12-port SPE vacuum manifold. The optimized SPE procedure was as follows: an aliquot of 25 mL of freshwater sample, previously acidified to pH 3, with 37% commercial concentrated HCl was placed in a volumetric flask and spiked at the desired analyte concentration levels. The two cartridges were conditioned and activated separately with 3 mL of MeOH followed by 3 mL of deionized water at pH 3. After that, the cartridges were connected in series: Strata-X cartridge (retaining low polar and moderately polar compounds such as MCs, NOD, CYN and ANA) was configured first in the line of sample flow, followed by the MCX cartridge (retaining the highly polar and water-soluble non-protein amino acids (NPAs) BMAA, DAB and AEG, as well as the rest of positively charged cyanotoxins that might pass through

Strata-X cartridge). Samples were loaded onto the dual-cartridge set-up. This step was vacuum-assisted in order to maintain a uniform and constant sample flow rate of 1 mL/min. Then, the cartridges were dried under vacuum for 5 min by passing low-pressure nitrogen. Before the elution step, the order of the cartridges was reversed, being MCX at the top and Strata-X at the bottom of the dual system. Elution of the analytes was carried out using 5 mL of 10% $NH_3 \cdot H_2O$ in MeOH. The eluate was evaporated to dryness in a heating block (30 °C) under a gentle stream of nitrogen and then the residue was re-dissolved with 250 μL of 60% MeCN with 0.1% of FA. The final extract was filtered through a CLARIFY-PTFE hydrophilic filter (0.2 μm × 13 mm), transferred to a glass insert and analyzed by the proposed HILIC-MS/MS method.

2.5. HILIC separation

Chromatographic separations were carried out on a SeQuant ZIC-HILIC column at 55 °C. Mobile phase flow rate was set at 0.2 mL/min. It consisted of water as eluent A and MeCN as eluent B, both containing 0.3% (v/v) of FA as volatile acid. Separation was accomplished under gradient elution conditions. The composition (expressed as a percentage of eluent B in mobile phase) was established as follows: 0 min, 60% (v/v); 3 min, 60% (v/v); 6 min, 40% (v/v); 12 min, 40% (v/v); and 13 min, 60% B (v/v). Finally, initial conditions were maintained for 22 min in order to guarantee column equilibration between runs (35 min of total analysis time). Injection volume was set to 20 μL (full loop injection).

2.6. MS/MS parameters

The detection of the target cyanotoxins was carried out using tandem mass spectrometry with electrospray ionization operated under positive mode (ESI (+)-MS/MS) in multiple reaction monitoring (MRM) conditions. Source parameters were set as follows: source temperature (TEM), 550 °C; ion spray (IS) voltage, 5500 V;

nebulizing and drying gases (GS1 and GS2, nitrogen), 50 psi (344.7 kPa); curtain gas (CUR, nitrogen), 25 psi (172.4 kPa); and collision gas (CAD, nitrogen) was set to 10 psi (69 kPa). The precursor and product ions of individual analytes were identified by tuning, after direct infusion of individual standard solutions into ESI-MS/MS system. One precursor ion and two product ions were monitored for all analytes. The protonated form of cyanotoxins (i.e., $[M+H]^+$) was selected as precursor ion for all analytes except for MC-RR, which tends to ionize as the diprotonated molecular ion ($[M+2H]^{2+}$). The fragment ion with the highest intensity was used for quantification (Q_{ion}) whereas the second one was acquired for identification (I_{ion}). Main MRM parameters, including declustering potential (DP), entrance potential (EP), collision entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP), are given in **Table 3**.

Table 3. MRM parameters for the analysis of cyanotoxins by HILIC-ESI-MS/MS.

Analyte	Retention time (min)	Molecular ion	Precursor ion (*m*/*z*)	DP (V)	EP (V)	CEP (V)	Product ions	CE (V)	CXP (V)
MC-LR	3.1	$[M+H]^+$	995.6	136	10.5	32	Q_{ion} 135.2	93	4
				136	10.5	32	I_{ion} 105.0	129	4
NOD	3.3	$[M+H]^+$	825.4	96	6.5	24	Q_{ion} 135.1	89	2
				96	6.5	100	I_{ion} 103.2	129	4
CYN	4.2	$[M+H]^+$	416.0	51	11.0	18	Q_{ion} 194.2	49	6
				51	11.0	18	I_{ion} 175.9	49	6
MC-RR	4.3	$[M+2H]^{2+}$	519.8	41	6.0	18	Q_{ion} 135.2	39	4
				41	6.0	18	I_{ion} 103.2	91	6
ANA	4.9	$[M+H]^+$	166.2	36	4.5	12	Q_{ion} 149.2	19	4
				36	4.5	12	I_{ion} 131.2	19	4
BMAA	9.6	$[M+H]^+$	119.1	26	4.0	6	Q_{ion} 44.1	27	2
				26	4.0	6	I_{ion} 102.1	15	4
DAB	10.6	$[M+H]^+$	119.1	21	3.0	14	Q_{ion} 101.0	11	4
				21	3.0	14	I_{ion} 56.0	31	2
AEG	11.8	$[M+H]^+$	119.1	26	3.5	10	Q_{ion} 102.0	11	4
				26	3.5	10	I_{ion} 56.0	25	4

Abbreviations: declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; collision energy, CE; collision cell exit potential, CXP; quantification ion, Q_{ion}; identification ion, I_{ion}.

3. Results and discussion

3.1. Optimization of the chromatographic separation and MS/MS detection

The development of suitable chromatographic separation conditions for the eight target cyanotoxins is challenging due to the large differences in analyte structure and polarity (log P ranging from 2.4 to -4.0, calculated by using Advanced Chemistry Development software V11.02 (ACD/Labs, Toronto, Ontario, Canada) [1]). Method optimization was carried out for a mixture of the eight cyanotoxins at concentrations between 30 and 400 μg/L, depending on the sensitivity for each analyte. BMAA, DAB and AEG are isomers, hence they have the same molecular ion $[M+H]^+$ at *m*/*z* 119. Product ion spectra show that they have very similar fragmentation patterns, with some minor differences, being the most intense fragments at *m*/*z* 102.1 for BMAA, at *m*/*z* 101.0 for DAB and at *m*/*z* 102.0 for AEG. However, although they were the most intense fragments obtained by direct infusion of individual standard solutions, for BMAA the fragment *m*/*z* 44.1 was finally selected as Q_{ion} because higher signal-to-noise ratio for this fragment was obtained in real samples. As described above, the challenge of developing a chromatographic separation method for the target cyanotoxins is to ensure that the baseline separation of isomers is successfully achieved to prevent misidentifications, while retaining the rest of the cyanotoxins, which are less polar than the NPAs.

Initially, several stationary phases were tested, including reversed phase (RP) columns (Zorbax RRHD Eclipse Plus C_{18}, Luna Omega C_{18} Polar and Kinetex Biphenyl) and two HILIC columns (Kinetex HILIC and SeQuant ZIC-HILIC). For RP columns, MeCN (eluent B) and H_2O (eluent A) were used as mobile phase employing isocratic mode at different percentages of eluent B (25%, 15% or 10%). Flow rate was 0.3 mL/min, temperature was set at 40 °C and an injection volume of 20 μL (full loop) was used. RP columns gave either no significant or slight retention of the compounds,

resulting in broad peaks. HILIC employs a polar stationary phase becoming an alternative approach to effectively separate small polar compounds such as NPAs. With the aim of testing HILIC columns, the same chromatographic conditions were employed in isocratic mode at 70% of eluent B. Among the two tested HILIC columns SeQuant ZIC-HILIC showed the best results in terms of resolution. ZIC-HILIC columns contain a zwitterionic sulfoalkylbetaine stationary phase bonded onto the silica gel or polymer support whose active layer consists of both strongly acidic sulfonic acid groups and strongly basic quaternary ammonium groups separated by a short alkyl spacer. The sulfoalkylbetaine phases adsorb water from the mobile phase that becomes part of the stationary phase, controlling the retention mechanism [2]. However, as the isomers were not fully eluted under the isocratic mode, a gradient elution program was investigated to provide the elution of BMAA, DAB and AEG. The final gradient program started at 60% B (held for 3 min), decreasing to 40% B in 3 min (held for 6 min), and back to 60% B in 1 min. An equilibration time of 22 min with the initial gradient conditions was needed after each sample run (35 min total analysis time) to ensure the reproducibility of the analysis. With these conditions, all cyanotoxins eluted as separate peaks. Commonly, the use of a volatile acid in the mobile phase leads to an improvement in the ionization step under ESI+ conditions. Therefore, the addition of FA to both, eluent A and B, was tested between 0% and 0.5% (v/v). It was observed that the presence of FA in the mobile phase improved the sensitivity and peak shape although signal suppression was observed at the highest concentrations of acid. In this sense, the best signal-to-noise ratio was achieved when 0.3% of FA (v/v) was added in both, eluent A and B. The effects of the mobile phase flow rate were investigated in the range between 0.2 and 0.35 mL/min. Although retention time decreased with high flow rates, system pressure increased above 400 bar. This was a constraint to the applicability of this method because it exceeded the maximum working pressure that the employed SeQuant HILIC column can withstand, so 0.2 mL/min was selected with a pressure of 315 bar at the initial

part of the gradient. Column temperature was evaluated in the range of 25 to 60 ˚C while 55 ˚C was found to be the optimum value as narrower peaks and slightly better sensitivity were obtained. Finally, injection solvent and injection volume were studied. Sample solvents with higher elution strength than the initial mobile phase composition cause a less effective retention on the stationary phase with undesirable peak distortion, peak broadening and earlier elution. This is even more relevant in HILIC separation as it is generally more sensitive to the solvent strength mismatch between the sample solvent and the mobile phase in comparison to RP chromatography. HILIC requires the injection of a low water content solution to maintain acceptable chromatographic peak shapes [3]. However, this can cause solubility issues with highly polar analytes, such as BMAA, DAB or AEG. Different percentages of aqueous MeCN were tested as sample solvent and we could check that the increase of MeCN up to percentages above the 60% improved the analyte peak shapes. Finally, it was observed that the addition of FA to the injection solvent increased peak areas and provided a significant improvement of peak shapes, being narrower for the isomers, especially for DAB, thus a 60% of aqueous MeCN with 0.1% of FA was selected as the optimum sample solvent.

The ionization source parameters as well as the CAD pressure were optimized to enhance sensitivity for each cyanotoxin. TEM was evaluated between 350 and 650 °C. The signals for most analytes increased with the temperature up to 550 °C, so this value was selected as optimum (**Figure 1A**). CAD was studied between 5 and 15 psi and 10 psi was finally selected as a compromise between all analytes (**Figure 1 B**). IS voltage was studied in the range of 4500 to 6000 V and it was observed that an increase in IS voltage produced a slightly decreased on the peak area for most of the analytes, while in the case of MC-LR, peak area dramatically increased. Thus, in order to have a better sensitivity for MC-LR, 5500 V was selected as optimum (**Figure 1C**). Pressure of CUR was studied between 10 and 40 psi. High values of CUR produced an increased peak area for most of the analytes except for MC-LR and NOD, which

disappeared when a pressure of 40 psi was applied. A pressure of 25 psi (172.4 kPa) was selected as optimum CUR as it was the highest value that did not impair the sensitivity of any analyte (**Figure 1 D**). GAS 1 and GAS 2 were studied between 40 and 60 psi and both were set to 50 psi (344.7 kPa). In **Figure 2** it can be observed an extracted ion chromatogram of a cyanotoxin standard solution using optimized HILIC-MS/MS conditions.

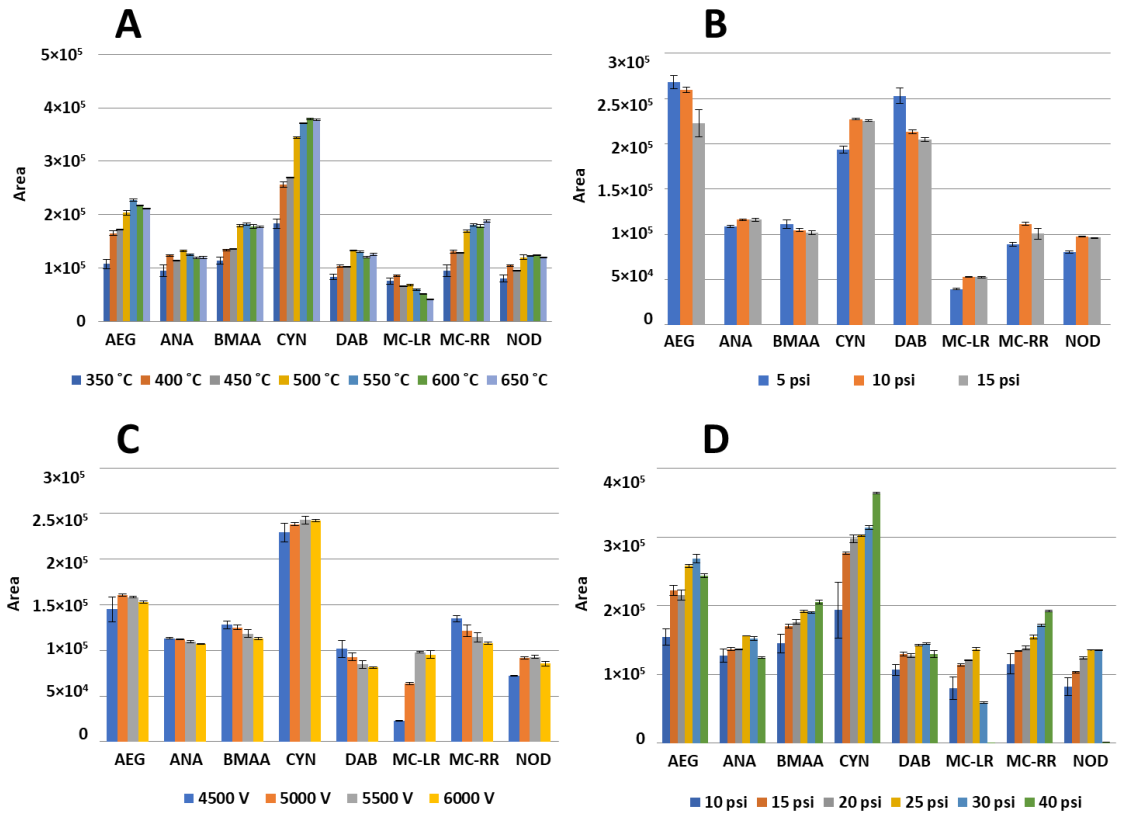


Figure 1. Optimization of the MS/MS ionization source parameters. A) Temperature (TEM); B) Collisionally activated dissociation (CAD) pressure; C) Ionization spray (IS) voltage; D) Curtain gas (CUR) pressure. Error bars represent the standard error (n=6).

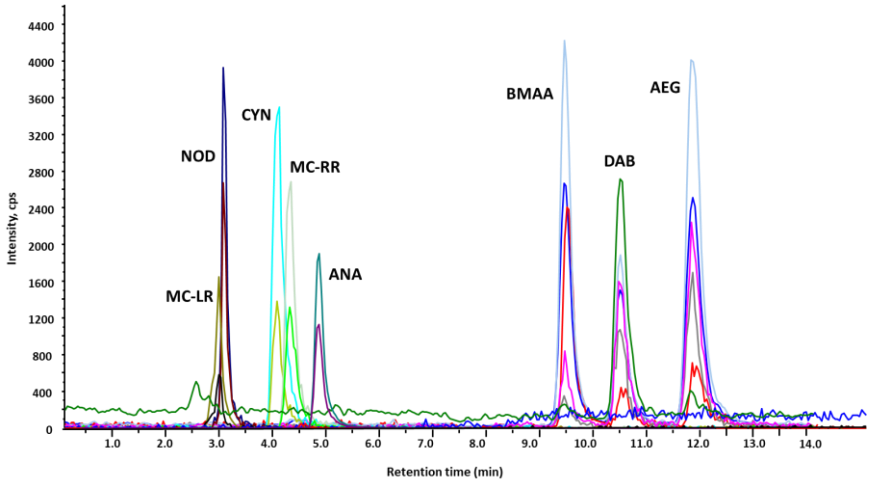


Figure 2. Extracted ion chromatogram of a mixture of cyanotoxins under the optimized HILIC-MS/MS conditions. Analyte concentration: 30 μg/L for MC-LR, NOD, CYN and MC-RR; 60 μg/L for ANA; 200 μg/L for AEG; 400 μg/L for BMAA and DAB.

3.2. Optimization of SPE procedure using standard solutions

Simultaneous SPE of the target cyanotoxins is a challenge due to their diverse physicochemical properties. MC-LR and MC-RR have a NPA named (all-S,all-E)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (ADDA) with a lipophilic side chain, two negatively charged carboxylic groups at neutral pH and a positive charge in the guanidine group (pKa > 12, calculated by using Advanced Chemistry Development software V11.02 (ACD/Labs, Toronto, Ontario, Canada) [1]) as both are arginine-containing MCs. NOD is structurally and toxicologically related to MCs and it also presents positively charged amino groups at neutral pH. CYN is highly hydrophilic and carries both a positive and a negative charge at neutral pH. ANA is also hydrophilic and has a positive charge at neutral pH. Isomers BMAA, DAB and AEG are small molecules and polar amino acids, so they can be in positive, negative or zwitterionic form, depending on the pH. At a pH below their isoelectric point, they carry a net positive charge, which facilitates their interaction with cation exchangers (**Table 1**).

Our study was focused on developing a SPE method and optimizing parameters that could affect the simultaneous extraction of the cyanotoxins from reservoir water samples. Thus, extraction parameters such as sorbent type and size, sample pH, composition and volume of the elution solvent and breakthrough volume were evaluated to select the optimum conditions by assessing the recovery of each step. For SPE optimization, samples were aqueous standard solutions containing 30 μg/L for MC-LR, NOD, CYN and MC-RR, 300 μg/L for ANA and AEG and 400 μg/L for BMAA and DAB. Samples were loaded into the cartridge without subsequent washing steps. After that, the cartridge was dried prior the elution step. Eluate was collected, evaporated to dryness, reconstituted in 250 μL of 60% MeCN with 0.1% FA (v/v), filtered with hydrophilic PTFE filters and injected into the HILIC-MS/MS system.

3.2.1. Type of sorbent

Taking into account that all the target cyanotoxins contain amino groups, which are positively charged at pH below their pKas, the use of cation exchangers was thoroughly investigated. Non-ionic cartridges were also evaluated. The selection of the appropriate cartridge was based on the evaluation and comparison of a wide variety of stationary phases from several suppliers. Preliminary studies were performed with 1 mL of deionized water spiked at the desired concentrations of each cyanotoxins and following the general SPE procedure recommended by the manufacturer for each sorbent. Non-ionic stationary phases such as Oasis HLB (60 mg, Waters) and Strata-X (60 mg, Phenomenex) were tested, eluting with MeOH. Weak cation exchange cartridges such as WCX (60 mg, Supelco) and Strata WCX (100 mg, Phenomenex) and a mixed-mode weak cation exchange Oasis WCX (60 mg, Waters) were tested, eluting with 2% HCl or $NH_3 \cdot H_2O$ in MeOH. Finally, the mixed-mode strong cation exchange cartridge Oasis MCX (60 mg, Waters) was tested, eluting with 5% $NH_3 \cdot H_2O$ in MeOH. In all cases, 5 mL of eluent was employed. The

non-ionic cartridges (HLB and Strata-X) showed an acceptable performance with non-polar compounds, although NPA isomers and ANA were scarcely retained. The weak cation exchange cartridges showed very low reproducibility and recoveries for most of the compounds, except for AEG, which was retained in both, Supelco and Strata WCX cartridges. However, the MCX cartridge provided satisfactory recoveries for the three isomers as well as for the rest of cyanotoxins, with values above 55% for all of them except for ANA (**Figure 3**).

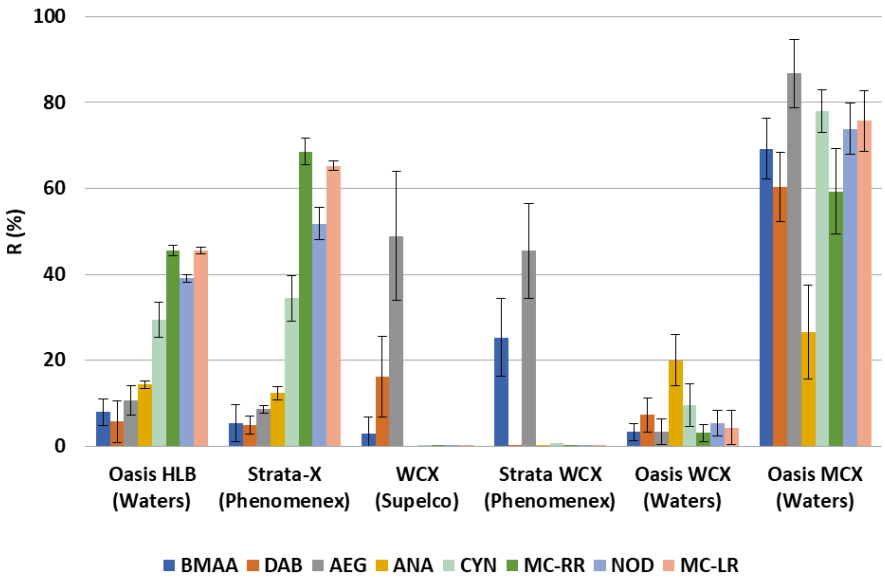


Figure 3. Efficiency (expressed as recovery, R (%)) of diverse SPE cartridges in the extraction of the selected cyanotoxins from a standard solution. Error bars represent the standard error (n=6).

In light of the results obtained from the overall assessment of individual SPE cartridges and in accordance with other author's suggestions [4], a more specific optimization was carried out using the mixed-mode strong cation exchange sorbent, Oasis MCX. These cartridges contain a mixed-mode polymeric sorbent with reversed-phase and strong cation functionalities (by sulfonated groups) that can interact selectively with neutral and basic compounds, therefore the retention mechanism of compounds by MCX includes both ion exchange and electrostatic interaction. Once the cartridge was selected, the sorbent mass of the cartridge (60 and 150 mg) was

evaluated showing that an increase in the amount of sorbent also increases the recoveries, especially for the NPA isomers, so a 150 mg MCX cartridge was selected.

3.2.2. Type of elution solvent

When using a MCX cartridge, a neutral organic eluate is not enough to desorb the analytes. Thus, an alkaline methanolic solution that helps to neutralize the charged cyanotoxins leading to their elution is necessary. Moreover, elution volume showed a crucial role especially for MC-RR elution [5]. To assess this influence, 1 mL of spiked deionized water was loaded onto the MCX cartridge and 5% $NH_3 \cdot H_2O$ in MeOH was selected as the elution solvent. Volumes from 1 to 10 mL were studied at a flow rate of 1 mL/min. It is noteworthy that when 1 mL of eluent was employed MC-LR, MC-RR, NOD and ANA were not eluted from the cartridge, showing higher interaction than the NPA isomers and CYN. However, increasing the elution volume up to 10 mL enhanced the recoveries for all analytes, so it was selected as the optimum value. Once the influence of elution volume was checked, the percentage of ammonia solution in MeOH was evaluated between 5-15% (v/v). Slightly better recoveries were obtained when increasing the percentage of aqueous ammonia up to 10%, so it was selected as the optimum composition. Then, the temperature of elution solvent was tested in order to improve the overall recoveries, however no significant differences were obtained between elution at room temperature or at 40 ˚C, so heating was not required.

3.2.3. Sample volume and sample pH

Sample volume was evaluated by spiking different volumes of deionized water (1, 10, 50, 75 and 100 mL) while keeping the cyanotoxin masses constant in all cases, loading them onto the MCX cartridge at a flow rate of 1 mL/min and eluting with 10 mL of 10% $NH_3 \cdot H_2O$ (v/v) in MeOH. In relation to the efficiency, expressed as recovery values, sample volume was not significant for most of the analytes except

for CYN, for which the recoveries highly decreased when the loading volume increased, which was in accordance with a previous publication [4]. This fact suggests that CYN is not strongly adsorbed in the stationary phase, probably due to its secondary amino group, which does not interact with the sulfonic groups as strongly as a primary amino group does, and also probably due to its high water solubility, which facilitates the early elution from the sorbent during the sample loading of relatively large volumes of water. Consequently, a volume of 10 mL was selected as sample loading volume.

The target cyanotoxins present amino groups positively charged at pH below their pKa (**Table 1**); therefore, acidification of water samples might be preferable prior to SPE to achieve satisfactory retention. We checked two situations: deionized water without any pH adjustment and pH 3 (adjusted with 37% HCl). Recoveries of NPA isomers slightly improved at pH 3 so it was selected as the optimum sample pH.

3.2.4. Evaporation and filtration

Evaporation after elution from the SPE cartridge permits preconcentration of the analytes and reconstitution of the sample with the most suitable solvent for injection into the HILIC column. Evaporation was carried out in a heating block under a gentle stream of N_2 at 30 °C. Filtration of the final extract is useful to extend column lifespan. However, this step may cause the loss of the target cyanotoxins. In order to reduce undesirable interactions between analytes and the filter membrane, different materials such as, CA, GF, PTFE, Nylon or PVDF from different suppliers, were tested by comparing in terms of recoveries filtrated standard solutions with unfiltered standard solutions. Hydrophilic PTFE filter membrane was the most reliable filter material for the elimination of suspended material as no significant differences were observed when comparing with unfiltered standard solution.

3.3. Optimization of the SPE procedure using reservoir water samples

After SPE optimization studies using spiked deionized water, reservoir water samples from "El Portillo" (Granada, Spain) were submitted to the final SPE protocol. Spiked reservoir water samples were prepared by adding the desired volume of standard working solutions in 10 mL of water sample to obtain concentrations of 30 μg/L for MC-LR, NOD, CYN and MC-RR, 300 μg/L for ANA and AEG and 400 μg/L for BMAA and DAB. Although the recoveries for all cyanotoxins with MCX cartridges were above 60% in deionized water, a decrease was observed for the NPA isomers, NOD and MCs when real reservoir water samples were analyzed (**Figure 4**). The main difference was related to MC-RR, the recoveries of which were negligible when spiked reservoir water was analyzed. This result could be explained by two main reasons: a practically non-existent retention of the target cyanotoxin in the sorbent, or an ineffective elution from the cartridge. The analysis of the collected reservoir water sample after the loading step showed that MC-RR was retained entirely on the Oasis MCX cartridge. Therefore, the elution of MC-RR was not occurring probably due to some interaction caused by compounds present in natural water samples. To verify the negative influence of the nature of the matrix in the elution of MC-RR from the cartridge, mixtures of different proportions of reservoir water and deionized water (from 0% to 100% of reservoir water) were spiked and submitted to the whole SPE procedure. In accordance with the aforementioned, the elution of MC-RR decreased when the proportion of reservoir water in the sample increased, being negligible when 100% of reservoir water sample was analyzed.

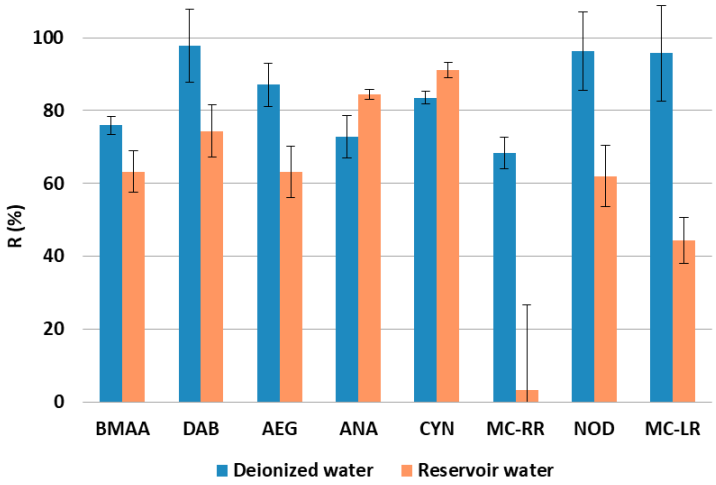


Figure 4. Efficiency (expressed as recovery, R (%)) of the optimized SPE at the selected conditions using standard solution compared to reservoir water samples. Error bars represent the standard error (n=6).

The obtained results indicated that a modified sample treatment approach was necessary. Alternatively, the use of a tandem SPE assembly with an extra cartridge could enhance the overall efficiency of the method, enabling the simultaneous extraction and elution of all cyanotoxins despite their different characteristics.

3.3.1. Evaluation of a tandem SPE assembly

Two types of non-ionic cartridges (Oasis HLB and Strata-X, 200 mg) were evaluated under MCX optimized conditions. The combination of the two sorbents could allow a successful recovery of MC-RR, since it could be retained on the non-ionic cartridge, which would be configured first in the line of the sample flow, followed by the MCX cartridge.

To that end, 10 mL of reservoir water sample spiked with 30 µg/L for MC-LR, NOD, CYN and MC-RR, 300 µg/L for ANA and AEG and 400 µg/L for BMAA and DAB were loaded onto the cartridges which were previously conditioned with 3 mL of MeOH and 3 mL of deionized water at pH 3. After drying the cartridges, analytes were eluted with 10 mL of 10% $NH_3 \cdot H_2O$ (v/v) in MeOH. The extract was dried under

a gentle stream of N_2 and reconstituted in 250 μL of 60% aqueous MeCN 0.1% FA prior to the injection into the HILIC-MS/MS system. Both sorbents showed a similar trend, however, the reversed phase functionalized polymeric sorbent Strata-X cartridge provided better overall recoveries for MCs, NOD, CYN and ANA while NPA isomers were scarcely retained in both Strata-X and HLB cartridges. Then, Strata-X and MCX cartridges were connected in series, being Strata-X cartridge configured at the top and MCX cartridge at the bottom of the tandem SPE system. After the conditioning step, spiked reservoir water sample was loaded at 1 mL/min onto the dual-cartridge set-up and then they were completely dried. The non-polar compounds were highly retained in the Strata-X cartridge, at the top of the tandem SPE assembly, while the more polar compounds leached through the first cartridge and were retained on the subsequent MCX cartridge. It is noticeable that compounds partially retained in the first cartridge like ANA and CYN, would be retained in the MCX cartridge, minimizing analyte losses. For analyte elution, the order of the cartridges was reversed, MCX being at the top and Strata-X at the bottom of the dual SPE system. This change is required to avoid MC-RR retention on the MCX sorbent. The elution step was performed using 10 mL of 10% $NH_3 \cdot H_2O$ in MeOH. Although usually the Strata-X cartridges are eluted with acidified methanol, an alkaline methanolic solution is necessary to neutralize and desorb the positively charged compounds from MCX cartridge. In spite of this, no negative effects were observed in the recoveries of non-polar compounds retained in Strata-X. In fact, overall recoveries highlighted that the proposed dual SPE strategy avoided the drawbacks resulting from the retention of MC-RR on MCX cartridge, achieving recoveries above 85% (**Figure 5**). At this point, some parameters affecting the dual SPE performance were re-optimized in natural reservoir water samples.

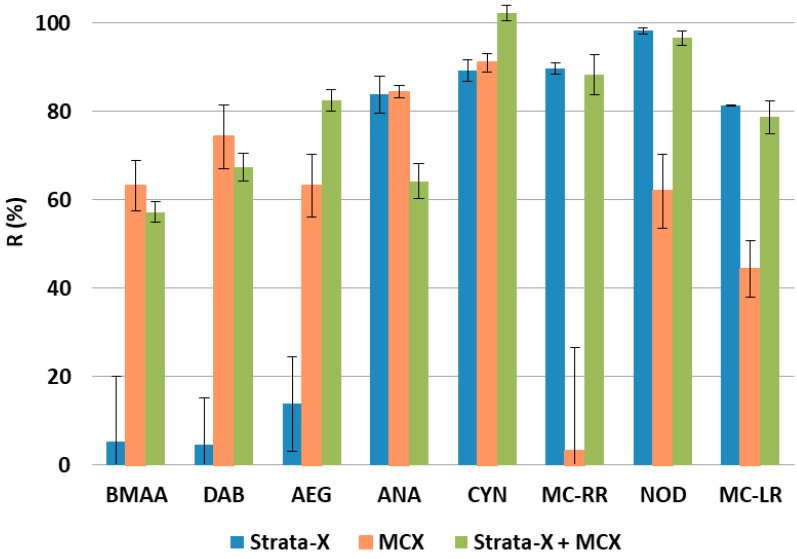


Figure 5. Efficiency of the proposed tandem SPE set-up compared with a single cartridge SPE mode, applied in reservoir water samples (expressed as recovery, R (%). Error bars represent the standard error (n=6).

3.3.2. Breakthrough volume

During the SPE procedure, a sudden drop in the recovery values can occur due to sorbent saturation when large volumes of sample are loaded. The breakthrough volume is important because it is related to the preconcentration factor, thus affecting the sensitivity of the method. To evaluate the breakthrough volume, a series of experiments with increasing volumes of spiked reservoir water samples (10, 25, 50 and 75 mL), maintaining the cyanotoxin masses constant in all cases, were carried out using the previously explained procedure. It was observed that an increase in the sample volume barely affected the retention of the non-polar MCs and NOD; however, volumes above 25 mL highly decrease the recoveries of the most polar compounds, especially for BMAA and DAB, although AEG and CYN were also negatively affected (**Figure 6**). The obtained results were in line with a previous work that highlighted that the competition with natural organic matter greatly reduced the adsorption capacity of cartridges and accelerated the leakage of BMAA and DAB [6]. Thus, the breakthrough volume of the entire method was set at 25 mL. Taking into account that 250 µL was the volume of the optimum injection solvent employed to

re-dissolve the dried extracts, a preconcentration factor of 100 was achieved. It is noteworthy that no washing step was needed.

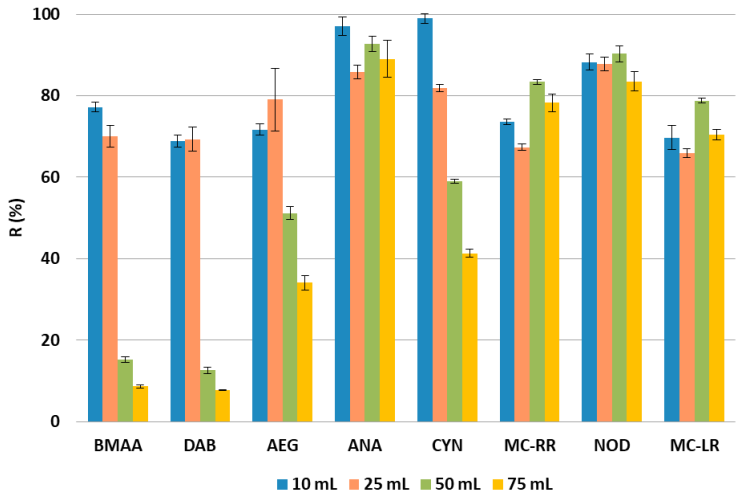


Figure 6. Effect of sample loading volume on the efficiency (expressed as recovery, R (%)) of the proposed tandem SPE procedure. Error bars represent the standard error (n=6).

3.3.3. Volume of elution solvent

With the aim of reducing the time of the sample treatment while maintaining the extraction efficiency, the volume of the elution solvent was evaluated using a natural reservoir water sample; therefore 5, 7.5 and 10 mL were tested. It was observed that the elution volume could be reduced to only 5 mL of 10% $NH_3 \cdot H_2O$ (v/v) in MeOH maintaining satisfactory recoveries. **Figure 7** shows the final optimized tandem SPE procedure.

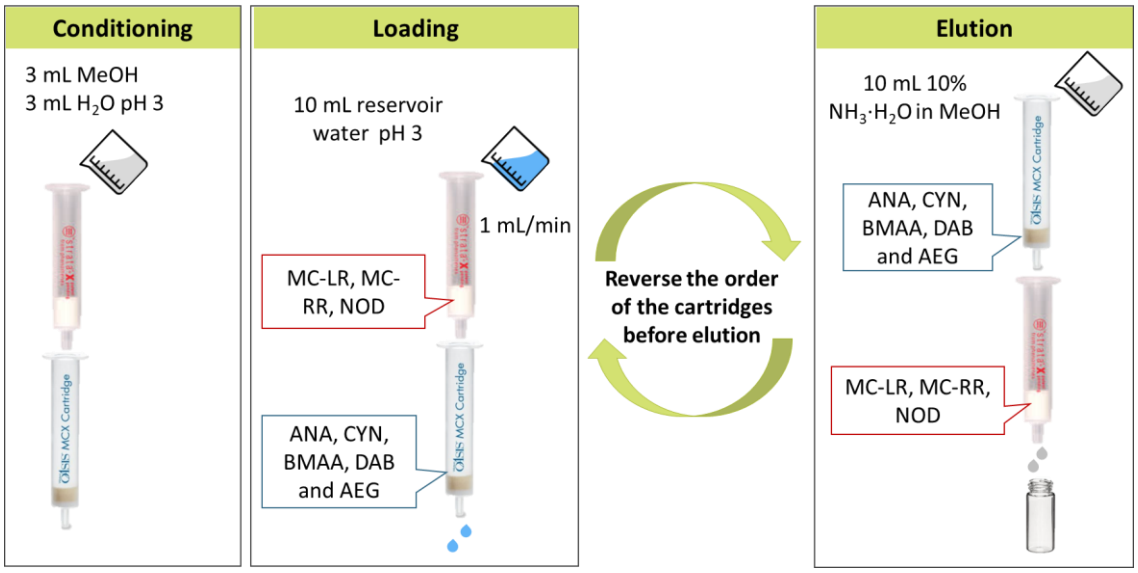


Figure 7. Proposed tandem SPE procedure for the extraction of multiclass cyanotoxins from natural reservoir waters.

3.4. Method characterization

The proposed tandem-SPE-HILIC-MS/MS method was validated in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), extraction efficiency, matrix effect and precision (i.e., repeatability and intermediate precision), using reservoir water samples from "El Portillo" (Granada, Spain). To assess specificity, blank samples were analyzed and no interfering peaks were observed comigrating at the retention time of the analytes.

3.4.1. Calibration curves and analytical performance characteristics

The calibration curves were established for the studied analytes in reservoir water samples according to the optimized tandem-SPE-HILIC-MS/MS procedure described above. Procedural calibration curves were established using reservoir water samples from "El Portillo", spiked at different concentrations of the eight studied cyanotoxins and submitted to the whole analytical procedure (from $7 \cdot 10^{-3}$ to 0.15 µg/L for MC-LR and NOD, from $4 \cdot 10^{-3}$ to 0.10 µg/L for CYN and MC-RR, from 0.1 to 2 µg/L for ANA, from 0.05 to 1 µg/L for BMAA, from 0.03 to 1 µg/L for DAB and from 0.02 to 0.4 µg/L

for AEG). Procedural calibration curves compensate both the matrix effect and the sample treatment losses. Six concentration levels were evaluated, with the lowest ones corresponding to the LOQ. Two samples per each concentration level were processed following the SPE method and injected in triplicate (n=6). Sample concentration levels were selected based on the preconcentration factor achieved by the sample preparation method. Peak area was considered as the response signal, being linearly dependent on the concentration of analyte in the sample. In all cases, acceptable linearity ($R^2 > 0.991$) was achieved.

Statistical parameters, calculated by least-square regression, and the performance characteristics of the tandem-SPE-HILIC-MS/MS method for reservoir water samples are shown in **Table 4**. LODs of each analyte were calculated as the lowest concentration for which the peak height was, at least, three times the signal-to-noise ratio while LOQs were calculated as the lowest concentrations, which fulfilled method performance acceptability criteria of a signal-to-noise ratio of at least 10. Very low LOQ values (between $7 \cdot 10^{-3}$ and 0.1 μg/L) were reached as a consequence of the high analyte preconcentration obtained during the sample treatment.

3.4.2. Matrix effect

The matrix effect (ME) is the result produced on the analytical response by the components of the sample other than the analytes. ME was assessed by comparing the analyte response from blank samples spiked after the tandem SPE procedure with the analyte response of standard solutions at the same concentration levels. Two concentration levels, L1 and L2, corresponding with the LOQ values and 7.5 x LOQ values, respectively, were studied for each analyte (**Table 4**). A ME higher than 100% means that the analytical response is enhanced while a ME lower than 100% means that the analytical response is suppressed. Significant signal suppression can be observed for ANA while a signal enhancement occurred for AEG at the lowest level

of concentration. However, procedural calibration curves take into account these matrix effects.

3.4.3. Precision studies

The precision of the method was evaluated in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the whole tandem-SPE-HILIC-MS/MS method to reservoir water samples spiked at L1 and L2 concentration levels. Repeatability was assessed over three samples for each concentration level and analyzed in triplicate (n=9) on the same day under the same conditions. Intermediate precision was evaluated with a similar procedure, with five samples analyzed in triplicate in five consecutive days (n=15). The results, expressed as relative standard deviation, RSD (%) of peak areas, are shown in **Table 4**.

Table 4. Statistical parameters and performance characteristics for the proposed tandem-SPE-HILIC-MS/MS method for the monitoring of cyanotoxins in reservoir waters.

Analyte	Linear range (μg/L)	LOD (μg/L)	LOQ (μg/L)	R^2	Intra-day precision (RSD %) n=9		Inter-day precision (RSD %) n=15		Matrix effect (%) n=9	
					L1	L2	L1	L2	L1	L2
MC-LR	$7{\cdot}10^{-3}$ - 0.15	$2.1{\cdot}10^{-3}$	$7{\cdot}10^{-3}$	0.997	10.8	6.9	10.6	7.5	130.7	123.1
NOD	$7{\cdot}10^{-3}$ - 0.15	$2.1{\cdot}10^{-3}$	$7{\cdot}10^{-3}$	0.995	5.7	5.6	9.3	7.3	92.0	90.8
CYN	$4{\cdot}10^{-3}$ - 0.10	$1.2{\cdot}10^{-3}$	$4{\cdot}10^{-3}$	0.997	5.5	3.3	6.4	5.1	69.8	62.0
MC-RR	$4{\cdot}10^{-3}$ - 0.10	$1.2{\cdot}10^{-3}$	$4{\cdot}10^{-3}$	0.999	8.2	8.0	6.1	9.0	78.9	67.6
ANA	0.10 - 2.00	0.03	0.10	0.997	4.5	3.6	6.4	14.0	11.4	16.8
BMAA	0.05 - 1.00	0.015	0.05	0.991	7.9	14.1	7.7	12.2	75.9	52.1
DAB	0.03 - 1.00	$9{\cdot}10^{-3}$	0.03	0.998	7.3	4.6	9.7	7.7	132.2	120.0
AEG	0.02 - 0.40	$6{\cdot}10^{-3}$	0.02	0.994	3.7	2.8	12.7	5.7	253.2	134.1

Concentration levels were established for each cyanotoxin as the LOQ (L1) and 7.5 x LOQ (L2). L1 and L2 were set to $7{\cdot}10^{-3}$ and 0.05 μg/L for MC-LR and NOD; $4{\cdot}10^{-3}$ and 0.03 μg/L for CYN and MC-RR; 0.1 and 0.75 μg/L for ANA; 0.05 and 0.375 μg/L for BMAA; 0.03 and 0.225 μg/L for DAB and 0.02 and 0.15 μg/L for AEG.

Satisfactory precision was obtained, as in all cases, RSD was lower than 14.1%. Chromatograms of a reservoir water sample analyzed by the proposed method, spiked at L2 concentration for each cyanotoxin, and its corresponding blank are shown in **Figure 8**.

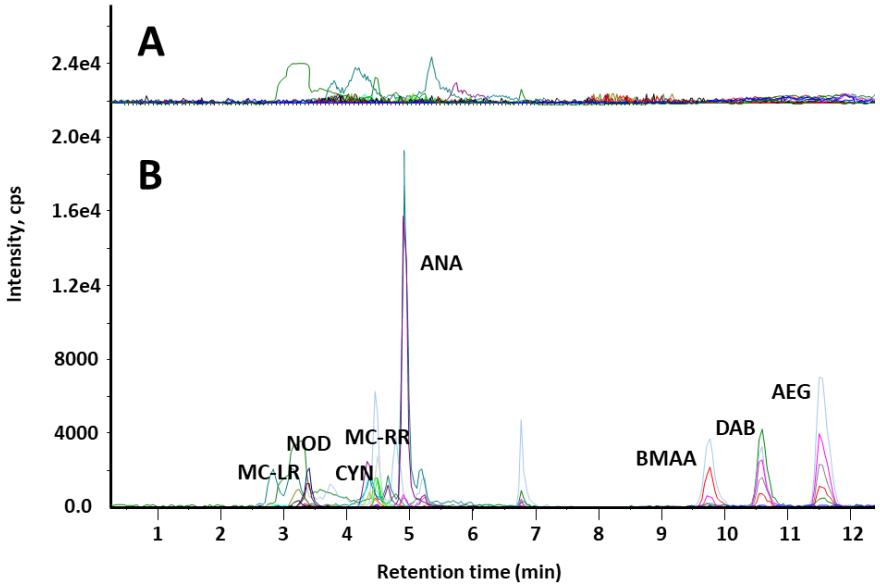


Figure 8. Extracted ion chromatogram of: A) Blank of reservoir water sample. B) A reservoir water sample spiked at L2 for each analyte, both obtained by application of the proposed tandem SPE-HILIC-MS/MS method.

3.4.4. Recovery study

Recovery experiments were carried out in reservoir water samples from different locations (see section 2.3) spiked at two concentration levels (L1 and L2). Three water samples from each reservoir were spiked at each concentration level, processed according to the tandem SPE procedure and subsequently analyzed by HILIC-MS/MS in triplicate (n=9). In all cases a blank sample was analyzed in order to check the absence of matrix compounds co-eluting with the cyanotoxin peaks. Recoveries were estimated as the relation between the peak area of samples spiked before the sample treatment and the peak area of samples spiked after sample treatment. Results

showed recoveries ranging between 70.9 and 94.7% with associated RSD < 15.3% for "El Portillo" reservoir water samples, and ranging between 75.4 and 93.6% with associated RSD < 17.3% for "Canales" reservoir water samples (**Table 5**). However, when water samples from "Bermejales" and "Cubillas" reservoirs were submitted to the recovery study, low recoveries were obtained for the isomers BMAA and DAB (16.2-33.3%). Similarly to our results, in a previous work where BMAA and DAB were extracted by an SPE protocol using Oasis MCX cartridges, an important reduction of the SPE performance occurred when natural water samples were analyzed instead of pure water [6]. This issue was attributed to the presence of cations, which compete for the active ion-exchange sites, and to the presence of natural organic matter, which causes pore blocking. In fact, the recoveries obtained from the diverse reservoir water samples in the present work are aligned with their content of total ions. Thus, "Bermejales" and "Cubillas" reservoir waters, which have the highest conductivity values (367 and 555 μS/cm, respectively) as a consequence of their highest number of ions, especially sulfates (153 and 124 μg/kg, respectively), presented the lowest recovery values for BMAA and DAB. On the contrary, "Canales" reservoir water samples, which has a much lower conductivity, similar to that of "El Portillo" (115 and 191 μS/cm, respectively), provided satisfactory recovery values with acceptable associated precision, showing that the present method could satisfactorily be applied to determine BMAA and DAB on waters with conductivities below 200 μS/cm.

Table 5. Recovery assessment for reservoir water samples of different origins in Granada province (Spain).

	El Portillo				Canales				Bermejales				Cubillas			
	L1		L2		L1		L2		L1		L2		L1		L2	
	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %	R %	RSD %
MC-LR	88.5	9.6	86.8	8.0	93.2	16.5	84.0	7.0	74.6	4.6	80.6	4.7	76.7	9.4	75.1	4.8
NOD	92.8	6.8	94.7	5.7	87.9	10.8	88.9	6.5	101.0	10.5	91.8	1.7	86.2	13.6	91.2	9.9
CYN	88.8	6.2	90.6	2.2	80.7	17.1	75.4	7.7	81.8	15.4	85.2	9.1	90.6	8.2	89.1	9.3
MC-RR	87.8	6.7	89.2	9.3	84.7	5.8	88.4	6.6	92.1	10.2	89.5	6.2	91.9	17.5	88.3	9.2
ANA	88.1	5.8	82.9	6.2	83.7	10.3	82.3	3.2	88.2	3.8	82.3	3.5	92.8	9.7	84.3	7.1
BMAA	74.6	15.3	70.6	7.8	87.1	17.3	80.4	7.1	16.2	34.0	26.1	11.9	27.2	36.6	26.7	10.5
DAB	70.9	10.9	74.5	5.6	93.6	8.4	80.9	7.5	26.2	35.9	26.9	11.3	33.3	15.8	25.5	10.7
AEG	71.6	4.8	80.7	4.1	87.6	15.9	76.6	14.0	91.4	13.7	75.6	5.3	76.0	12.4	94.7	15.0

Concentration levels were established for each cyanotoxin as the LOQ (L1) and 7.5 x LOQ (L2). L1 and L2 were set to $7{\cdot}10^{-3}$ and 0.05 μg/L for MC-LR and NOD; $4{\cdot}10^{-3}$ and 0.03 μg/L for CYN and MC-RR; 0.1 and 0.75 μg/L for ANA; 0.05 and 0.375 μg/L for BMAA; 0.03 and 0.225 μg/L for DAB and 0.02 and 0.15 μg/L for AEG. BMAA and DAB for reservoir water samples from Bermejales and Cubillas cannot be quantified.

4. Concluding remarks

The presence of cyanobacteria and their toxins in drinking or recreational waters may present a risk for human health. In this context, our study was focused on the development of a new method for the determination of 8 multiclass cyanotoxins (MC-LR, MC-RR, NOD, ANA, CYN, BMAA, DAB and AEG) in reservoir water samples. Separation based on HILIC coupled with tandem MS detection was carried out in less than 12 minutes. Prior the chromatographic separation, a tandem SPE procedure was developed for the simultaneous extraction of all cyanotoxins, despite their different physicochemical properties. For this purpose, a Strata-X and a MCX cartridges were connected in series, being the Strata-X cartridge at the top and the MCX cartridge at the bottom of the dual system. Before the elution step, the order of the cartridges was reversed in order to fully elute MC-RR. In this manner, it was possible to extract the

target cyanotoxins from reservoir water samples by means of a single sample loading step and the use of a single eluent in the elution step. This makes the process much more efficient, in contrast with previous studies where different procedures are employed for each family of cyanotoxins [7,8,9]. Furthermore, this is an innovative work as it includes the joint extraction of NPA isomers with other cyanotoxins from different families such as cyclic peptides and alkaloids. The method was validated reporting adequate linearity, repeatability, reproducibility, recoveries and matrix effect yields for the considered cyanotoxins. The resulting LOQs, ranging from $7 \cdot 10^{-3}$ to 0.1 μg/L, were significantly lower than the guideline limit value proposed by WHO for MC-LR in drinking water. In addition, LOQs were lower than those obtained previously with diverse dual SPE systems prior to the application of LC-MS/MS for the quantification of cyanotoxins [10,11,12,13]. This method was satisfactorily applied to reservoir water samples from different locations and characteristics, suggesting its possible application in the monitoring of the presence of target analytes in these matrices. Only BMAA and DAB showed a reduction of their recoveries when reservoir water samples with high content of ions were submitted to the tandem SPE procedure. In conclusion, this work will advance our knowledge of cyanotoxins in natural waters, allowing, for the first time, the simultaneous extraction and separation of the target multiclass cyanotoxins using an HILIC-MS/MS method.

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CHAPTER 4

DETERMINATION OF MULTICLASS CYANOTOXINS IN BLUE-GREEN ALGAE (BGA) DIETARY SUPPLEMENTS USING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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1. Summary

In recent years the consumption of blue-green algae (BGA) dietary supplements is increasing because of their health benefits. However, cyanobacteria can produce cyanotoxins, which present serious health risks. In this work we propose hydrophilic interaction liquid chromatography coupled with tandem mass spectrometry (HILIC-MS/MS) to determine cyanotoxins in BGA dietary supplements. Target toxins, including the microcystins (MCs) MC-LR and MC-RR, nodularin, anatoxin-a and three non-protein amino acids β-N-methylamino-L-alanine (BMAA), 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl)glycine (AEG), were separated using a SeQuant ZIC-HILIC column. Cyanotoxin extraction was based on solid-liquid extraction (SLE) followed by a tandem-solid phase extraction (SPE) procedure using Strata-X and MCX cartridges. The method was validated for BGA food supplements obtaining quantification limits from 60 to 300 μg/kg. Nine commercial BGA dietary supplements were analyzed, and DAB, AEG, and MCs were found in some of the samples, highlighting the relevance of monitoring these substances as precaution measures for the safe consumption of these products.

2. Experimental

2.1. Material and reagents

Commercial standards for microcystin-leucine-arginine (MC-LR ≥ 99%), microcystin-arginine-arginine (MC-RR ≥ 99%), nodularin (NOD ≥ 95%) and anatoxin-a (ANA ≥ 98%) were supplied by Enzo Life Sciences, Inc. (Lausen, Switzerland). Isomers β-N-methylamino-L-alanine hydrochloride (BMAA ≥ 97%), 2-4-diaminobutyric acid dihydrochloride (DAB ≥ 95%) and N-(2-aminoethyl)glycine (AEG ≥ 98%) were supplied by Sigma Aldrich (Darmstadt, Germany). The physicochemical properties of the compounds have been included in in **Chapter 3**.

Stock standard solutions were prepared by adding 1 mL of the desired solvent directly into the vial of the toxin supplied by the manufacturer, which was gently swirling to dissolve the analyte. The obtained solutions were: 50 μg/mL MC-LR in methanol, 50 μg/mL MC-RR in 80% aqueous MeOH, 50 μg/mL NOD in 50% aqueous MeOH, 1000 μg/mL ANA in water. Stock solutions of 1000 μg/mL for the three standard isomer molecules (BMAA, DAB and AEG) were prepared by dissolving the desired amount of analyte in water. All of them were stored in the dark at −20 °C. Intermediate standard solutions of each compound at 2.5 μg/mL were prepared by dilution of the stock solutions with the corresponding solvent for each toxin. Working standard multi-toxin solutions containing the seven cyanotoxins were prepared when needed in 50% aqueous MeOH at the desired concentrations, depending on the experiment. These solutions were stored at 4 °C and equilibrated to room temperature before use.

HPLC grade acetonitrile (MeCN) and methanol (MeOH) were purchased from VWR (Radnor, PA, USA), ammonia solution ($NH_3 \cdot H_2O$) (30% assay) was purchased from Merck (Darmstadt, Germany), trichloroacetic acid was purchased from Panreac (Barcelona, Spain) and formic acid (FA) was purchased from Sigma-Aldrich (St. Louis,

MO, USA). Ultra-pure water (18.2 MΩ·cm resistivity) was obtained from an ultrapure water purification system (Milli-Q plus system, Millipore, Bedford, MA, USA).

Oasis MCX cartridges (150 mg) from Waters (Milford, MA, USA) and Strata-X cartridges (200 mg) from Phenomenex (Torrance, CA, USA) were used for the cyanotoxin extraction procedure. SPE tube adapters from Supelco Inc. (Bellefonte, PA, USA) were employed. CLARIFY Polytetrafluoroethylene (PTFE) hydrophilic syringe filter (0.2 μm × 13 mm) were supplied by Phenomenex (Torrance, CA, USA).

2.2. Instrumentation

Chromatographic separation was performed on an Agilent 1290 Infinity II System (Agilent Technologies; Waldbronn, Germany) equipped with a quaternary pump, a degasser, an autosampler (with 20 μL injection loop) and a column thermostat. Separation was achieved using a SeQuant ZIC-HILIC column (2.1 × 250 mm, 3.5 μm diameter, EMD Millipore; Billerica, MA). The HPLC system was coupled to an API 3200 triple quadrupole (QqQ) mass spectrometer (SCIEX; Darmstadt, Germany) equipped with a Turbo V electrospray ionization source. Instrumental data were collected by the Analyst® Software (version 1.5) using the Scheduled MRMTM Algorithm (SCIEX).

For sample treatment, an analytical balance with 0.001 g resolution (Sartorius; Goettingen, Germany), a vortex-2 Genie (Scientific Industries; Bohemia, NY, USA), an Universal 320R centrifuge (Hettich Zentrifugen; Tuttlingen, Germany), a nitrogen dryer EVA-EC System (VLM GmbH; Bielefeld, Germany), a high speed solid crusher (Model 250A from Hukoer, China), an ultrasonic bath USC-300 model (VWR; Radnor, PA, USA), and a Visiprep solid-phase extraction unit from Supelco (Bellefonte, PA, USA) were used.

2.3. BGA dietary supplements

BGA dietary supplements were composed by the BGA *Spirulina* and *Aphanizomenon flos-aquae*, the microalgae *Chlorella*, and the brown algae *Fucus*, in different ratios or in pure form. They were sold as tablets, capsules and powder and they were obtained from local retail stores in Granada (Spain) and via Internet. Detailed information on all samples with their form, suppliers and BGA content are listed in **Table 1**. All the samples were analyzed before their expiration date.

Table 1. List of analyzed BGA dietary supplements.

Sample	Supplier	Form	Composition	Daily dose
1	Local store	Powder	Pure spirulina (A*throspira Platensis*)	6000 mg spirulina
2	Online	Capsule	Spirulina (*Platensis Gomont*), Hydroxypropyl methylcellulose, chlorella (*chlorella vulgaris Beijerinck*), fucus (*fucus vesicculosus L.*), vitamin C, magnesium stearate	891 mg spirulina, 445.5 mg chlorella, 445.5 mg fucus
3	Online	Tablet	Spirulina (*Arthrospira platensis*), Chlorella (*chlorella vulgaris*), ferrous fumarate, folic acid, vitamin B12	750 mg spirulina, 750 mg chlorella
4	Online	Tablet	Klamath (*Aphanizomenon flos-aquae*), spirulina (*Spirulina Platensis Geitler*), microcrystalline cellulose, silicon dioxide	600 mg klamath, 600 mg spirulina
5	Online	Tablet	Chlorella (C*hlorella Pyrenoidosa Chick*), silicon dioxide	3268 mg chlorella
6	Online	Tablet	Spirulina (S*pirulina Platensis Geitler*), dioxide de silício	2562 mg spirulina
7	Local Store	Capsule	Spirulina (*Arthrospira Platensis*), hypromellose	2160 mg spirulina
8	Local Store	Powder	Pure spirulina	Not available
9	Local Store	Powder	Pure klamath	Not available

2.4. SLE procedure

Each food supplement was ground individually with a grinder to obtain a fine powder, and a representative subsample was taken for extraction. BGA products (75 mg) were weighed into a 15-mL conical centrifuge tube. Then, 4 mL of the first extraction solvent, which consists of 5% aqueous FA, were added to the sample and it was mixed by vortex for 1 min and placed in an ultrasonic bath for 20 min. Afterwards, the sample was centrifuged for 10 min at 9000 rpm and 4 °C and the supernatant was transferred to a 50 mL centrifuge tube. The residual pellet was reextracted with 4 mL of 80% aqueous MeOH. The mixture was shaken thoroughly by vortex 1 min followed by sonication for 20 min in ultrasonic bath. After centrifuging at 9000 rpm and 4 °C for 10 min, the supernatant was collected and transferred to the 50 mL centrifuge tube containing the previous supernatant to combine both. The mixture was made up to a final volume of 16 mL with deionized water to obtain a 20% of MeOH in the solution. The crude extract is then purified on a combined "in-series" SPE system.

2.5. SPE procedure

For the SPE procedure, the previously reported method for the determination of cyanotoxins in reservoir water samples included in **Chapter 3**, was employed with some modifications [1]. The SPE procedure was carried out using an assembly of two cartridges, a Strata-X (200 mg, 6 mL) and Oasis® MCX (150 mg, 6 mL), connected in series. The two cartridges were conditioned and activated separately with 3 mL of MeOH followed by 3 mL of deionized water. Then, the cartridges were connected in series, being Strata-X cartridge at the top followed by the MCX cartridge, and the 16 mL extraction solution was loaded at a flow rate of 1 mL/min. Thereafter, the cartridge assembly was washed with 2 mL of 30% aqueous MeOH and dried under vacuum for 2 min. Before the

elution step, the order of the cartridges was reversed, thus MCX cartridge was configured first followed by the Strata-X cartridge. Elution was carried out using 5 mL of 10% $NH_3 \cdot H_2O$ in MeOH. The eluate was evaporated to dryness under a gentle stream of nitrogen in a heating block (30 °C) and the residue was re-dissolved with 250 μL of 60% MeCN with 0.1% of FA. The final extract was filtered through a CLARIFY-PTFE hydrophilic filter (0.2 μm × 13 mm), transferred to a 0.3 mL glass insert and analyzed by the proposed HILIC-MS/MS method.

2.6. HILIC separation

Chromatographic separation was performed in a SeQuant ZIC-HILIC (2.1 × 250 mm, 3.5 μm particle size) column using a mobile phase consisted of water as eluent A and MeCN as eluent B, both containing 0.3% (v/v) of FA as volatile acid, at a flow rate of 0.2 mL/min. Gradient program was established as follows (expressed as a percentage (v/v) of eluent B in mobile phase): 0 min, 60%; 3 min, 60%; 6 min, 40%; 15 min, 40%. At 15 min, mobile phase composition went back to initial conditions in 1 min, and it was maintained for 20 min in order to guarantee column equilibration and reproducibility between runs. Column temperature was set at 55 °C and a volume of 20 μL (full loop injection) was selected as injection volume.

2.7. MS/MS parameters

The determination of the target cyanotoxins was carried out using electrospray ionization tandem mass spectrometry (ESI-MS/MS) system operated under positive mode in scheduled multiple reaction monitoring (MRM) conditions. The source parameters were set as follows: source temperature (TEM), 550 °C; ion spray (IS) voltage, 5500 V; nebulizing and drying gases (GS1 and GS2, nitrogen), 50 psi (344.7 kPa), curtain gas (CUR, nitrogen), 25 psi (172.4 kPa) and collision gas (CAD, nitrogen) was set to 10 psi

(69.0 kPa). Main MRM parameters, including declustering potential (DP), entrance potential (EP), collision entrance potential (CEP), collision energy (CE) and collision cell exit potential (CXP), as well as precursor ions, product ions (used for quantification and identification purposes) and retention time are given in **Table 2**.

Table 2. MRM parameters for the analysis of cyanotoxins in BGA dietary supplements by HILIC-ESI-MS/MS.

Analyte	Retention time (min)	Molecular ion	Precursor ion (*m*/*z*)	DP (V)	EP (V)	CEP (V)	Product ions (*m*/*z*)	CE (V)	CXP (V)
MC-LR	2.8	$[M+H]^+$	995.6	136.0	10.5	32.0	Q_{ion} 135.2	93.0	4.0
				136.0	10.5	32.0	I_{ion} 105.0	129.0	4.0
NOD	3.0	$[M+H]^+$	825.4	96.0	6.5	24.0	Q_{ion} 135.1	89.0	2.0
				96.0	6.5	100.0	I_{ion} 103.2	129.0	4.0
MC-RR	4.0	$[M+2H]^{2+}$	519.8	41.0	6.0	18.0	Q_{ion} 135.2	39.0	4.0
				41.0	6.0	18.0	I_{ion} 103.2	91.0	6.0
ANA	4.8	$[M+H]^+$	166.2	36.0	4.0	10.0	Q_{ion} 131.1	21.0	4.0
				36.0	4.0	10.0	I_{ion} 43.1	39.0	4.0
BMAA	8.9	$[M+H]^+$	119.2	26.0	4.0	6.0	Q_{ion} 44.1	27.0	2.0
				26.0	4.0	6.0	I_{ion} 102.1	15.0	4.0
DAB	9.7	$[M+H]^+$	119.1	21.0	3.0	14.0	Q_{ion} 101.0	11.0	4.0
				21.0	3.0	14.0	I_{ion} 56.0	31.0	2.0
AEG	11.3	$[M+H]^+$	119.1	26.0	3.5	10.0	Q_{ion} 102.0	11.0	4.0
				26.0	3.5	10.0	I_{ion} 56.0	25.0	4.0

Abbreviations: declustering potential, DP; entrance potential, EP; collision entrance potential, CEP; collision energy, CE; collision cell exit potential, CXP; quantification ion, Q_{ion}; identification ion, I_{ion}.

3. Results and discussion

3.1. Optimization of chromatographic and MS/MS conditions

The chromatographic separation and MS/MS detection conditions employed were based on our previous work included in **Chapter 3**, for the analysis of these toxins in reservoir

water samples [1]. In this case, we have considered spirulina as matrix for the optimization study, a well-known BGA dietary supplement. A slightly gradient composition adjustment was considered in relation to this previous study, due to the complexity of spirulina matrix. Regarding the MS/MS detection, the precursor and product ions of individual analytes were those previously identified in **Chapter 3** by tuning after direct infusion of individual standard solutions of 1 mg/L in 0.1% aqueous FA:MeOH (50:50, v/v) into ESI(+)-MS/MS system. All the target cyanotoxins were determined using the protonated form ($[M+H]^+$) as precursor ion except for MC-RR, which tends to ionize as the diprotonated molecular ion ($[M+2H]^{2+}$). The fragment ion with the highest intensity was used for quantification (Q_{ion}) whereas the second one was acquired for identification (I_{ion}). However, it is worth highlighting the fact that in the case of ANA the most abundant MRM transition in standard solution, corresponding to *m*/*z* 166.1>149.0 (**Figure 1A**), could not eventually be used as Q_{ion} for the analysis of BGA dietary supplement samples because an interference peak with this same transition appeared at the same retention time than ANA (4.8 min). This peak did not correspond to ANA because it did not present any of the other characteristic transitions of ANA (**Figure 1B**). Thus, the fragment ion with the second highest intensity was selected as Q_{ion} (166.1>131.1) and the one with the third highest intensity was used as I_{ion} (166.1>43.1) for ANA. Furthermore, an intense peak with the same precursor and product ions than ANA but with different ion ratios appeared at 4.0 min just before ANA peak (**Figure 1C**). According to the literature, this peak corresponds to the essential amino acid Phenylalanine (Phe), which is an isobaric compound of ANA and presents a similar fragmentation pattern and LC retention [2]. In fact, misidentification of Phe as ANA has been reported in forensic investigation, especially in the presence of naturally occurring Phe. The assumption that this peak corresponds to Phe is also supported by the fact that

spirulina contains a moderately high amount of both essential and non-essential amino acids, being the content of Phe around 28 mg/g [3].

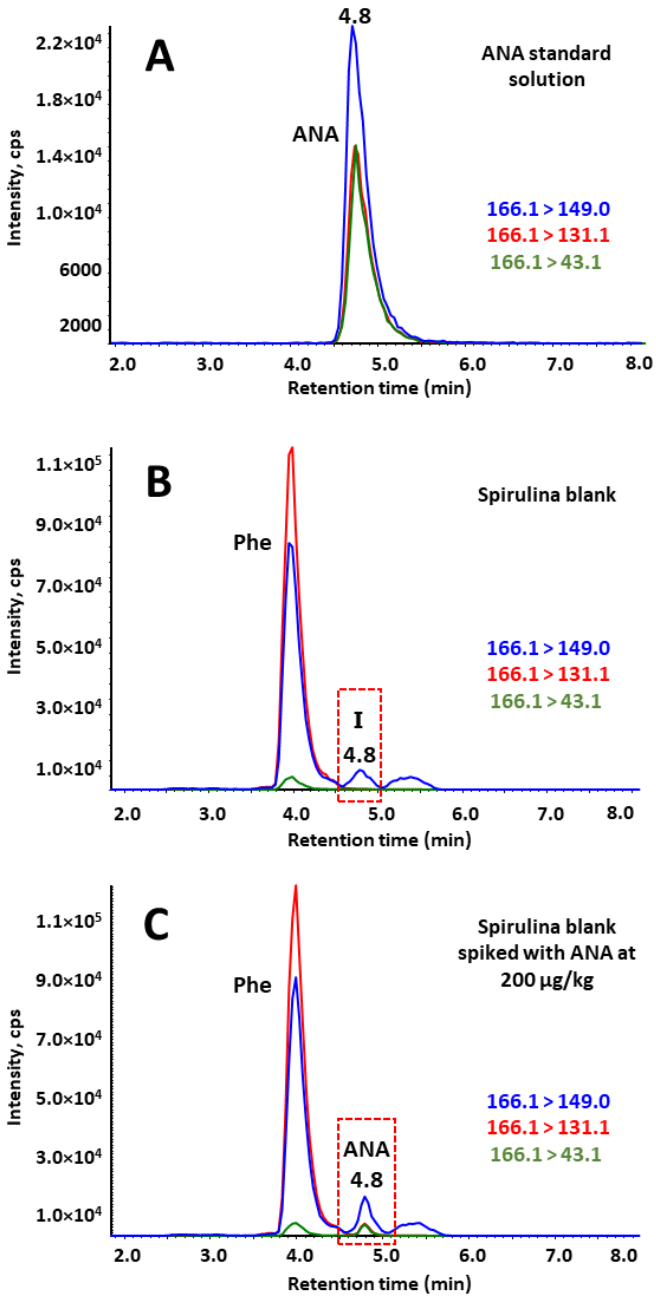

Figure 1. Extracted ion chromatograms of the three most intense transitions of ANA (blue 166.1 > 149.0, red 166.1 > 131.1 and green 166.1 > 43.1). A) Standard solution of ANA at 100 µg/kg; B) Spirulina blank sample; C) Spirulina blank sample spiked with ANA at 200 µg/kg. Phe: Phenylalanine; I: interference.

3.2. Multi-toxin extraction procedure

Cyanotoxin extraction from BGA supplements is strongly influenced by a wide range of features such as physicochemical properties and nature of the matrix. Because of this, most of the developed analytical methods do not include the extraction of a multitude of different congeners, and several approaches are usually employed for each family of toxins including differences in the extraction procedure but also in the separation method.

To extract the target cyanotoxins showing different polarities from the BGA dietary supplements, a SLE was firstly proposed, followed by an isolation and clean-up step based on a tandem SPE. Thus, it is possible to remove the remaining matrix minimizing interferences, and to achieve a preconcentration of the analytes increasing sensitivity, prior their injection into the HILIC-MS/MS system. For the sample treatment optimization, a dehydrated organic spirulina sample free of cyanotoxins was spiked with a mixture of the target toxins at concentration levels of 200 μg/kg for MC-LR, MC-RR, NOD and ANA and 1500 μg/kg for the isomers BMAA, DAB and AEG, depending on the sensitivity of the method for each analyte. Optimum extraction conditions were selected for achieving the highest recoveries, which were calculated by comparing the peak area of the analytes detected from a BGA dietary supplements spiked before and after the extraction procedure. Several parameters were assessed with the aim of improving the efficiency of the SLE procedure, being the more significant the extraction solvents. BMAA and its isomers can be associated in the cell substrate mainly as free or protein-bound forms. For the purpose of this paper, only the free cyanotoxin fractions were considered.

3.2.1. Study of solvents for SLE

Free BMAA and its isomers can be extracted from lyophilized samples or raw powder with a suitable solvent, typically 0.1-0.3 M trichloroacetic acid (TCA), although other solvents have also been used with this purpose, such as hydrochloric acid (HCl), acetonitrile (MeCN), methanol (MeOH) and acidified water with citric acid [4]. Regarding MCs, polar extractants such as mixtures of water with a polar or mid-polar organic solvent showed high recoveries [5], and when cyanobacteria were treated with methanol and hydrochloric or acetic acid, good recovery yields were obtained for anatoxins. Firstly, mixtures of MeOH, MeCN and EtOH with water (80:20, v/v) were tested as extraction solvents in BGA samples. This ratio was chosen according to the literature, where around 75-90% methanolic-water mixtures are usually employed to extract MCs and NOD with optimal recoveries from dietary supplements [6,7,8,9]. With this purpose, 50 mg of organic spirulina spiked with a mixture of cyanotoxins were extracted with 4 mL of the extraction mixtures, stirred 10 min and placed in an ultrasonic bath for 15 min. After centrifuging at 9000 rpm and 4 ˚C during 10 min the supernatant was transferred to a 50 mL centrifuge tube and made up to 32 mL with deionized water to achieve a 10% organic solvent (MeOH) in the extract before loading onto the tandem-SPE set-up. The aqueous extract was submitted to the tandem-SPE procedure previously described in **Chapter 3** [1]. Among all the tested solvents, 80% MeOH showed the best results with recoveries above 70% for MCs and NOD, and about 60% for ANA (**Figure 2A**). However, a deficient non-protein amino acid (NPA) extraction was obtained, with recoveries below the 50%. No improvement in recoveries was obtained when two extractions were made halving the extraction volume, so one extraction with 4 mL of 80% aqueous MeOH was maintained. Afterwards, with the aim of improving extraction efficiency of the NPAs, an acidic aqueous extraction was evaluated before the methanolic one as well as increasing

the sonication time. With this purpose, 4 mL of TCA 0.1 M and 20 min of sonication bath were employed. After centrifuging, the supernatant was transferred to a 50 mL centrifuge tube and stored. Then, 4 mL of 80% MeOH were added to the remaining pellet to repeat the extraction. The collected supernatants were combined and made up to 32 mL with water. The aqueous extract was submitted to the tandem-SPE procedure previously described and injected into the HILIC-MS/MS system. As expected, the application of a first 0.1M TCA extraction step before the methanolic one significantly increased the recoveries of the NPA isomers up to around 70% while recoveries values for the MCs and NOD remained unchanging around 80% (**Figure 2B**). However, it is remarkable that under these conditions, the peak corresponding to ANA, which should have appeared at 4.8 min, was missing and only the intense peak of Phe was observed between 3.0 and 3.7 min (**Figure 3A**). It was supposed that the remaining TCA present in the injected sample negatively affected the interaction of ANA with the column, reducing its retention time and causing its coelution with the big peak of Phe, precluding its determination. In the light of the obtained results, it seems clear that an aqueous acidic extraction enhances the extraction of NPAs, so the next step was to compare the extraction performance of a variety of acidic solvents. With this purpose, 4 mL of 0.1M TCA, 0.1M oxalic acid (OA) and 5% formic acid (FA) aqueous solution were evaluated as the first extraction step in the SLE procedure while keeping constant the rest of the procedure. Similar recoveries were obtained when using TCA and OA, being over 70% for NPAs and about 80% for MCs and NOD while ANA peak remained missing when both extraction solvents were employed. On the contrary, when 4 mL of 5% FA solution was employed, ANA peak was observed at 4.8 min, just after the large Phe peak (**Figure 3B**), with an acceptable recovery value of 60% (**Figure 2C**).

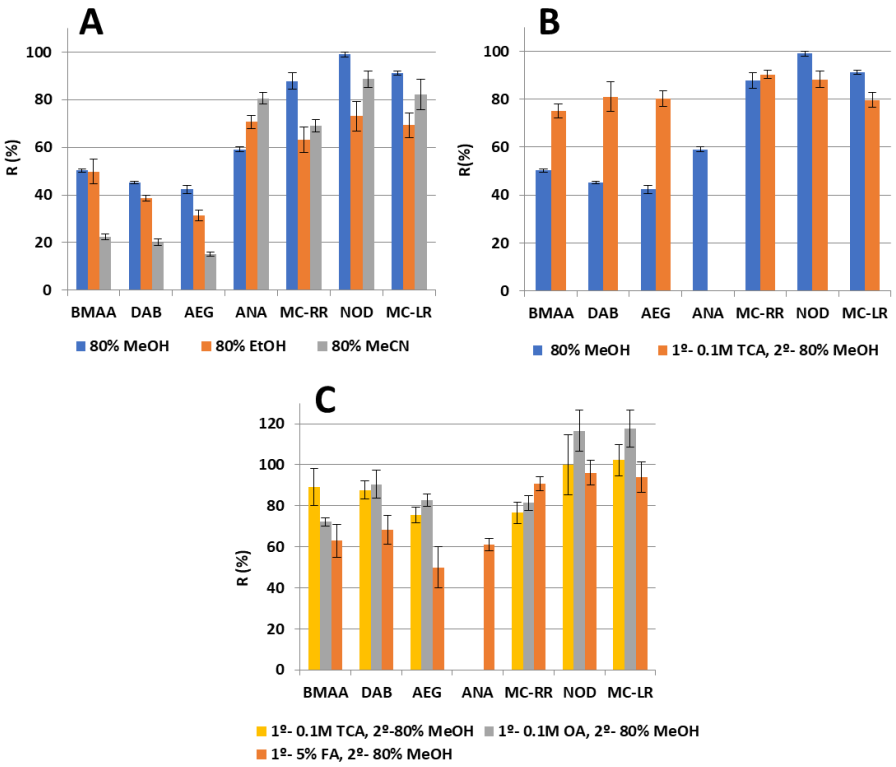

Figure 2. Study of the SLE procedure in relation to the extraction efficiency (R(%)): (A) Influence of the nature of the extraction solvent; (B) Efficiency of the SLE extraction when using aqueous 80% MeOH extraction versus when adding a previous extraction step with 0.1M TCA; (C) Influence of different acidic extractions in the first step of the SLE procedure. Error bars represent the standard error.

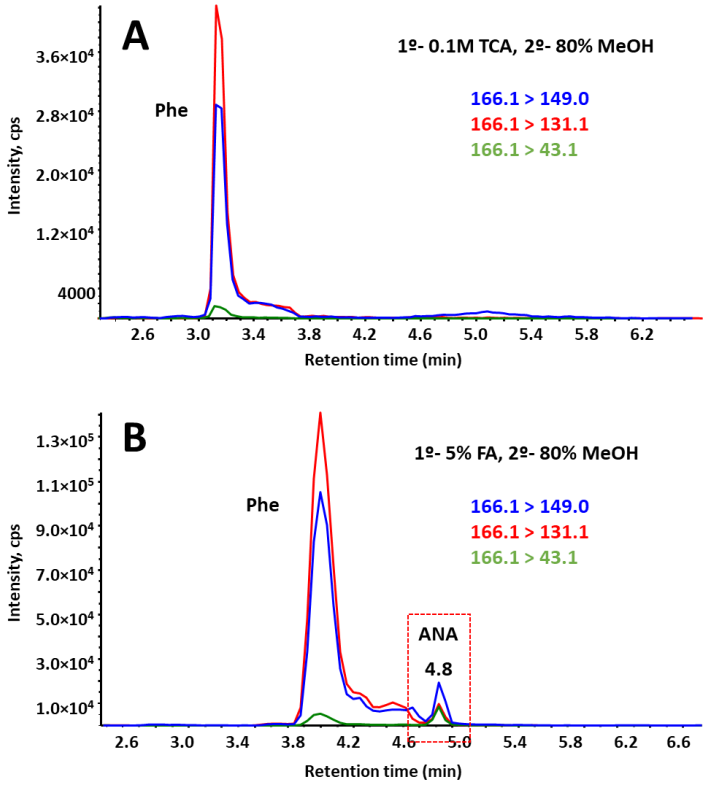

Figure 3. Extracted ion chromatograms of the three most intense transitions of ANA when the SLE is done with (A) 0.1M TCA followed by 80% MeOH; (B) aqueous 5% FA followed by 80% MeOH.

However, recoveries for NPAs declined below 60% and the reproducibility worsened so these problems were explored. Moreover, the final reconstituted extract after the SPE procedure tended to show a two-phase separation (**Figure 4**). This may be attributed to the remaining salts from the BGA dietary supplement in the dried extract, which caused a sort of salting-out effect when re-dissolving it in the 60% aqueous MeCN with 0.1% FA aqueous solution before the injection.

Figure 4. Final reconstituted extract after tandem-SPE procedure without a washing step.

3.2.2. SPE washing step

In this respect, the addition of a washing step after sample loading in the tandem-SPE procedure was critical for matrix removal and for the complete re-dissolution of the dried extract in the injection solvent. The washing solvent optimization study was performed for each cartridge separately. On the one hand, for Strata-X cartridge, 2 mL of different percentages of MeOH in water (from 0 to 50%) were tested to determine the maximum percentage that can be tolerated without any significant analyte loss. A 30% aqueous MeOH yielded the best results, as a higher amount of MeOH resulted in a decrease in MC-LR recoveries (**Figure 5A**). On the other hand, the washing solvents evaluated for MCX cartridge were 2 mL of different solvents such as: 100% MeOH, 30% MeOH, 2% FA in MeOH, and a two-step washing procedure with 2% aqueous FA followed by 100% MeOH, which is commonly used according to the protocol specified by the manufacturer for MCX cartridge. As expected, when analytes are strongly held by an ionic attraction, a 100% of MeOH proved to be a powerful washing step to remove matrix interferences. According to the data obtained, a 30% aqueous MeOH was selected as the optimum washing solvent for MCX cartridge because similar recoveries to those using 100% MeOH were obtained for all analytes (**Figure 5B**) and in this way the dual-cartridge system could be washed in a single step. The washing volume passing through the cartridges was

evaluated between 1 and 3 mL. A volume of 2 mL of 30% aqueous MeOH was selected as a satisfactory matrix removal was attained while limiting analyte breakthrough. With this washing step a single-phase final extract was achieved, overcoming the drawback of the two-phase separation in the final re-dissolved extract, which involved injection irreproducibility.

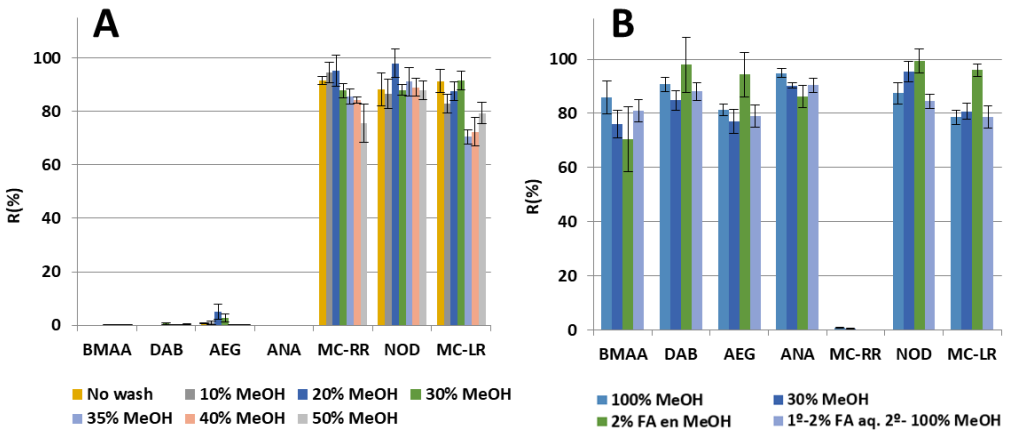

Figure 5. Evaluation of washing solvents in the SPE procedure: A) Strata-X cartridge ; B) MCX cartridge.

3.2.3. SPE loading volume and sample amount

After that, considering that a percentage up to 30% of MeOH was tolerated in the washing solvent, the percentage of MeOH in the SPE load solvent was modified in order to speed up the loading step. Therefore, when supernatants from SLE extraction were combined, they were filled with deionized water up to only 16 mL to reach a 20% MeOH in the final extract to be loaded. Finally, the amount of BGA dietary supplement sample to be treated was studied between 50 and 100 mg, being 75 mg the optimum value as higher amounts of sample decreased the MCs and NOD recoveries. The final procedure is described in sections 2.4 and 2.5.

A chromatogram of a BGA dietary supplement sample of spirulin spiked with the studied analytes submitted to the optimized sample treatment and analyzed by the proposed SLE-SPE-HILIC-MS/MS method is shown in **Figure 6**.

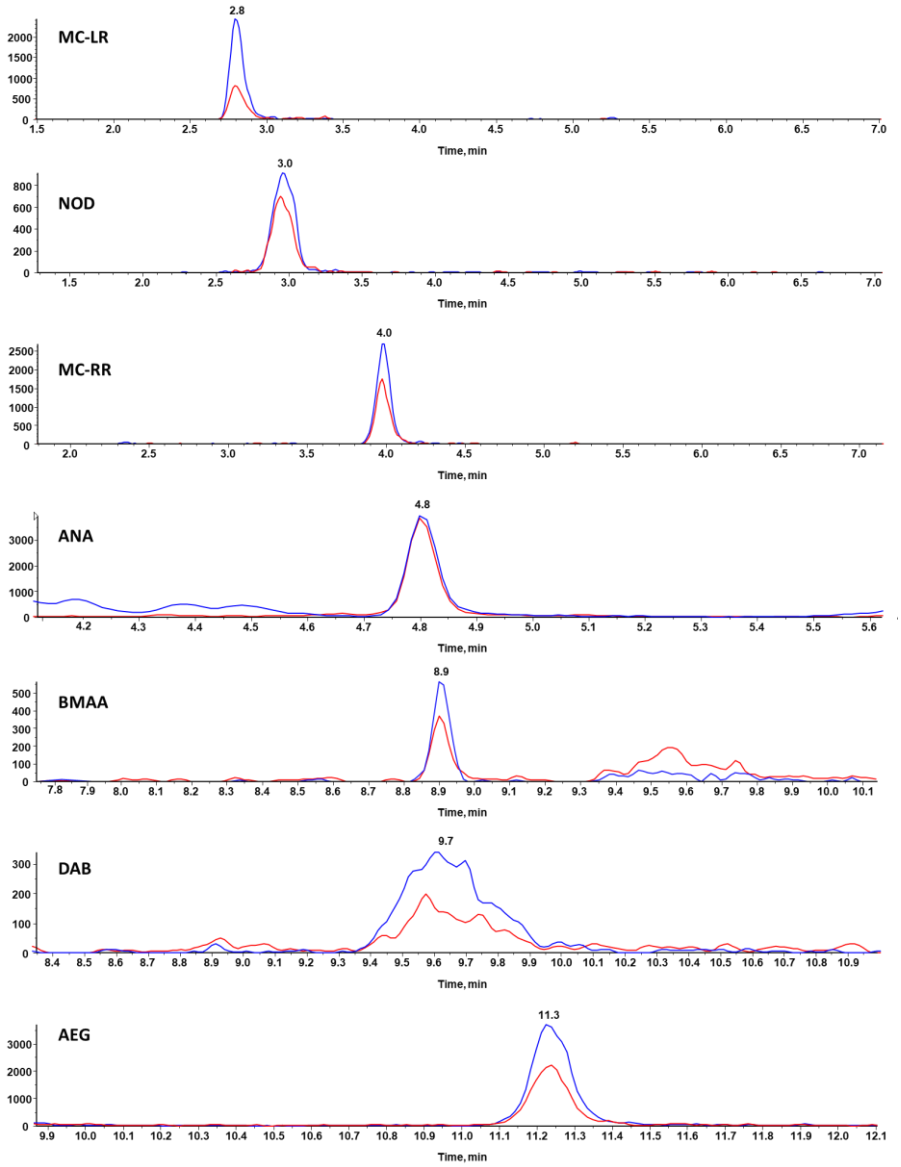

Figure 6. Chromatogram from a blank BGA dietary supplement sample spiked with a mixture of cyanotoxins at concentrations corresponding with the fourth point of the calibration curve and submitted to the whole proposed SLE-tandem SPE-HILIC-MS/MS method. Blue and red lines represent the Q_{ion} and I_{ion} transitions, respectively.

3.3. Method characterization

The proposed method was validated in terms of linearity, sensitivity, limit of detection (LOD) and limit of quantification (LOQ), extraction efficiency, precision (i.e., repeatability and intermediate precision) and matrix effect, using organic spirulina purchased from a local retail store. To assess specificity, blank samples were analyzed and no interferences of endogenous substances were observed comigrating at the retention times of the analytes of interest. Three concentration levels (L1, L2 and L3) were evaluated for precision, recovery, and matrix effect studies, which corresponded with the first (LOQ values), third and fourth concentration levels of the calibration curves for all analytes.

3.3.1. Calibration curves and analytical performance characteristics

Procedural calibration curves were established in BGA food supplement samples. In this regard, 0.5 g of blank sample were spiked at the beginning of the extraction procedure with the desired concentration of the target cyanotoxins, left to stand 15 min and then they were submitted to the optimized SLE-tandem-SPE-HILIC-MS/MS method previously described. Five concentration levels were evaluated, with the lowest level corresponding to the LOQ, ranging from 60 to 500 μg/kg for MC-LR, from 50 to 500 μg/kg for NOD and MC-RR, from 150 to 750 μg/kg for ANA, from 300 to 2500 μg/kg for BMAA and DAB and from 150 to 2500 μg/kg for AEG. Three samples for each concentration level were processed and injected in triplicate (n=9) following the optimized extraction procedure.

Peak area was considered as response signal, being linearly dependent on the analyte concentration in the sample. In all cases, satisfactory linearity was achieved over the working range (with determination coefficients $R^2 > 0.9817$). Statistical parameters

calculated by least-square regression and the performance characteristics of the method can be seen in **Table 3**. LODs were calculated as the lowest concentration for which the peak height of the Q_{ion} was, at least, three times the signal-to-noise ratio while LOQs were calculated as the lowest concentration with a signal-to-noise ratio of at least 10, which fulfilled method performance acceptability criteria of precision and extraction efficiency. For some analytes, such as the amino acids, the LOQ values obtained are higher than those provided by other studies. This is due to the complexity of the approach in terms of sample treatment, as there are different toxins with different properties that limit the simultaneous extraction to some extent. In any case, the obtained LOQs are in the low μg/kg levels, which allows the detection and quantitation in real samples.

3.3.2. Matrix effect

The matrix effect (ME) was assessed at three concentration levels (L1, L2 and L3) by comparing the analyte response from a blank extract of BGA food supplement sample spiked with the analytes after sample treatment and just before injection and the analyte response from a solvent standard solution spiked with the analytes after sample treatment and just before injection, both at the same concentrations. This procedure eliminates the contribution of reagents and solvents to the matrix effect. ME was calculated over three samples for each concentration level and analyzed in triplicate (n=9) according to the following equation.

$$\mathrm{ME}(\%) = \frac{(\text{Signal of analyte in sample extract spiked after treatment})}{(\text{Signal of analyte in standard solution spiked after treatment})} \times 100 \quad (1)$$

From this equation, a ME equal to 100% indicates the absence of a matrix effect whereas a value greater or lower than 100% implies signal enhancement or ion suppression, respectively. Ion suppression was observed for almost all analytes to a greater or lesser extent, being the isomers BMAA and DAB the most affected, with ME values down to 9.1 and 21.5% respectively, as it is shown in **Table 3**. Furthermore, it was noteworthy the fact that the ionization of MC-LR is the most matrix-dependent, as it exhibited a signal enhancement over 350 %, even though in the blank extracts no peak was observed at its retention time. MC-LR is the first analyte that elutes from the HILIC column as it is barely retained. Thus, it was hypothesized that endogenous matrix components poorly retained in the column and elute along with MC-LR, modifying in some way the ionization state of this analyte. Previous works has also shown a signal enhancement of MC-LR when analyzing food supplements [10], but not at such high levels as we obtained. Enhancement of ionization has the potential to be used in a favorable manner, improving sensitivity. With this purpose, further studies would be needed in this area to help understand the behavior of certain compounds in the presence of some additives. However, this limitation was compensated by performing a procedural calibration curve for quantification purposes, which considers the correction of bias introduced from the matrix effect and from losses in sample treatment.

Table 3. Performance characteristics, precision and matrix effect for the proposed SLE-tandem-SPE-HILIC-MS/MS method in spirulina sample.

Analyte	Linear range (μg/kg)	LOD (μg/kg)	LOQ (μg/kg)	R^2	Intra-day precision (RSD %) n=9			Inter-day precision (RSD %) n=15			Matrix effect (%) n=9		
					L1	L2	L3	L1	L2	L3	L1	L2	L3
MC-LR	60-500	18	60	0.9906	8.3	11.5	11.4	14.9	14.7	14.8	467.8	401.2	356.4
NOD	50-500	15	50	0.9872	11.9	13.1	16.3	15.2	15.3	13.9	138.2	118.7	123.3
MC-RR	50-500	15	50	0.9910	8.8	7.0	3.7	8.2	10.1	5.6	41.0	43.7	38.7
ANA	150-750	45	150	0.9900	9.1	8.0	7.1	8.5	8.6	8.2	33.0	27.6	26.0
BMAA	300-2500	90	300	0.9817	15.2	19.5	19.2	25.1	19.6	18.8	23.0	18.1	9.1
DAB	300-2500	90	300	0.9938	9.8	15.4	9.2	17.3	16.3	11.9	24.4	29.7	21.5
AEG	150-2500	45	150	0.9940	18.3	9.5	15.2	16.3	16.1	19.3	88.0	86.0	63.8

Concentration levels (L1, L2 and L3) for the precision and matrix effect studies were established as follows: 60, 150 and 350 μg/kg for MC-LR; 50, 150 and 350 μg/kg for NOD and MC-RR; 150, 300 and 600 μg/kg for ANA; 300, 800 and 1800 μg/kg for BMAA and DAB; and 150, 600 and 1800 μg/kg for AEG.

3.3.3. Precision studies

The precision of the method was assayed in terms of repeatability (intra-day precision) and intermediate precision (inter-day precision) by the application of the whole procedure to organic spirulina samples spiked at three different concentration levels. Repeatability was assessed over three samples for each concentration level and analyzed in triplicate (n=9) on the same day under the same conditions. Similarly, intermediate precision was evaluated during five consecutive days by analyzing one sample per day

in triplicate (n=15). Results, expressed as relative standard deviation (% RSD) of peak areas, are shown in **Table 3**. Acceptable precision (RSD < 20%) was obtained with RSD values lower than 19.6% in all cases, except for BMAA, which reported the highest RSD values, reaching 25.1% at the lowest concentration level in the inter-day precision.

3.3.4. Recovery study

Recovery experiments were carried out at the same L1, L2 and L3 concentration levels, by analyzing three real samples for each concentration level in triplicate (n=9). Recovery at each concentration level was estimated by comparing the analyte concentrations obtained from samples spiked before and after the whole sample treatment (SLE-SPE procedure) using peak area as analytical signals. Results showed satisfactory recoveries, ranging between 64.2% and 102.9%, with an associated RSD < 19.2% for all analytes (**Table 4**).

Table 4. Recovery assessment for the BGA dietary supplement sample containing spirulina (n=6).

	L1		L2		L3	
	R %	**RSD %**	**R %**	**RSD %**	**R %**	**RSD %**
MC-LR	74.0	15.1	76.6	9.9	81.6	9.2
NOD	102.9	16.1	94.1	19.2	77.7	15.4
MC-RR	91.2	11.6	89.9	5.1	88.0	4.4
ANA	87.8	11.8	80.1	10.2	78.7	9.0
BMAA	70.2	17.5	64.2	16.8	80.7	16.5
DAB	91.0	11.8	76.6	12.5	72.9	11.7
AEG	82.7	11.1	67.9	18.2	81.6	14.0

Concentration levels (L1, L2 and L3) were established as follows: 60, 150 and 350 μg/kg for MC-LR; 50, 150 and 350 μg/kg for NOD and MC-RR; 150, 300 and 600 μg/kg for ANA; 300, 800 and 1800 μg/kg for BMAA and DAB; and 150, 600 and 1800 μg/kg for AEG.

3.4. Determination of cyanotoxins in BGA dietary supplements

The SLE-tandem-SPE-HILIC-MS/MS method developed was satisfactorily applied to determine the natural occurrence of the target toxins in the BGA dietary supplements described in section 2.3. For each sample, three replicates were analyzed in triplicate (n=9). To the best of our knowledge this is the first time that this simultaneous analysis of multiclass cyanotoxins is carried out and applied in real samples. Eight of 9 samples were found positive for at least one cyanotoxin (**Table 5**).

Table 5. Determination of cyanotoxins in BGA dietary supplements from Table 1.

	MC-LR	NOD	MC-RR	ANA	BMAA	DAB	AEG
1	n.d.	n.d.	n.d.	n.d.	n.d.	**432**	n.d.
2	n.d.	n.d.	n.d.	n.d.	n.d.	**1065**	n.d.
3	n.d.	n.d.	n.d.	n.d.	n.d.	**2408**	**194**
4	n.d.	n.d.	n.d.	n.d.	n.d.	**900**	n.d.
5	n.d.	n.d.	n.d.	n.d.	n.d.	**605**	n.d.
6	n.d.	n.d.	n.d.	n.d.	n.d.	**2038**	**71**
7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	n.d.	n.d.	n.d.	n.d.	n.d.	**331**	n.d.

Concentration expressed in μg/kg; n.d.: Non detected.

Among all the target cyanotoxins, DAB, AEG, MC-LR and MC-RR were present in some of the BGA supplements. DAB was the most frequent one, being found on seven out of nine samples at concentrations ranging from 331 to 2480 μg/kg. Three of these samples exceeded the concentration value of 1 mg/kg. AEG was detected in two samples but was only quantified in one of them (194 μg/kg). In general, the concentrations present in the samples did not exceed from 2.5 mg/kg. However, MC-LR and MC-RR were present in one sample of *Aphanizomenon flos-aquae* at levels higher than 5 mg/kg (**Figure 7**), which is similar to what has been found in previous works [11,17].

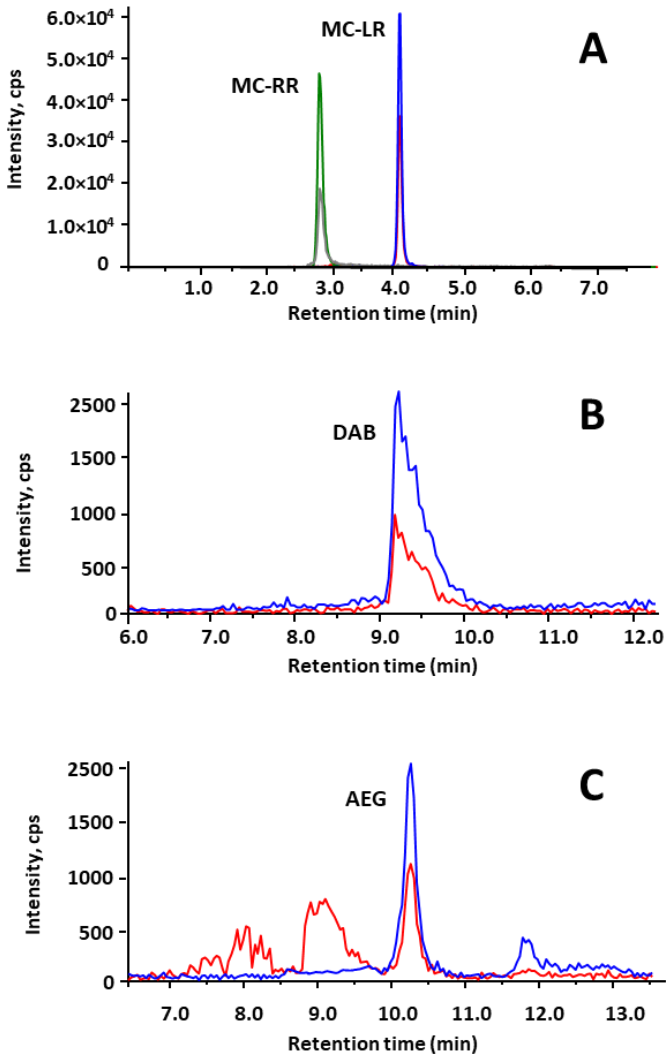

Figure 7. Extract ion chromatograms of different BGA dietary supplement samples contaminated with A) MC-LR and MC-RR; B) DAB; and C) AEG.

In the present work, based on the daily dose recommended by each manufacturer for each BGA supplement, 2.4 g was chosen as the average daily ration. Considering this daily dose value and the tolerable daily intake (TDI) for the only regulated cyanotoxin, the MC-LR (2.4 μg per day), a value of 1 mg/kg was obtained as maximum tolerable concentration of MC-LR in BGA dietary supplements. This does not entail that MC-LR is the only toxic or that the rest of cyanotoxins are less harmful. It merely reflects the lack

of toxicological information on these compounds. Due to the lack of data and according to precautionary principle, the same maximum tolerable value of 1 mg/kg has been taken as a reference for the rest of cyanotoxins to conclude the exposure risk in BGA dietary supplements, even though they do not have a fixed TDI. Under this premise, results showed that three samples exceeded this value for DAB, reaching concentrations up to 2.48 μg/g. Furthermore, the high concentrations, up to 5 μg/g for MCs, found in one sample of *Aphanizomenon flos-aquae* far outstripped the considered maximum tolerable value of 1 μg/g. These results are in line with those previously observed in other studies, where MCs were not present in spirulina samples but they were repeatedly detected in *Aphanizomenon flos-aquae* (also known as Klamath) dietary supplements [6,7,9,11,12,13].

4. Concluding remarks

In this work, a multi-toxin method has been developed for the first time to determine simultaneously cyanotoxins from several common families with different chemical characteristics in BGA dietary supplements. For this purpose, a SLE procedure followed by a tandem SPE method were applied as sample treatment, using HILIC mode coupled to MS/MS as analytical technique. From a critical point of view, the main challenge posed in this work has been to achieve for the first time the analysis of multiclass cyanotoxins in such a complicate matrix in a single run, performing a single extraction of the analytes belonging to different chemical families and being able to quantify concentrations of each toxin at extremely low levels. It was possible to validate the method in spirulina-based dietary supplements, showing its usefulness for this kind of samples. Positive samples found for MCs and NPAs set a point of attention toward the need of a satisfactory quality control in the production of these food supplements and envisaged the importance of international legislation to ensure product safety and to protect consumer health from cyanotoxin contamination.

5. References

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CHAPTER 5

APPLICATION OF ION MOBILITY SPECTROMETRY- LIQUID CHROMATOGRAPHY- HIGH RESOLUTION MASS SPECTROMETRY FOR THE CREATION OF A COLLISION CROSS SECTION DATABASE FOR PHYCOTOXINS SCREENING

This work is currently in drafting:

Aparicio-Muriana, M. M., Lara, F. J., Hernández-Mesa, M., del Olmo-Iruela, M., García-Campaña, A. M., Dall'Asta, C., Righetti, L**. Implementing the use of collision cross section database for phycotoxin screening analysis**.

1. Summary

The increased consumption of blue-green algae (BGA) based dietary supplements has raised concern about their food safety, especially about cyanotoxins presence. The hyphenation of ion mobility spectrometry (IMS) with traditional liquid chromatography-tandem mass spectrometry (LC-MS/MS) represents a relevant tool for the screening of several compounds in a large variety of food matrices. As stated in the **Introduction**, in IMS the separation of charged analytes is governed by their mobilities in a carrier gas under the influence of an electric field. Analytes are separated according to their size, charge and geometric conformation, and in the case of traveling wave ion mobility spectrometry (TWIMS), high charged and compact ions travel faster than the ones with an open structure, as they interact more with the drift gas molecules slowing their speed. TWIMS is able to report the collision cross section (CCS, Ω), which is defined as the affected area from the interaction between the charged analyte and the neutral buffer gas molecule. The CCS is considered a molecular descriptor as it is an intrinsic property of each molecule, i.e., its value is directly linked to the chemical structure of the charged molecule and the buffer gas used in the measurement experiment. The implementation of IMS and CCS databases in LC-MS/MS workflows brings several benefits to the analysis of a wide variety of samples, including improvements in the identification and quantification of ions. However, IMS remains as an unexplored technique in the phycotoxin analysis field.

In this work, ultra-high performance liquid chromatography coupled to traveling wave ion mobility spectrometry/quadrupole time-of-flight mass spectrometry (UPLC-TWIMS-QTOF) was employed to establish the first comprehensive TWIMS-derived collision cross section ($^{TW}CCS_{N2}$) database for phycotoxins. The database included 20

cyanotoxins and one marine biotoxin. Accurate *m*/*z*, retention times and $^{TW}CCS_{N2}$ values were obtained for 81 adducts in positive and negative electrospray (ESI^+/ESI^-) modes. Reproducibility and robustness of the $^{TW}CCS_{N2}$ measurements were determined to be independent of the matrix. A screening analysis was carried out on 19 commercial BGA dietary supplements of different composition. Cyanotoxins were confidently identified in 5 samples based on retention time, *m*/*z* and $^{TW}CCS_{N2}$.

2. Experimental

2.1. Material and reagents

HPLC-grade methanol (MeOH) was purchased from Sigma-Aldrich (Taufkirchen, Germany); bidistilled water was obtained using Milli-Q System (Millipore, Bedford, MA, USA); MS-grade formic acid (FA) was purchased from Fisher Chemical (Thermo Fisher Scientific Inc., San Jose, CA, USA and ammonium formate was obtained from Sigma–Aldrich (St. Luis, MO, USA). Leucine-enkephalin [186006013] used as lock mass solution and Major Mix IMS/TOF Calibration Kit [186008113] for mass and CCS calibration were purchased from Waters (Manchester, UK).

Analytical standards of phycotoxins were purchased individually from Enzo Life Sciences, Inc. (Lausen, Switzerland), Sigma Aldrich (Darmstadt, Germany) and Cayman Chemicals (Michigan, USA). Detailed information such as common names, toxicity, structure and physicochemical properties of the targeted toxins are included in **Annex (Table A1)**. Stock standard solutions of 50 or 25 μg/mL were prepared by adding 1 mL of the desired solvent directly into the vial of toxin supplied by the manufacturer and gently swirling the vial to dissolve the toxin. Cylindrospermopsin (CYN), microcystin-leucine-arginine (MC-LR), microcystin-tyrosine-arginine (MC-YR), microcystin-tryptophan-arginine (MC-WR), microcystin-leucine-alanine (MC-LA), microcystin-leucine-tyrosine (MC-LY), microcystin-leucine-tryptophan (MC-LW), microcystin-leucine-phenylalanine (MC-LF), microcystin-homoisoleucine-Arginine (MC-HliR), microcystin-homotyrosine-Arginine (MC-HtyR), [D-Asp3]microcystin-leucine-arginine ([DAsp3]MC-LR), Anabaenopeptin A (APa) and Anabaenopeptin B (APb) were prepared in 100% MeOH. Nodularin (NOD) was prepared in H_2O:MeOH (1:1). Microcystin-arginine-arginine (MC-RR) was prepared in H_2O:MeOH (20:80). Okadaic

acid (OA) was prepared in 100% ethanol. Anatoxin-a (ANA) and the isomers β-methylamino-L-alanine (BMAA), 2,4-diaminobutyric acid (DAB) and N-(2-aminoethyl)glycine (AEG) were prepared in H_2O. Finally, saxitoxin (SAX) was prepared in 0.003M HCl. Stock solutions were stored in the dark at -20 °C. Intermediate standard solutions of each compound at 2.5 μg/mL were prepared by dilution of the stock solutions with the corresponding solvent for each toxin.

2.2. BGA dietary supplement samples

BGA derived dietary supplements were obtained from several brands and different sources, largely on internet but also from local retail stores in Granada (Spain) and Parma (Italy). They were sold as tablets, capsules, powder and liquid form, thus presenting different matrices, as they were composed by different cyanobacteria species in different ratios. In addition, some of them were also formulated with separate excipients along with other components. Detailed information on samples with their form, composition and daily dose are listed in **Annex (Table A2)**. All the samples were analyzed before their expiration date.

2.3. Sample preparation

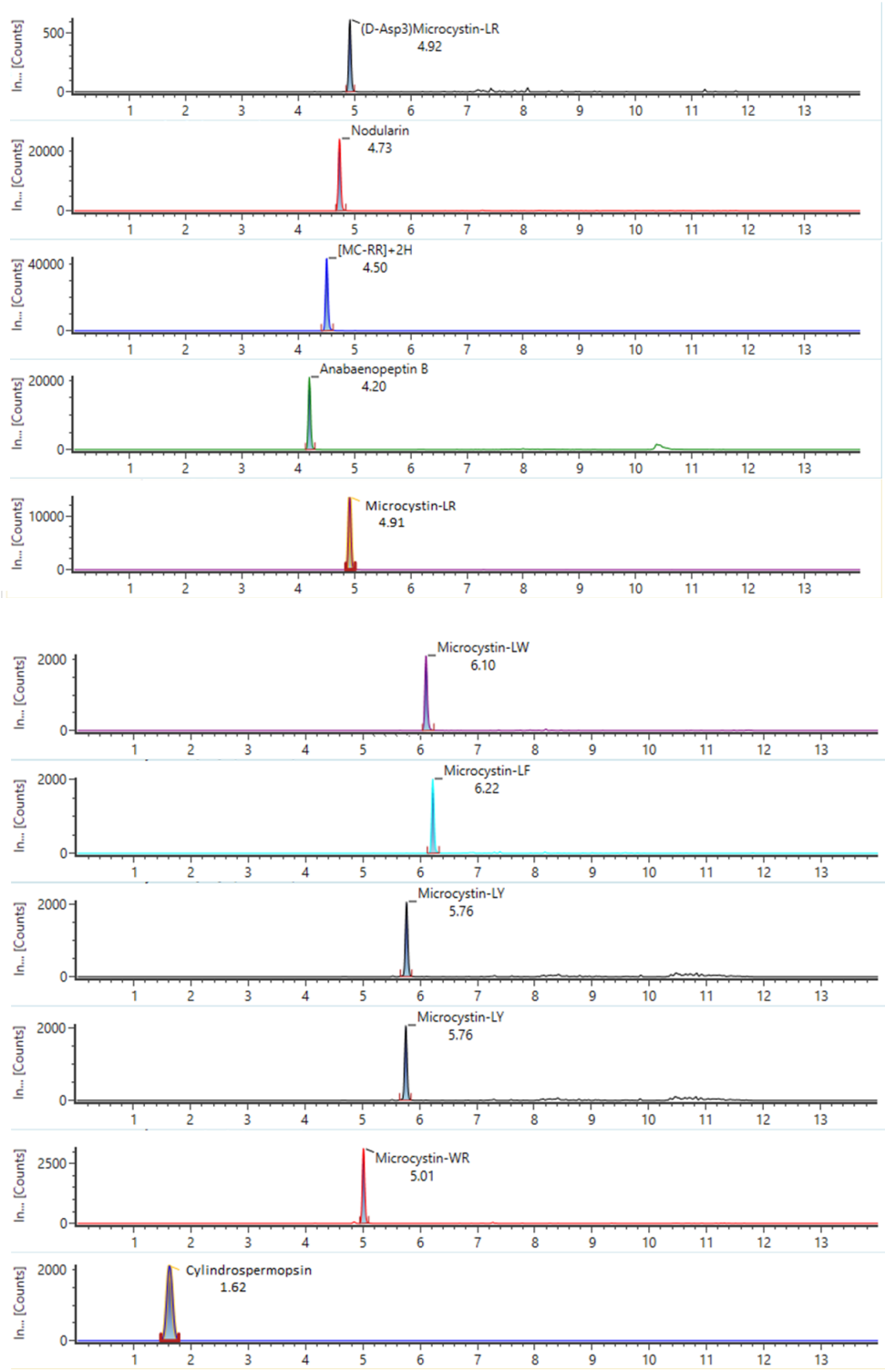
Tablets were grounded to make a fine powder and capsules were opened and mixed. Aliquots of powdered sample were submitted to the protocol previously employed by Van Pamel et al. for the extraction of plant toxins and cyanotoxins in dietary supplements [1]. Briefly, 0.5 g of each sample was weighted and introduced in a conical polypropylene centrifuge tube and 5 mL of 75% MeOH in water were added (sample:extraction solvent, 1:10 (w/v)). After vortex shaking for 1 min, samples were mechanically shaken for 10 min, placed in ultrasonic bath for 15 min at room temperature and mechanically shaken again for 10 min. After that, the extracts were centrifuged for 10 min at 9000 rpm at room

temperature and finally 100 μL of the supernatant was diluted up to 375 μL of H_2O with 0.1% FA solution to obtain a ratio 80:20 H_2O:MeOH. The diluted extract was transferred to a vial and injected into the UHPLC-TWIMS-QTOF system.

2.4. UHPLC analysis conditions

An ACQUITY I-Class UPLC (Waters, Manchester, U.K.) separation system was employed. For the chromatographic separation, two different columns and methods were employed depending on the nature of the toxins. On the one hand, an Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size; Waters, Manchester, U.K.) was employed for all the MC congeners, NOD, APa, APb, CYN, SAX and OA. The reversed phase (RP) chromatographic method, based on a published application note from Waters, employed water with 0.1% FA acid as solvent A and MeCN with 0.1% of FA as solvent B [2]. The separation was achieved using the following gradient mode: 0 min, 0% B flow rate 0.4 mL/min; 1.5 min 0% B flow rate 0.4 mL/min; 6.5 min, 80% B flow rate 0.4 mL/min; 6.6 min, 100% B flow rate 0.5 mL/min; 11 min 100% B flow rate 0.5 mL/min; 11.1 min 0% B flow rate 0.4 mL/min and 14 min, 0% B flow rate 0.4 mL/min. The column and autosampler were maintained at 45 and 10 °C, respectively, and 2 μL of the extract were injected. On the other hand, an Atlantis Premier BEH Z-HILIC column (2.1 mm × 100 mm, 1.7 μm particle size) from Waters (Manchester, U.K.) was used for ANA and for the non-protein amino acid (NPA) isomers BMAA, DAB and AEG. The mobile phase consisted of 10 mM ammonium formate with 0.3% FA in water (A) and 0.3% FA in MeCN (B). The separation was performed using the following gradient mode: 0 min, 95% B; 2 min, 95% B; 10 min 50% B; 11 min 50% B; 12 min 95% B and 15 min, 95% B at a flow rate of 0.4 mL/min. The column and autosampler were maintained at 45 and 10 °C, respectively, and 4 μL of the extract were injected. **Figure 1** and **Figure 2** show

extracted ion chromatograms of all compounds using both, RP and HILIC methods, respectively.



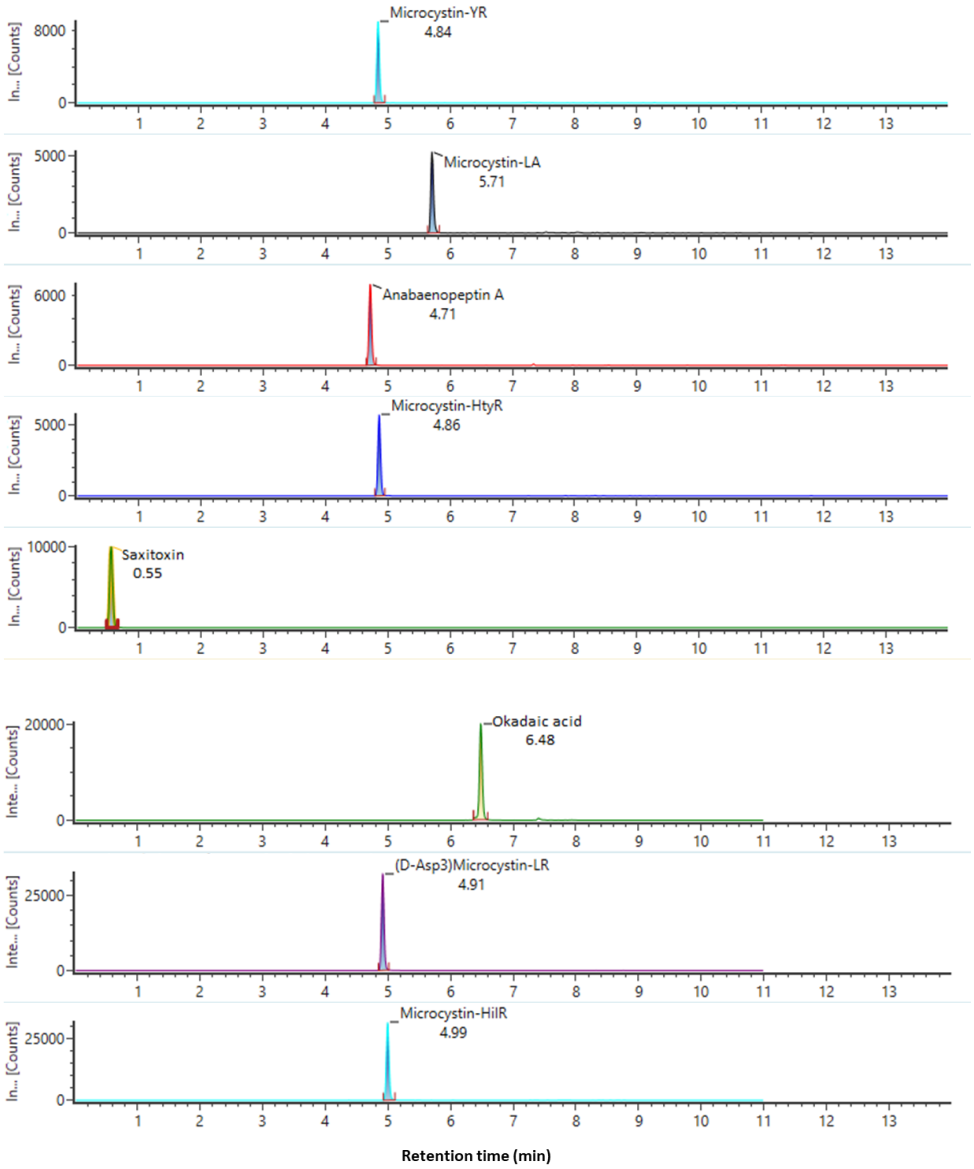


Figure 1. Chromatographic separation of toxins using reversed phase method.

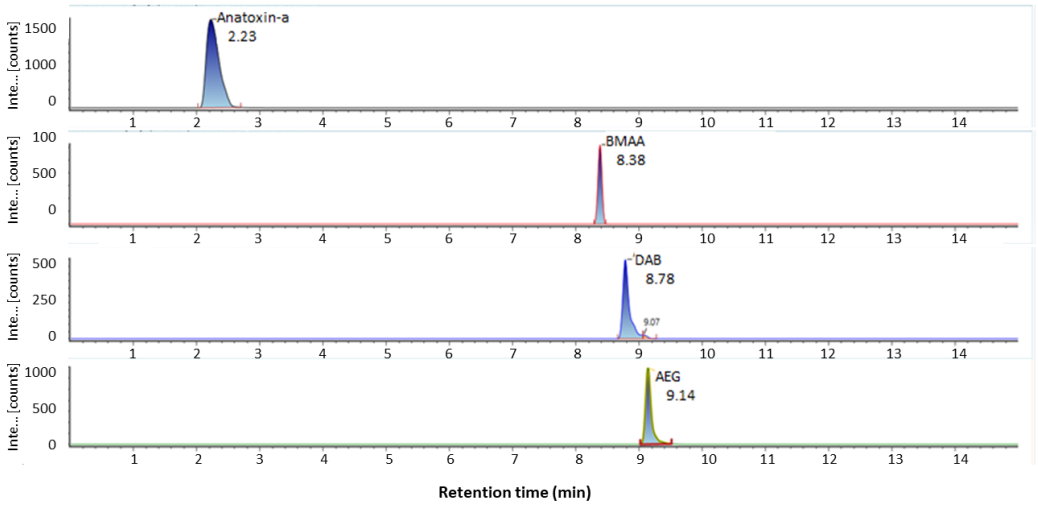


Figure 2. Chromatographic separation of toxins using HILIC method.

2.5. TWIMS-QTOF conditions

The ACQUITY I-Class UPLC separation system was coupled to a Vion IMS-QTOF mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) interface. The IMS-MS system consists of a hybrid quadrupole orthogonal acceleration time-of-flight (Q-TOF) mass spectrometer, where the mobility cell, which is a stacked ring ion guide, is placed before the quadrupole mass filter. The mass spectrometry (MS) detection was conducted in both positive and negative ESI mode in the mass range of *m*/*z* 50-1100 with a scan time of 0.15 and 0.30 s for the reversed phase and the HILIC method, respectively. Argon was used as the collision gas and nitrogen was used as the ion mobility gas. IMS gas flow rate was 90 mL/min (3.2 mbar), a wave velocity of 650 m/s, and a wave height of 40 V.

For the reversed phase method, parameters related to source conditions were set as follows: capillary voltage, 2.5 kV; cone voltage, 40 V; source temperature, 150˚C; desolvation temperature, 600˚C; desolvation gas flow rate, 950 L/h and cone gas flow

rate, 50 L/min. In data-independent high-definition MS^E ($HDMS^E$) mode two data channels are acquired simultaneously in a single run. The fragmentation of precursor ions is minimized in the low-energy channel, so it is used to monitor the protonated and deprotonated molecules and other formed adducts. A collision energy ramp is applied in the high energy channel to induce fragmentation of precursor ions travelling through the collision cell. The low-energy spectra were acquired at CE of 6 V for both ESI^+ and ESI^- while high-energy spectra were acquired with a ramp of the transfer CE from 30 to 80 V.

For the HILIC method, parameters related to source conditions were set as follows: capillary voltage, 1 kV; cone voltage, 30V; source temperature 150°C; desolvation temperature 450°C; desolvation gas flow rate, 800 L/h and cone gas flow rate, 50 L/min. The low-energy spectra were acquired at a CE of 6 eV for both ESI^+ and ESI^-, while high energy spectra were acquired with a CE ramp from 10 to 50 eV.

Lock mass correction was performed by infusing a solution of leucine-encephalin $[M+H]^+$ (*m*/*z* 556.2766, calibration kit from Waters) at a concentration of 200 pg/μL (flow rate 10 μL/min) and acquired every 2.5 min to provide a real-time single-point mass and CCS calibration.

2.6. Creation of the CCS database

Phycotoxins standard mixes were prepared at four different concentration levels (10, 50, 100 and 500 μg/L) and 2 and 4 μL were injected in reversed phase and HILIC method, respectively. $^{TW}CCS_{N2}$ values were obtained from the average of nine replicates for 500 and 100 μg/L standard mixtures plus three replicates for 50 and 10 μg/L standard mixtures, employing a Vion IMS-QTOF instrument (resolution ~20 Ω/ΔΩ fwhm). $^{TW}CCS_{N2}$ values were measured using nitrogen as drift gas and were experimentally

determined by the application of CCS calibration curves created using the Major Mix CCS calibration solution for both ESI^+ and ESI^- mode. TWIMS calibration procedure has been previously described [3], and it is automatically performed by UNIFI 1.8 software (Waters; Manchester, UK). The Major Mix calibration solution contained poly-DL-alanine, Ultramark 1621, low molecular weight acids and other small molecules. The calibrants covered a *m*/*z* range from 152.0706 Da to 1921.9459 Da, and CCS range from 130.4 to 372.6 $Å^2$ in positive mode and a mass range from 150.0561 Da to 1965.9369 Da, and a CCS range from 131.5 to 367.2 $Å^2$ in negative mode. Major Mix was prepared in 50:50 (v:v) water:acetonitrile with 0.1% FA. The exact composition of the different calibration solutions is reported in **Annex (Table A3** and **Table A4)**. CCS calibration was carried out considering singly charged ions, so TWIMS derived CCS values were only applicable to singly charged ions. All the ionized species detected for each toxin were identified with a deviation lower than 5 ppm in relation to their exact mass.

2.7. Software and data analysis

Data acquisition was conducted using UNIFI 1.8 software (Waters; Manchester, UK), which also provides the $^{TW}CCS_{N2}$ values. Theoretical CCS values were also predicted by three different machine learning tools named AllCCS (http://allccs.zhulab.cn/) [4], CCSbase (https://ccsbase.net/) [5], and MetCCS Predictor (http://www.metabolomics-shanghai.org/MetCCS/) [6,7]. The molecular descriptors required for CCS prediction were obtained from the human metabolome database (HMDB, http://www.hmdb.ca/) [8], and PubChem database (https://pubchem.ncbi.nlm.nih.gov/) [9].

3. Results and discussion

In order to implement IMS in routine MS-based phycotoxins workflows, searchable databases with CCS values and accurate *m*/*z* values need to be generated. This work reports the first $^{TW}CCS_{N2}$ database for cyanotoxins which encompasses compounds from different families, including cyclic peptides (n=14), alkaloids (n=3) and non-protein amino acids (n=3). In addition to cyanotoxins, the OA, which is the main representative of the marine biotoxins [10], was also characterized because they often coexist in marine environments. Overall, 21 phycotoxins were characterized in terms of $^{TW}CCS_{N2}$. All $^{TW}CCS_{N2}$ values were collected for commercially available standards (see **Annex**, **Table A1**). Various parameters and instrumental conditions were tested to validate the database, as well as $^{TW}CCS_{N2}$ values were also measured in spiked dietary supplement extracts to prove the reliability of the CCS measurements. Moreover, its applicability to the phycotoxin screening analysis of BGA derived dietary supplements was investigated.

3.1. Phycotoxins $^{TW}CCS_{N2}$ database

All phycotoxins (n=21) were characterized in both positive and negative ionization mode. $^{TW}CCS_{N2}$ measurements were carried out through several replicates (nine times the standard solutions of 500 and 100 μg/L plus three times the standard solutions of 50 and 10 μg/L). The developed database provides the $^{TW}CCS_{N2}$ of the most abundant ion observed for each toxin but it also offers information about all the identified adducts observed for each compound in both positive and negative ionization mode (e.g., $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$, $[M+H-H_2O]^+$, $[M-H]^-$ and $[M-H-H_2O]^-$) as well as their influence in the drift time. The CCS of the most intense adduct was observed at all injected concentration levels; however, the $^{TW}CCS_{N2}$ of all other adducts could not be

determined for the 50 and 10 μg/L standard solutions due to their low peak intensity. Overall, a total of 81 ions (considering protonated and deprotonated molecules, cationic and anionic adducts) have been identified and characterized in terms of *m*/*z* and CCS. In detail, protonated adducts were detected for all compounds except for the marine biotoxin okadaic acid and the three amino acid isomers cyanotoxins. Complete information of the investigated toxins, the observed ions under positive and negative ESI conditions, their *m*/*z* as well as $^{TW}CCS_{N2}$ values can be found in **Table 1**. In all cases, high reproducibility was observed with relative standard deviations (RSDs) lower than 0.3% for 71% of the compounds, being 0.72% and 0.55% the highest values obtained for positive and negative ionization mode, respectively.

Table 1. $^{TW}CCS_{N2}$ database for phycotoxins (n=24).

Compound	Adduct	Theoretical exact *m*/*z*	Experimental $^{TW}CCS_{N2}$ (Å²)	SD	RSD (%)
β-methylamine-L-alanine	$[M-H]^-$	117.0670	**140.6**	0.56	0.40
2,4-diaminobutyric acid	$[M-H]^-$	117.0670	**140.7**	0.78	0.55
N-(2-aminoethyl)glycine	$[M-H]^-$	117.0670	**141.9**	0.55	0.39
Anatoxin-a	$[M+H]^+$	166.1227	**136.1**	0.32	0.24
Anatoxin-a	$[M+H-H_2O]^+$	148.1121	**132.6**	0.19	0.14
Saxitoxin	$[M+H]^+$	300.1415	**159.6**	0.31	0.19
Saxitoxin	$[M+H-H_2O]^+$	282.1309	**157.2**	0.29	0.18
Cylindrospermopsin	$[M+H]^+$	416.1235	**198.6**	0.42	0.21
Cylindrospermopsin	$[M-H]^-$	414.1089	**198.9**	0.28	0.14
Okadaic acid	$[M-H]^-$	803.4582	**308.4**	0.36	0.12
Okadaic acid	$[M+Na]^+$	827.4558	**296.9**	0.26	0.09
Okadaic acid	$[M+K]^+$	843.4297	**298.9**	0.36	0.12
Okadaic acid	$[M+H-H_2O]^+$	785.4476	**275.5**	0.40	0.14
Nodularin	$[M+H]^+$	825.4505	**296.5**	0.68	0.23
Nodularin	$[M-H]^-$	823.4359	**288.8**	0.54	0.19

Nodularin	$[M+Na]^+$	847.4325	**274.9**	0.99	0.36
Nodularin	$[M+K]^+$	863.4070	**277.3**	0.90	0.32
Nodularin	$[M-H-H_2O]^-$	805.4249	**291.9**	1.43	0.49
Anabaenopeptin B	$[M+H]^+$	837.4618	**278.3**	0.45	0.16
Anabaenopeptin B	$[M-H]^-$	835.4472	**286.4**	0.86	0.30
Anabaenopeptin B	$[M+Na]^+$	859.4438	**282.2**	1.14	0.41
Anabaenopeptin B	$[M-H-H_2O]^-$	817.4361	**280.5**	0.99	0.35
Anabaenopeptin A	$[M+H]^+$	844.4240	**279.2**	0.53	0.19
Anabaenopeptin A	$[M-H]^-$	842.4094	**278.3**	0.44	0.16
Anabaenopeptin A	$[M+Na]^+$	866.4060	**285.5**	0.55	0.19
Anabaenopeptin A	$[M+K]^+$	882.3804	**286.7**	1.01	0.35
Anabaenopeptin A	$[M+H-H_2O]^+$	826.4134	**277.8**	1.10	0.39
Microcystin-LA	$[M+H]^+$	910.4921	**296.0**	0.79	0.27
Microcystin-LA	$[M-H]^-$	908.4775	**317.2**	0.49	0.15
Microcystin-LA	$[M+Na]^+$	932.4741	**301.3**	0.74	0.25
Microcystin-LA	$[M+K]^+$	948.4485	**303.2**	0.87	0.29
Microcystin-LA	$[M+H-H_2O]^+$	892.4815	**296.0**	1.81	0.61
[D-Asp3]Microcystin-LR	$[M+H]^+$	981.5409	**305.9**	1.17	0.38
[D-Asp3]Microcystin-LR	$[M-H]^-$	979.5253	**324.8**	0.41	0.13
[D-Asp3]Microcystin-LR	$[M+Na]^+$	1003.5229	**304.7**	1.27	0.42
Microcystin-LF	$[M+H]^+$	986.5234	**309.2**	0.80	0.26
Microcystin-LF	$[M-H]^-$	984.5088	**329.7**	0.51	0.15
Microcystin-LF	$[M+Na]^+$	1008.5054	**316.3**	0.63	0.20
Microcystin-LF	$[M+K]^+$	1024.4798	**319.2**	1.03	0.32
Microcystin-LF	$[M+H-H_2O]^+$	968.5128	**310.2**	1.41	0.45
Microcystin-LR	$[M+H]^+$	995.5561	**309.3**	0.60	0.19
Microcystin-LR	$[M-H]^-$	993.5415	**326.9**	0.50	0.15
Microcystin-LR	$[M+Na]^+$	1017.5381	**307.3**	0.68	0.22
Microcystin-LR	$[M+K]^+$	1033.5125	**318.9**	1.02	0.32
Microcystin-LR	$[M+H-H_2O]^+$	977.5455	**310.2**	1.41	0.45
Microcystin-LY	$[M+H]^+$	1002.5183	**313.5**	0.71	0.23
Microcystin-LY	$[M-H]^-$	1000.5037	**326.1**	0.53	0.16

Microcystin-LY	$[M+Na]^+$	1024.5003	**320.0**	0.61	0.19
Microcystin-LY	$[M+H-H_2O]^+$	984.5077	**313.8**	1.33	0.43
Microcystin-HilR	$[M+H]^+$	1009.5722	**314.7**	0.86	0.27
Microcystin-HilR	$[M-H]^-$	1007.5566	**331.5**	0.40	0.12
Microcystin-HilR	$[M+Na]^+$	1031.5542	**312.1**	0.65	0.21
Microcystin-LW	$[M+H]^+$	1025.5343	**317.3**	0.69	0.22
Microcystin-LW	$[M-H]^-$	1023.5197	**332.1**	0.38	0.11
Microcystin-LW	$[M+Na]^+$	1047.5163	**320.7**	0.49	0.15
Microcystin-LW	$[M+K]^+$	1063.4907	**322.0**	0.96	0.30
Microcystin-LW	$[M+H-H_2O]^+$	1007.5237	**317.2**	1.03	0.32
Microcystin-RR	$[M+2H]^{2+}$	514.7550	**177.5**	0.23	0.13
Microcystin-RR	$[M+H]^+$	1038.5731	**316.4**	0.62	0.20
Microcystin-RR	$[M-H]^-$	1036.5585	**327.4**	0.69	0.21
Microcystin-RR	$[M+Na]^+$	1060.5551	**307.4**	0.75	0.24
Microcystin-YR	$[M+H]^+$	1045.5353	**318.0**	0.63	0.20
Microcystin-YR	$[M-H]^-$	1043.5207	**322.7**	0.44	0.14
Microcystin-YR	$[M+Na]^+$	1067.5173	**316.6**	0.75	0.24
Microcystin-YR	$[M-H-H_2O]^-$	1025.5096	**313.6**	0.58	0.18
Microcystin-HtyR	$[M+H]^+$	1059.5510	**316.4**	0.69	0.22
Microcystin-HtyR	$[M-H]^-$	1057.5364	**331.2**	0.48	0.14
Microcystin-HtyR	$[M+Na]^+$	1081.5330	**312.0**	0.86	0.28
Microcystin-HtyR	$[M+K]^+$	1092.5244	**319.7**	2.30	0.72
Microcystin-WR	$[M+H]^+$	1068.5513	**320.0**	0.77	0.24
Microcystin-WR	$[M-H]^-$	1066.5367	**328.2**	0.46	0.14
Microcystin-WR	$[M+Na]^+$	1090.5333	**319.5**	0.92	0.29

Being the present one the first database developed for phycotoxins, the comparison of CCS measurements presented here with other previously reported using TWIMS, drift tube ion mobility spectrometry (DTIMS) or trapped ion mobility spectrometry (TIMS) technology is not possible to this day. The only cyanotoxin that had already been characterized in terms of CCS is SAX. The CCS value of the protonated SAX adduct

obtained in the present work varied by less than 1.1% from the value previously reported using the TWIMS cell of the Synapt G2 HDMS instrument and N_2 as buffer gas [11], even though the calibration mix employed previously was polyalanine instead of Major Mix. Another study that took advantage of IMS to enhance cyanotoxins determination employed differential mobility spectrometry (DMS) as an ion filter after HILIC separation and ESI and before MS/MS detection for the separation of BMAA and its isomers [12]. However, no CCS values are determined with this IMS instrumentation.

As the CCS is a molecular characteristic closely related to *m*/*z* ratio, correlation between both parameters is often expected for compounds belonging to the same chemical family or with similar structures [13]. To analyze the correlation between CCS values and *m*/*z*, the experimentally determined CCSs of all singly charged adducts detected were plotted as a function of *m*/*z* (**Figure 3**).

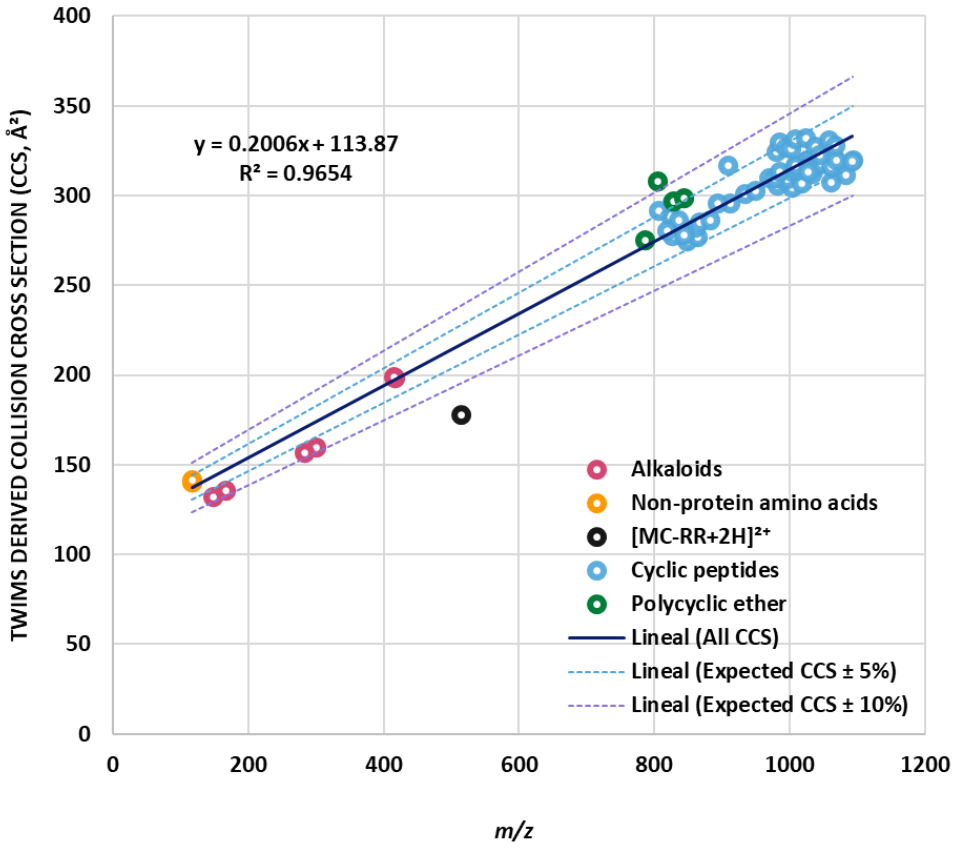


Figure 3. Correlations of m/z and measured TWIMS-derived CCS values of 21 phycotoxins determined in ESI$^+$ and ESI$^-$ modes.

The range of *m*/*z* and CCS values obtained showed the structural diversity among the target toxins, as compounds that share structural characteristics showed a trend in their ion mobilities. Thus, two main group of data were observed depending on their toxin family. The first one had lower values of CCS and *m*/*z* (below 198.6 Å^2 and 416.1, respectively), which corresponds with the alkaloid group of toxins (anatoxin, cylindrospermopsin and saxitoxin) (**Figure 4**).

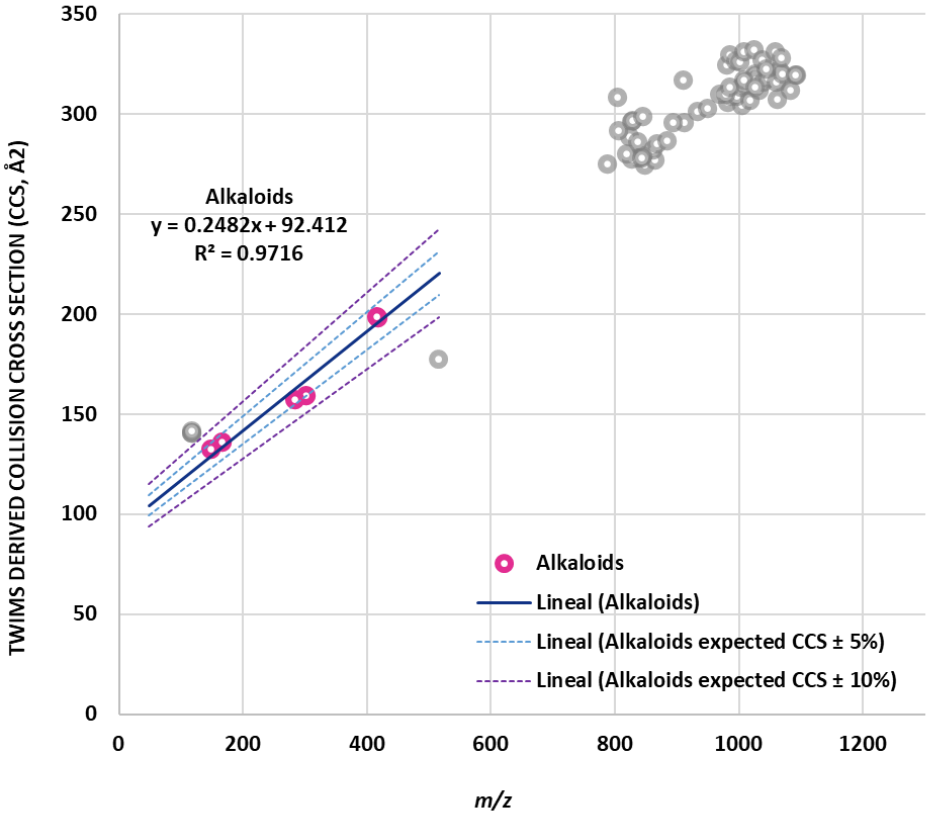


Figure 4. Correlations of m/z and measured TWIMS-derived CCS values of alkaloid group of cyanotoxins.

The second group of data (**Figure 5**), which encompasses the cyclic peptides toxins (microcystins, nodularin and anabaenopeptins), presented higher values of CCS (above 274.9 Å^2), in accordance with their higher molecular mass and *m*/*z* ratio (above 785.4).

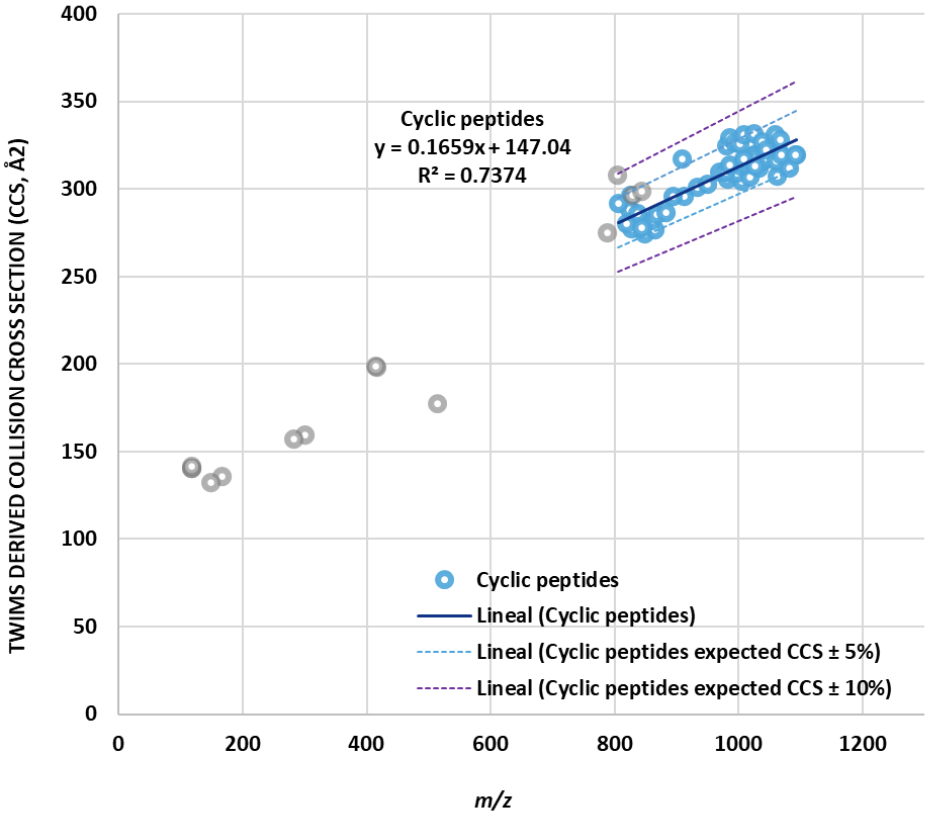


Figure 5. Correlations of m/z and measured TWIMS-derived CCS values of cyclic peptide group of cyanotoxins.

However, no trendline was obtained from the polycyclic ether and amino acid group of toxins due to sample size issue. For instance, the okadaic acid was the only compound belonging to the polycyclic ether family, thus, obtaining a trendline for a group of compounds from a single compound is impractical. Likewise, only three CCS data were obtained for the NPA toxin group. In addition to the trend observed for alkaloids and cyclic peptides, when the experimental CCS values of all singly charged adducts were plotted as a function of *m*/z, a general trend was observed for all of them regardless the group of toxins to which they belonged (**Figure 3**). A great correlation between *m*/z and CCS (R^2=0.9655; n=71) and low dispersion of data points were obtained, according to the

linear regression model proposed. The dashed lines represent approximately ±5% and ±10% from the center of the data (blue and purple dashed lines, respectively) as determined by the linear fit of the main trendline (solid line).

The dispersion of CCS values at any *m*/*z* region can be appreciated by the curves of ±5% and ±10 %. A remarkably low dispersion was obtained as most adducts fall within the established threshold (81% of them fall within ±5%, and only one adduct, the deprotonated okadaic acid, fall slightly above the ±10% threshold), resulting in an interval of CCS predictability, as previously reported by other authors [14,15,16]. This is of particular relevance in non-targeted analysis, in which previously uncharacterized phycotoxins can be detected and their identification can be carried out based on the observed *m*/*z* and CCS prediction.

A relevant point is noteworthy when referring to the linear trend line of CCS values versus *m*/*z*, particularly in positive ESI mode. It can be observed a black point at *m*/*z* 514.7 and CCS 177.5 $Å^2$ that lies far below the -10% curve. This value corresponds to the CCS of the doubly-protonated molecule of microcystin-RR ($[M+2H]^{2+}$). This fact is in accordance with previously reported works, where multiply-charged species have also been found far above the +10% curve [14]. As explained before, the CCS calibration was carried out considering singly charged ions, so CCS measurements are only applicable as valid reference values to single charged ions. In the case of MC-RR, although the monoprotonated and sodium adducts were also observed, the most predominant adduct found by far in both, the analysis of standard solutions and the analysis of the spiked and positive dietary supplement samples, was the doubly charged molecule. In positive ESI mode, MCs with two arginine residues (R) appear primarily as doubly-charged ions while those with one or no R residue are mostly singly protonated [17], which is in accordance with the work presented in **Chapter 3** where MC-RR was determined using

ESI^+ [18]. Hence, the CCS value obtained for the diprotonated molecular ion of MC-RR was represented in the graph, taking into account that this value could be considered as a reference in the MC-RR identification when using the Major Mix calibration kit employed here. In that sense, although represented in the graph, the linear curve fit to the CCS-*m*/*z* trendline of cyanotoxins excludes the multiply-charged $[MC\text{-}RR+2H]^{2+}$ specie from the trendline equation.

3.1.1. Limitations to CCS measurements of BMAA, DAB and AEG isomers

As mentioned above, when measuring the CCS of amino acid isomers in negative ionization mode, the CCS values corresponding to the deprotonated adduct of all isomers were determined. Those $[M-H]^-$ adducts, with an exact mass of 117.0670, presented $^{TW}CCS_{N2}$ values of 140.6, 140.7 and 141.9 $Å^2$ for BMAA, DAB and AEG, respectively. These results showed differences between them less than 2%, which correspond to the instrumental variation. To make the separation of the three isomers possible, a higher resolving power instrument such as the cyclic IMS (Waters) or the TIMS-TOF (Bruker) would be necessary. On the other hand, when we refer to positive ionization mode, it was found that in flow injection analysis, the major adduct for the three amino acid isomers was not the protonated specie, but the double sodium with proton loss, $[M-H+2Na]^+$ (*m*/*z* 163.0452) (**Figure 6**).

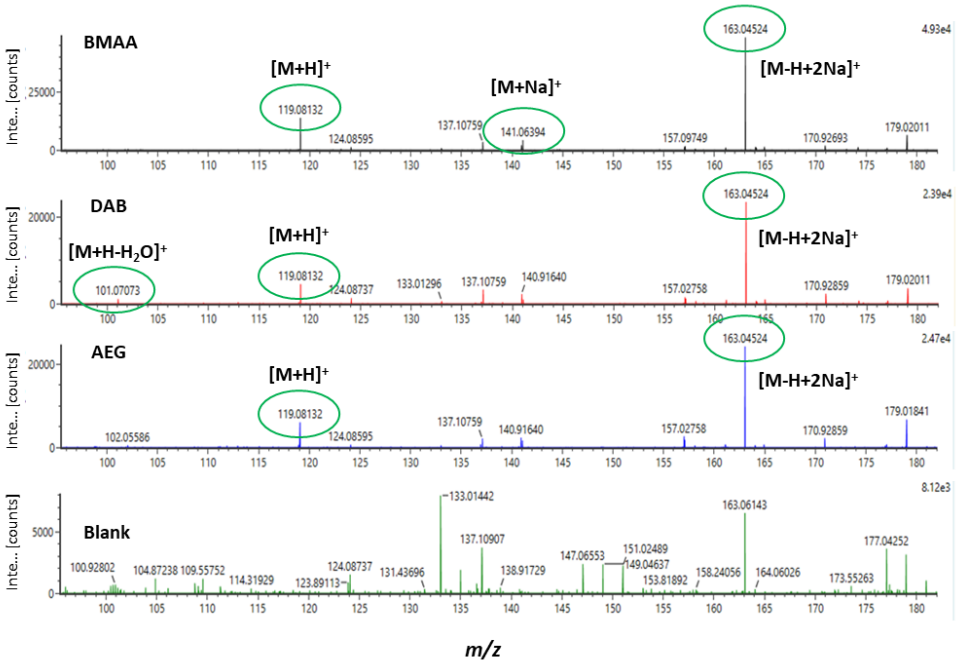


Figure 6. Adducts observed for the non-protein amino acids BMAA, DAB and AEG using flow injection analysis.

As far as it is known, this adduct has not been reported before in the literature for these analytes. In fact, the most common adduct formed in positive electrospray ionization mode is generally the protonated specie, whose CCS value could not be determined under these experimental conditions and with the employed Vion IMS-QTOF instrument. The presence of three broad peaks was observed in the ion mobilogram of each amino acid (**Figure 7**), each of them corresponding with a different specie of the $[M-H+2Na]^+$ adduct, and yielding to three major CCS values.

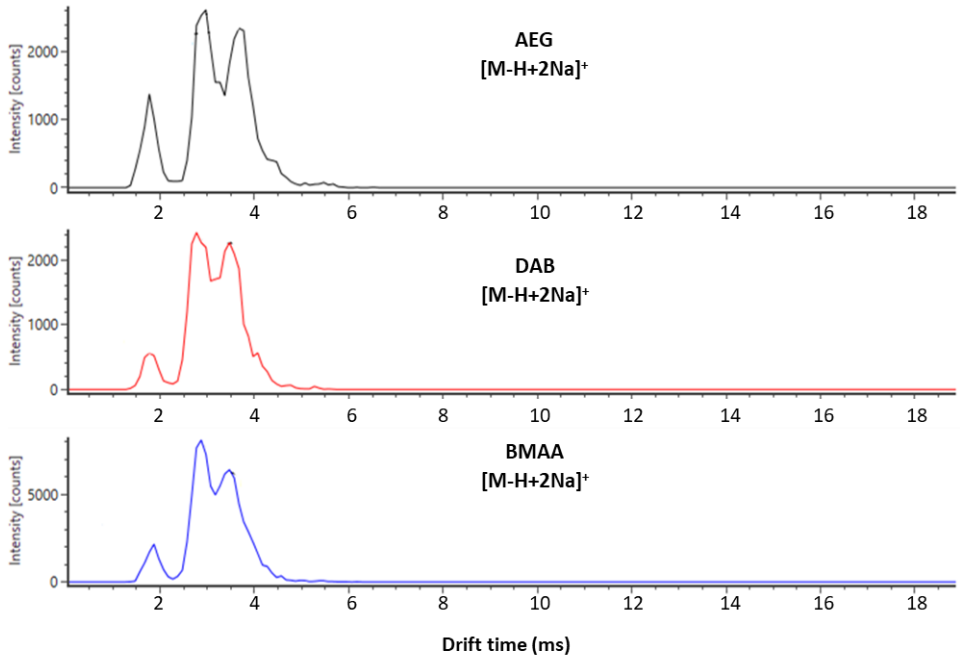


Figure 7. Mobilograms obtained from the [M-H+2Na]+ adduct of the non-protein amino acid isomers AEG, DAB and BMAA.

These values (see **Annex**, **Table A5**) are very similar for the three amino acids and because their CCS percent difference is lower than ±2%, they will be aligned in the drift time dimension so they will be processed as the same ions as long as they had not been baseline resolved in the chromatographic dimension. From the obtained results, it can be inferred that the different shapes of the amino acid isomer ions were not intensified by the coordination of two sodium atoms within the molecular structures thus no distinction between the target analytes can be done through the CCS measurements. In that sense, computational studies would be of great interest to understand the adduct states. In addition, the next step would be to conduct additional studies with different IMS instrumentation to find more convenient adducts which may present CCS values significantly distinguishable and whose percentage difference are > ±2% allowing the identification of the isomers. In the present work, the integration of IMS in the LC-MS

workflow did not increase the detection selectivity for BMAA, DAB and AEG and CCS cannot be taken as a novel parameter for compound identification between these cyanotoxins. Therefore, according to present results, the chromatographic separation provided by HILIC would be necessary to distinguish and identify BMAA, DAB and AEG isomers (**Figure 2**).

3.2. Adduct effect on CCS values

As previously mentioned, small molecules such as the algal toxins present in this work (118-1067 Da) might form different adducts besides the protonated and deprotonated molecule and they can also form protomers, leading to various ion mobility drift times. As CCS values depend on the charge and on the multiple adduct states, these facts influence the consistency of the developed database for toxins identification in real and complex matrix samples. In fact, different adduct formation can provide additional selectivity and can also show practical utility for identification of analytes and isomers, as not all isomeric structures form the same ionic specie in electrospray ionization mode [19]. For that reason, it is important to include in databases not only the CCS of the most abundant ions formed but also of all the adducts observed. In order to check the impact of the formed adducts on the CCS values, adducts formed under ESI^+ mode conditions were considered and displayed in **Figure 8**.

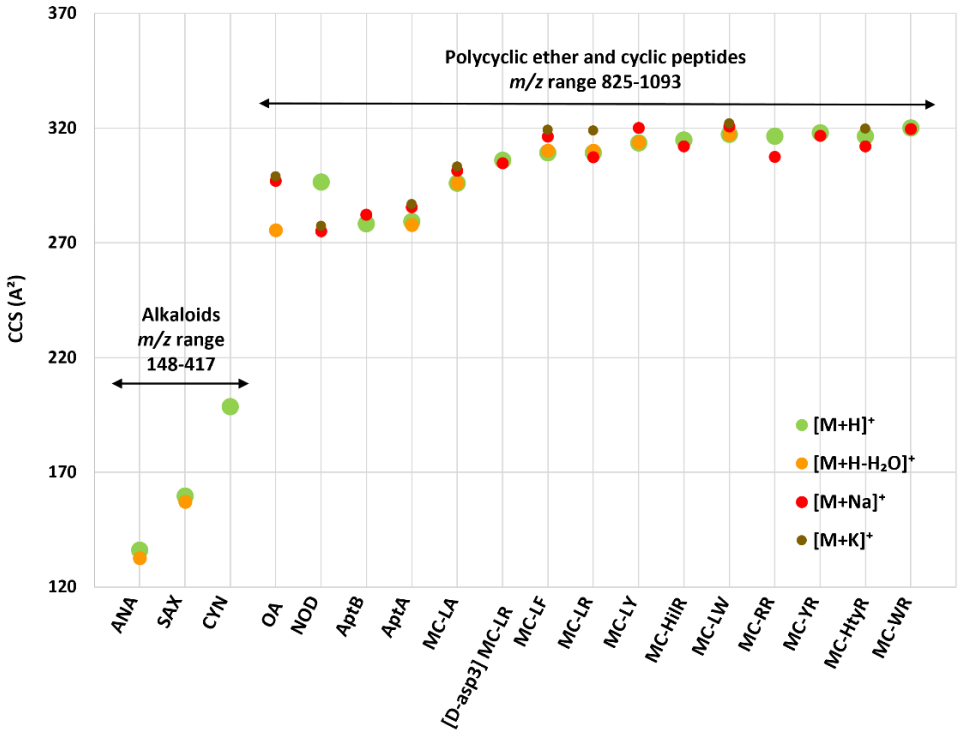


Figure 8. Comparison of experimentally derived CCS in four commonly observed ion adduct states ($[M+H]^+$, $[M+H-H_2O]^+$, $[M+Na]^+$, $[M+K]^+$).

Generally, sodium and potassium adducts are expected to have higher CCS values than the protonated molecule, as these cations are larger than the proton and consequently the formed adduct interacts more with the nitrogen buffer gas giving rise to a higher drift time value. This trend has been observed in literature when the CCS values of molecules such as mycotoxins [20] or steroids [15] have been investigated. The difference observed between CCS values of protonated and cationated toxins shows the influence in the structural properties of the adducts in comparison to the protonated molecules. Alkaloids such as ANA, CYN and SAX did not tend to form sodium or potassium adducts, but they formed the dehydrated adduct (except for CYN) in positive electrospray ionization mode. However, only ANA showed a difference between this CCS and that from the protonated adduct slightly higher than the 2% threshold. For the

polycyclic ether marine biotoxin OA, the sodium, potassium and dehydrated adducts were detected, showing a difference in CCS values between the cationated adducts and the dehydrated adduct far higher than 2%. In that case, the protonated molecule was not observed. In fact, its most intense adduct was the deprotonated molecule observed in ESI^- mode.

On the other hand, all cyclic peptides cyanotoxins (microcystins, anabaenopeptins and nodularin) leaded to the formation of the sodium adduct apart from the protonated molecule, but only some of them tended to form the potassium adduct and/or the dehydrated adduct (see **Table 1**). In addition, as explained above, only MC-RR leads to the formation of the double-charged $[M+2H]^{2+}$ adduct. For most of the compounds it was observed that $\Omega(K^+) > \Omega(H^+)$, which is in line with the increase in the size of the potassium cation compared with the proton. Furthermore, the differences CCs values observed between potassium and protonated adducts were higher than 2% for all cyanotoxins excepting MC-LW and MC-HtyR. However, when referring to sodium adducts, there was no consistent trend in the CCS values obtained as it was expected due to the increase in the radii of the ionic metal compared with the proton. Instead, the CCS values of the sodium adducts were quite similar to the protonated molecules and only the sodium adducts of NOD, APa, MC-LF, MC-LY and MC-RR showed differences in CCS values greater than 2%. The most remarkable case is nodularin, in which it was observed that both, the potassium and sodium adducts presented CCS values well below those of the protonated molecule, contrary to that was expected. This can be explained by an increased ion compaction and stability after cation binding. However, the CCS differences were higher than 6% for both adducts when comparing with the protonated nodularin. Similarly, the dehydrated adducts lead to a decrease in the size of the molecule, so the CCS values are generally lower than those obtained for the protonated

molecule $\Omega(-H_2O+H^+) < \Omega(H^+)$. Differences in CCS values higher than 2% was observed only in the anatoxin alkaloid; however, for all other toxins the CCS values of the protonated and dehydrated adducts are not significantly different, i.e., less than 2%.

3.3. CCS prediction

The experimentally derived $^{TW}CCS_{N2}$ can also be compared with corresponding predicted values. In this work, CCS values were predicted by different machine learning online tools such as AllCCS, CCSbase and MetCCS. The achievement of good predictions of CCS values would allow a greater reliability in the identification process, as new phycotoxins can emerge and be characterized by matching predicted and experimental CCS values, despite the lack of analytical standards. Overall, 61, 59 and 60 ions (anions plus cations) were considered for AllCCS, CCSbase and MetCCS, respectively. Considering protonated, deprotonated, potassium, sodium and dehydrated adducts, the differences among the machine learning tools lie in the fact that AllCCS does not predict potassium adducts, CCSbase does not predict the dehydrated adducts but it does predict potassium adducts and MetCCS do not predict neither potassium adducts nor dehydrated adducts in negative mode.

Values predicted by the machine learning approaches showed a Pearson correlation coefficient higher than 0.9220. Despite the great power of these tools, large deviation was observed. For instance, prediction errors were observed within ±2% only for 16%, 17% and 7% of the ions when dealing with AllCCS, CCSbase and MetCCS prediction models, respectively, regardless the phycotoxin specie considered. It is remarkable that high deviations were found for the predicted values of CCS in ESI^- mode, especially for AllCCS and CCSbase prediction models, where only 5% of the predicted adducts showed a difference within ±2% with respect to the experimental value. Results of

predicted CCS values and differences compared to the experimental values can be observed in **Figure 9** and Table A6.

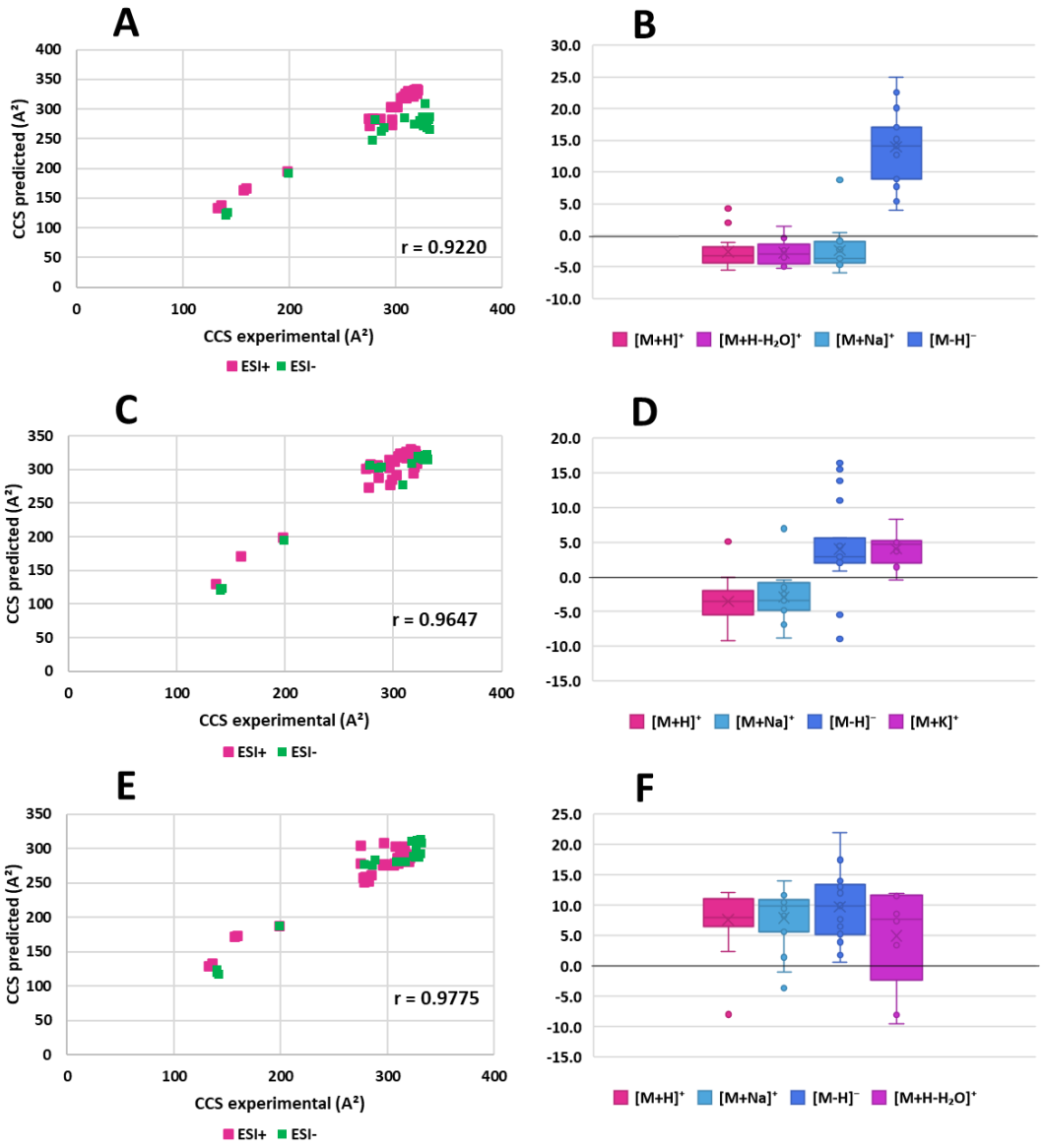


Figure 9. Predicted vs. experimentally observed TWCCSN2 values obtained with: A) AllCCS; C) CCSbase; E) MetCCS online tools (A: Pearson r = 0.9220, p < 0.001; C: Pearson r = 0.9647, p < 0.001; E Pearson r = 0.9775, p < 0.001). Spread of CCS percent deviations (ΔCCS) according to most abundant adduct ions monitored with: B) AllCCS; D) CCSbase; F) MetCCS

One of the possible reasons for these high errors might be that the CCS data used to develop the training set were $^{DT}CCS_{N2}$ employing the stepped field method [7]. Thus, our results seem to suggest that it would be necessary a training set composed by $^{TW}CCS_{N2}$ in order to obtain more accurate results. According to results previously reported by Righetti et al., it was observed that the CCSbase prediction model provided more accurate CCS values, as it includes measurements on TWIM platforms [21]. Despite this fact, it can be concluded that the predictive models are not yet completely accurate for every molecule and formed adducts, thus making necessary the employment of the same class of chemical compounds and the same IMS technology.

3.4. CCS measurements in BGA samples

Once the $^{TW}CCS_{N2}$ values were obtained in standard solutions, an extract of BGA dietary supplement free of toxins was spiked with a mixture of toxins and analyzed to evaluate the influence of the matrix on CCS measurements. The robustness of CCS measurements was carried out by comparing the average $^{TW}CCS_{N2}$ values obtained with standard solutions with those obtained in a spiked spirulina dietary supplement. For that purpose, dietary supplement was spiked with a mixture of the phycotoxins at 500 μg/L and analyzed following the procedure detailed in the Experimental Section 2.3.

As a proof of concept, the toxins investigated in this study were the ones analyzed by the reversed phase method (i.e., all of them except anatoxin and the amino acid isomers), and the selected concentration level ensured that the peak intensity was high enough to be detected under both positive and negative ESI mode. In addition to spiked samples, blanks of BGA dietary supplements were also analyzed.

Among all CCS values measured in BGA samples, high accuracy and robustness was generally achieved when compared with those derived in pure solvent. As can be seen

in **Figure 10**, more than 87% of the CCS values obtained in matrix showed a deviation lower than 0.5% with respect to the standard solutions measurements, around 10% of the values presented an error between 0.5 and 1%, and only one measurement, corresponding with the potassium adduct of NOD showed an error higher than 1%, which was in any case lower than the established threshold value of 2%. The small differences in CCS values between standards and spiked BGA extracts, ranging from 0.0 to 1.3%, proved the reliability in CCS measurements.

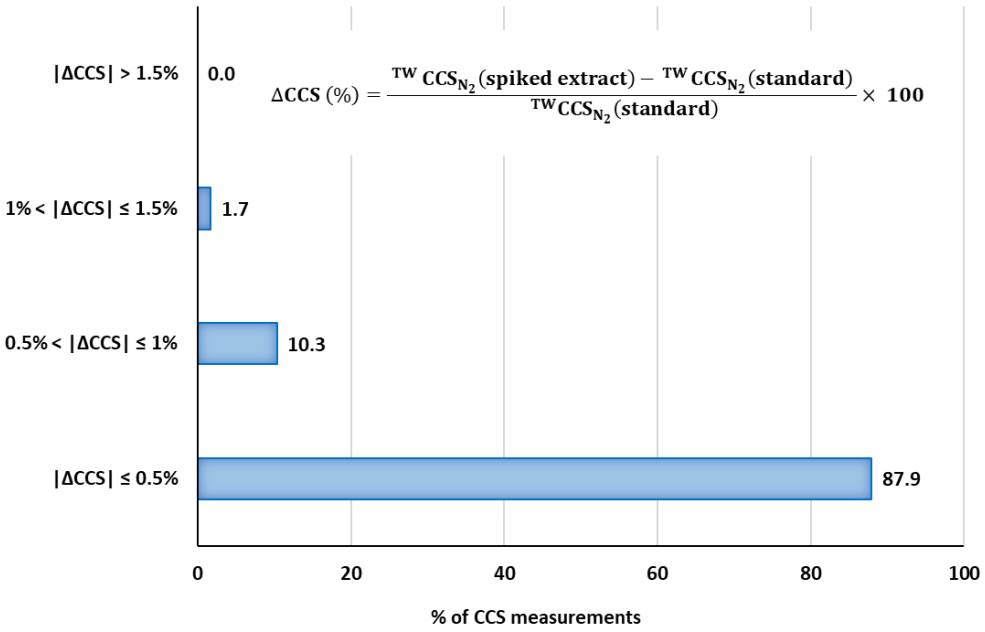


Figure 10. Accuracy of CCS measurements of cyanotoxins in BGA derived dietary supplements.

3.5. Application of ion mobility derived information to the analysis of BGA dietary supplements

As stated above, the implementation of IMS in LC-HRMS workflows enhances the detection of compounds in both targeted and non-targeted analysis because CCS provides an additional parameter for compound identification which may enhance sensitivity and selectivity. In this sense, the proposed UHPLC-TWIMS-QTOF method

was applied to the analysis of dietary supplements containing BGA to demonstrate for the first time the applicability of the IMS technology for the determination of phycotoxins in BGA dietary supplements.

As a proof of concept, nineteen samples based on diverse BGA (listed in **Annex**, **Table A2**) from different markets were analyzed applying the reversed phase UHPLC-TWIMS-QTOF workflow in a suspect screening approach.

The results showed that five out of nineteen analyzed samples were positive for at least one cyanotoxin. Overall, five different cyanotoxins were identified in the analyzed samples as they matched retention time, accurate *m*/*z* and $^{TW}CCS_{N2}$ values with the ones obtained from the standard solutions. MC-LA was positively identified in 4 out of the 5 positive samples. In this line, earlier studies carried out in algal dietary supplements from the Belgium market have also reported MC-LA as one of the most frequently detected [22]. On the other hand, MC-LR and MC-RR were found in two samples while MC-YR and APb were identified in one sample. One of the positive BGA dietary supplement contained up to four different MCs. The extracted ion chromatograms and low mass spectra of this positive sample can be observed in **Figure 11**.

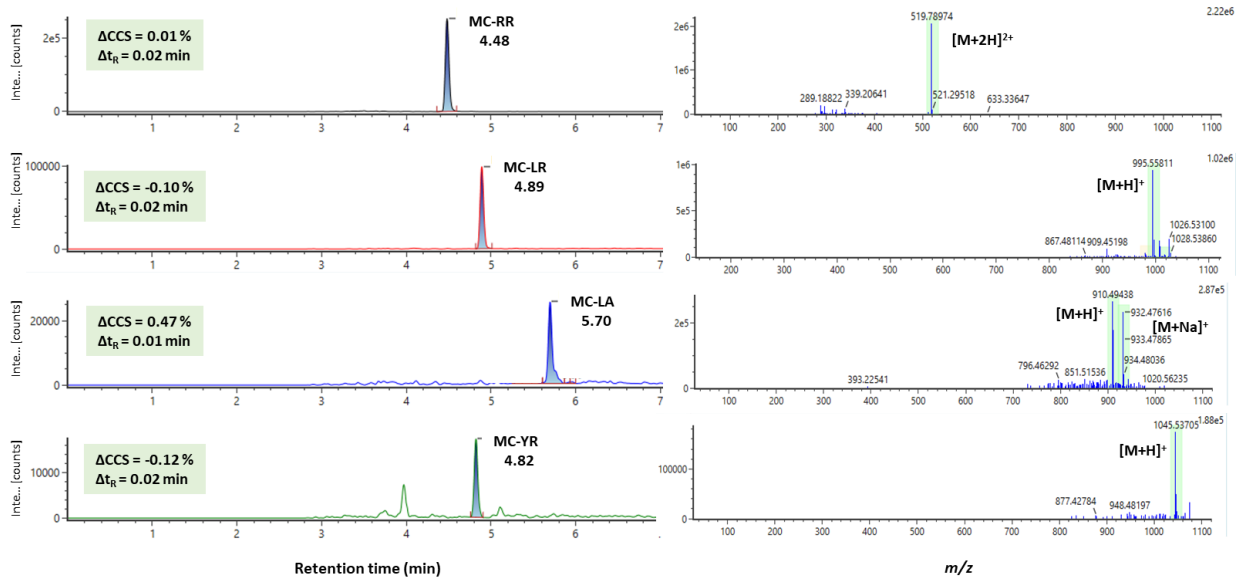


Figure 11. Extracted ion chromatograms and low energy mass spectra of cyanotoxins found in one of the positive BGA dietary supplement samples.

It has also been specified the Δt_R value, which is the difference between the retention time of the analyte in standard solution and the one found in the positive sample, and the ΔCCS value, which represents the percentage difference of the CCS value observed in the positive BGA dietary supplement sample compared with the experimental CCS value measured in standard solutions, following the equation (1).

$$\Delta CCS = \frac{(\text{CCS observed in positive sample} - \text{CCS of standard solution})}{\text{CCS of standard solution}} \times 100 \qquad (1)$$

As it can be observed, the ΔCCS values are below the threshold of 2%, so it is verified that, indeed, the CCS can be taken as an additional identification point. Moreover, these results are in line with those previously reported by other authors, where MCs were not present in spirulina samples, but they were almost exclusively detected in products containing *Aphanizomenon flos-aquae* [22,23,24,25,26,27].

4. Concluding remarks

The comprehensive $^{TW}CCS_{N2}$ database developed provided reliable and reproducible *m*/*z* values, retention times, and $^{TW}CCS_{N2}$ values for 81 adducts (including ESI^+ and ESI^- modes), extending the limited information currently available about the CCS characterization of natural toxins. The reliability and robustness of the $^{TW}CCS_{N2}$ measurements was also demonstrated, as their values were constant and independent from the sample matrix (87% of the CCS values obtained in spiked matrix showed a deviation less than 0.5% with respect to the standard solution measurements used to obtain the database). Nevertheless, further studies would be highly recommended to extend this investigation and verify CCS measurements in an interlaboratory study and among different IMS systems, such as DTIMS, TIMS or differential mobility analyzers. Despite the trend observed regardless the toxins classes, the characterization of a larger number of compounds for each group would be very useful to identify the structural family distribution trends more clearly. Moreover, it would improve the reliability of identification of unknown substances in IMS-MS by determining the chemical categories in complex samples. In addition, while the separation in the drift time dimension of the critical trio of cyanotoxin isomers was addressed, further computational and experimental studies would be advisable to achieve significantly different CCS values that could be used as identification parameters. As a proof of concept, the applicability of this approach was evaluated in the screening of cyanotoxins by analyzing various commercial BGA dietary supplements. Several positive samples were found, being MC-LA the most frequently detected toxin, confirming that such products should be subject to routine monitoring before being distributed. The obtained CCS values in positive analyzed samples exhibited small deviations ($\Delta CCS < 2\%$) compared with database, which verifies CCS as an additional identification parameter, adding confidence in

cyanotoxins identification. The availability of this approach is relevant also from the perspective of the expected and ongoing increase in applications for novel food registrations based on cyanobacteria and algae, that will sustain the need for punctual, reliable, and flexible analytical approaches to cyanotoxin analysis.

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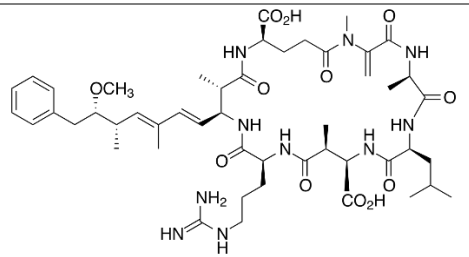
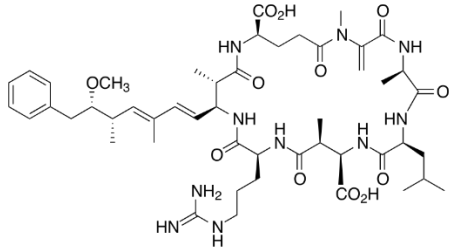
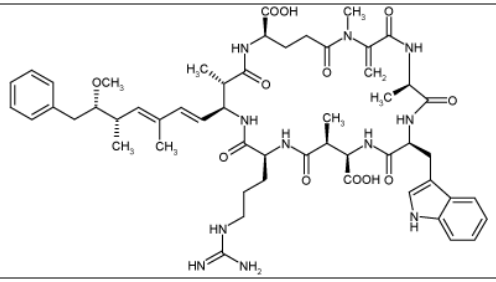
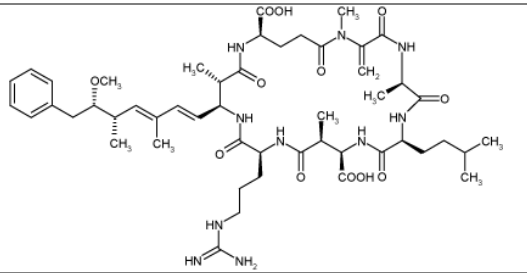
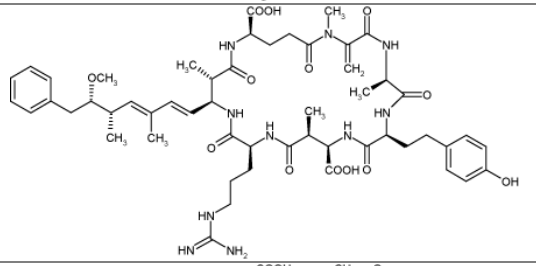
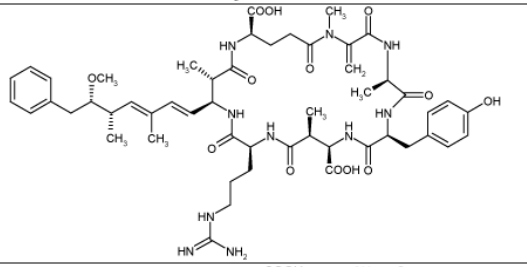
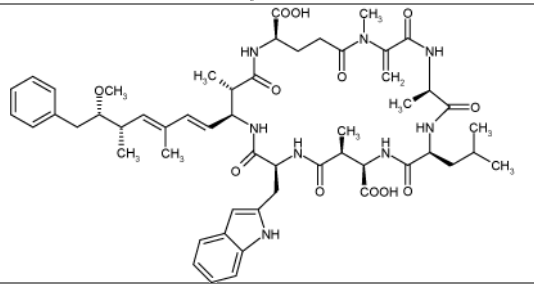
ANNEX

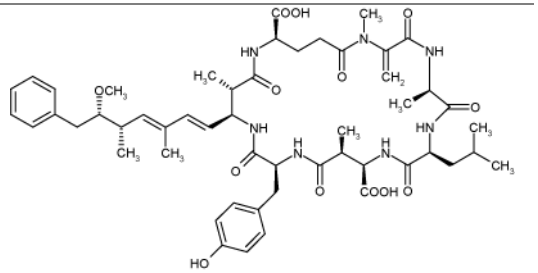
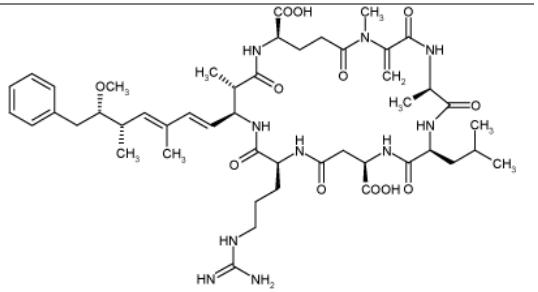
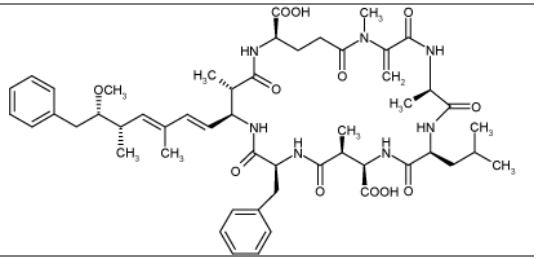
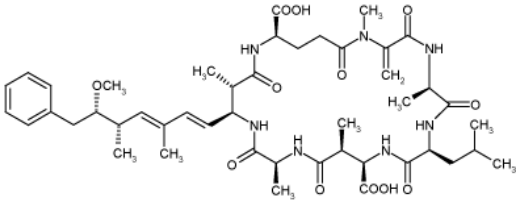
CHAPTER 5

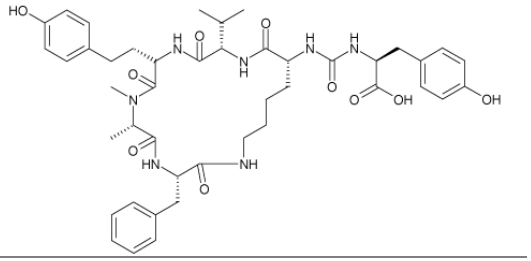
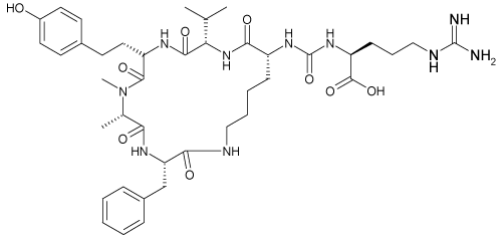
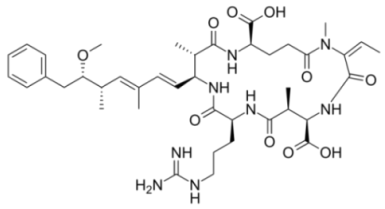
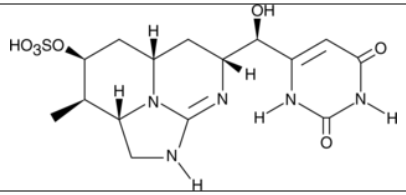
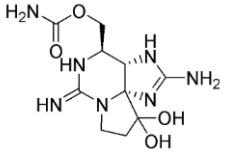
APPLICATION OF ION MOBILITY SPECTROMETRY- LIQUID CHROMATOGRAPHY- HIGH RESOLUTION MASS SPECTROMETRY FOR THE CREATION OF A COLLISION CROSS SECTION DATABASE FOR PHYCOTOXINS SCREENING

Table A1. Toxicity, structure and physicochemical properties of target toxins.

Name	Toxin group	Toxicity	Formula	Molecular weight	Purity	Seller	Chemical structure
Microcystin-Leucine-Arginine (MC-LR)	**Cyclic peptides**	Hepatotoxin	$C_{49}H_{74}N_{10}O_{12}$	994.5488	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Microcystin-Arginine-Arginine (MC-RR)	**Cyclic peptides**	Hepatotoxin	$C_{49}H_{75}N_{13}O_{12}$	1037.5658	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Microcystin-Tryptophan-Arginine (MC-WR)	**Cyclic peptides**	Hepatotoxin	$C_{54}H_{73}N_{11}O_{12}$	1067.5440	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	

Microcystin-Homoisoleucine-Arginine (MC-HilR)	**Cyclic peptides**	Hepatotoxin	$C_{50}H_{76}N_{10}O_{12}$	1008.5644	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Microcystin-Homotyrosine-Arginine (MC-HtyR)	**Cyclic peptides**	Hepatotoxin	$C_{53}H_{74}N_{10}O_{13}$	1058.5437	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Microcystin-Tyrosine-Arginine (MC-YR)	**Cyclic peptides**	Hepatotoxin	$C_{52}H_{72}N_{10}O_{13}$	1044.5280	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Microcystin-Leucine-Tryptophan (MC-LW)	**Cyclic peptides**	Hepatotoxin	$C_{54}H_{72}N_8O_{12}$	1024.5270	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	

Microcystin-Leucine-Tyrosine (MC-LY)	**Cyclic peptides**	Hepatotoxin	$C_{52}H_{71}N_7O_{13}$	1001.5110	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
[D-Asp3]Microcystin-Leucine-Arginine ([D-Asp3]-MC-LR)	**Cyclic peptides**	Hepatotoxin	$C_{48}H_{72}N_{10}O_{12}$	980.5331	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Microcystin-Leucine-Phenilalanine (MC-LF)	**Cyclic peptides**	Hepatotoxin	$C_{52}H_{71}N_7O_{12}$	985.5161	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Microcystin-Leucine-Alanine (MC-LA)	**Cyclic peptides**	Hepatotoxin	$C_{46}H_{67}N_7O_{12}$	909.4848	≥ 99%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	

Anabaenopeptin A (APa)	**Cyclic peptides**	Hepatotoxin	$C_{44}H_{57}N_7O_{10}$	843.4167	≥ 95%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Anabaenopeptin B (APb)	**Cyclic peptides**	Hepatotoxin	$C_{41}H_{60}N_{10}O_9$	836.4545	≥ 95%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Nodularin (NOD)	**Cyclic peptides**	Hepatotoxin	$C_{41}H_{60}N_8O_{10}$	824.4432	≥ 95%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Cilindrospermopsin (CYN)	**Alkaloids**	Cytotoxin	$C_{15}H_{21}N_5O_7S$	415.1162	≥ 95%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
Saxitoxin (STX)	**Alkaloids**	Neurotoxin	$C_{10}H_{17}N_7O_4$	299.1342	≥ 97%	CIGFA (Lugo, Spain)	

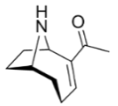
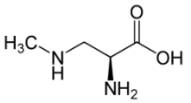
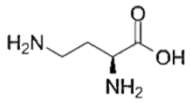
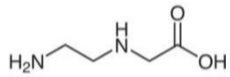
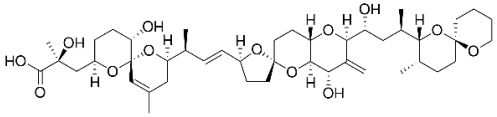
Anatoxin-a (ANA)	**Alkaloids**	Neurotoxin	$C_{10}H_{15}NO$	165.1154	≥ 98%	Enzo Life Sciences, Inc. (Lausen, Switzerland)	
β-methylamino-L-alanine (BMAA)	**Non-protein amino acids**	Neurotoxin	$C_4H_{10}N_2O_2$	118.0742	≥ 97%	Sigma Aldrich (Darmstadt, Germany)	
2,4-diaminobutyric acid (DAB)	**Non-protein amino acids**	Neurotoxin	$C_4H_{10}N_2O_2$	118.0742	≥ 95%	Sigma Aldrich (Darmstadt, Germany)	
N-(2-aminoethyl)glycine (AEG)	**Non-protein amino acids**	Neurotoxin	$C_4H_{10}N_2O_2$	118.0742	≥ 98%	Sigma Aldrich (Darmstadt, Germany)	
Okadaic Acid (OA)	**Polycyclic ether**	Neurotoxin	$C_{44}H_{68}O_{13}$	804.4660	≥ 95%	Cayman Chemicals (Michigan, USA)	

Table A2. List of analyzed BGA dietary supplements.

Sample	Acquired in	Supplier	Origin	Form	Composition	Daily dose
1	Spain	Local store (Granada)	Costa Rica	Powder	Pure spirulina (*Arthrospira platensis*)	6 g product, i.e, 6000 mg spirulina
2	Spain	Online	USA	Capsule	Spirulina (*Arthrospira platensis* Gomont), hydroxypropyl methylcellulose, chlorella (*Chlorella vulgaris* Beijerinck), fucus (*Fucus vesiculosus* L.), vitamin C, magnesium stearate	6 capsules, i.e, 891 mg spirulina, 445.5 mg chlorella, 445.5 mg fucus
3	Spain	Online	Spain	Tablet	Spirulina (*Arthrospira platensis*), chlorella (*Chlorella vulgaris),* ferrous fumarate, folic acid, vitamin B12	3 capsules, i.e, 750 mg spirulina, 750 mg chlorella
4	Spain	Online	Spain	Tablet	Klamath (*Aphanizomenon flos-aquae*), spirulina (*Spirulina platensis* Geitler), microcrystalline cellulose, silicon dioxide	3 capsules, i.e, 600 mg klamath (Aphanizomenon flos aquae), 600 mg spirulina
5	Spain	Online	Agriculture non-UE	Tablet	Chlorella (*Chlorella pyrenoidosa* Chick), silicon dioxide	8 capsules, i.e, 3268 mg chlorella
6	Spain	Online	Agriculture non-UE	Tablet	Spirulina (*Spirulina platensis* Geitler), dioxide de silício	6 capsules, i.e, 2562 mg spirulina
7	Spain	Local Store (Granada)	Unknown	Capsule	Spirulina (*Arthrospira platensis*), hydroxypropyl methylcellulose	6 capsules, i.e, 2160 mg spirulina

8	Spain	Local Store (Granada)	?	Powder	Pure Spirulina (*Arthrospira platensis*)	Not available
9	Spain	Local store	?	Powder	Pure Klamath (*Aphanizomenon flos-aquae*)	Not available
10	Italy	Online	Agriculture non-UE	Tablet	Pure Klamath (*Aphanizomenon flos-aquae*)	3 capsules, i.e, 1500 mg klamath
11	Italy	Online	Agriculture non-UE	Tablet	Klamath (*Aphanizomenon flos-aquae*) 99.5%, anti-caking: talc 0.5%	4 capsules, i.e, 1990 mg kalmath
12	Italy	Online	USA	Capsule	Klamath (*Aphanizomenon flos-aquae*), vegetal capsule: hydroxipropyl methylcellulose, potato starch	6 capsules, i.e, 2400 mg klamath
13	Italy	Online	France	Liquid	Klamath (*Aphanizomenon flos-aquaea*) 40%, *Spirulina platensis* extract (phycocyanin) 50%, stabilizer: glycerol, Flavour: tea tree essential oil	5 mL product, i.e., 2 mL klamath
14	Italy	Online	Italy	Tablet	Klamath (*Aphanizomenon flos-aquae*), adjuvants: hydroxypropyl cellulose, silicium dioxide, microcrystalline cellulose, magnesium stearate, vegetal capsule; hydroxipropyl methylcellulose, polyethylenglicol	3 capsules, i.e, 2700 mg klamath
15	Italy	Online	USA	Powder	Pure klamath (*Aphanizomenon flos-aquae*)	1 g product, i.e., 1000 mg klamath
16	Italy	Local Store (Parma)	Italy	Tablet	*Spirulina platensis*, anti-caking agent: talc	6 capsules, i.e, 2985 mg spirulina

17	Italy	Online	Italy	Capsule	Pure *Spirulina platensis*	6 capsules, i.e, 2400 mg spirulina
18	Italy	Online	Italy	Capsule	Chlorella (*Chlorella pyrenoidosa* H. Chick), inlunin, anti-caking agent: magnesium salt from fatty acid, vegetal capsule (hydroxipropyl methylcellulose)	4 capsules, i.e, 1400 mg chlorella and 170 mg inulin
19	Italy	Online	USA	Powder	Pure Klamath (*Aphanizomenon flos-aquae*)	2 g product, i.e., 2000 mg klamath

Table A3. Composition of CCS calibration solution used for positive ionization mode.

Compound	*m/z*	CCS (Å²)
Acetaminophen	152.0706	130.4
Reserpine fragment	195.0877	138.2
Sulfaguanidine	215.0597	146.8
Sulfadimethoxine	311.0809	168.4
Val-Tyr-Val	380.2180	191.7
Verapamil	455.2904	208.8
Terfenadine	472.3210	228.7
Polyalanine	516.2776	211.0
Leucine Enkephalin	556.2766	229.8
Polyalanine	587.3148	252.3
Reserpine	609.2807	252.3
Polyalanine	658.3519	243.0
Polyalanine	729.3890	256.0
Polyalanine	800.4261	271.0
Polyalanine	871.4632	282.0
Polyalanine	942.5003	294.0
Polyalanine	1013.5374	306.0
Polyalanine	1084.5746	321.5
Polyalanine	1155.6117	333.6
Ultramark 1621	1022.0034	263.1
Ultramark 1621	1121.9970	276.5
Ultramark 1621	1221.9843	291.2
Ultramark 1621	1321.9843	304.0
Ultramark 1621	1421.9779	316.7
Ultramark 1621	1521.9715	329.0
Ultramark 1621	1621.9651	340.1
Ultramark 1621	1721.9587	351.3
Ultramark 1621	1821.9523	362.1
Ultramark 1621	1921.9459	372.6

Table A4. Composition of CCS calibration solution used for negative ionization mode.

Compound	*m/z*	CCS (Å²)
Acetaminophen	150.0561	131.5
Theophylline	179.0575	132.4
Sulfaguanidine	213.0452	145.2
Sulfadimethoxine	309.2034	170.1
Val-Tyr-Val	378.2034	192.5
Leucine Enkephalin	554.2620	225.3
Perfluoroheptanoic acid-CO2	318.9766	130.1
Perfluoroheptanoic acid-CO2	368.9766	137.2
Polyalanine	585.3002	227.7
Reserpine	607.2661	265.2
Polyalanine	656.3373	242.1
Polyalanine	727.3744	255.9
Polyalanine	798.4115	268.5
Polyalanine	869.4487	280.2
Polyalanine	940.4856	294.6
Polyalanine	1011.5288	308.8
Polyalanine	1082.5600	322.4
Ultramark 1621	1165.9880	275.8
Ultramark 1621	1265.9816	288.0
Ultramark 1621	1365.9752	299.7
Ultramark 1621	1465.9688	311.7
Ultramark 1621	1565.9624	323.7
Ultramark 1621	1665.9560	334.7
Ultramark 1621	1765.9496	346.2
Ultramark 1621	1865.9432	357.1
Ultramark 1621	1965.9369	367.2

Table A5. $^{TW}CCS_{N2}$ values obtained for BMAA, DAB and AEG.

Compound	Adduct	Theoretical exact *m*/*z*	Experimental $^{TW}CCS_{N2}$	SD	RSD (%)
β-methylamine-L-alanine	$[M-H+2Na]^+$	163.0459	**133.4**	0.75	0.56
β-methylamine-L-alanine	$[M-H+2Na]^+$	163.0459	**161.8**	0.80	0.50
β-methylamine-L-alanine	$[M-H+2Na]^+$	163.0459	**182.7**	2.72	1.49
2,4-diaminobutyric acid	$[M-H+2Na]^+$	163.0459	**132.7**	-	-
2,4-diaminobutyric acid	$[M-H+2Na]^+$	163.0459	**159.4**	0.91	0.57
2,4-diaminobutyric acid	$[M-H+2Na]^+$	163.0459	**177.9**	4.66	2.62
N-(2-aminoethyl)glycine	$[M-H+2Na]^+$	163.0459	**132.4**	0.30	0.23
N-(2-aminoethyl)glycine	$[M-H+2Na]^+$	163.0459	**164.2**	0.83	0.50
N-(2-aminoethyl)glycine	$[M-H+2Na]^+$	163.0459	**185.3**	1.13	0.61

Table A6. Theoretical CCS of target toxins obtained using AllCCS, CCSbase and MetCCS online tools.

Compound	Adduct	Experimental $^{TW}CCS_{N2}$	AllCCS prediction	ΔCCS (%) AllCCS	CCSbase prediction	ΔCCS (%) CCSBase	MetCCS prediction	ΔCCS (%) MetCCS
β-methylamine-L-alanine	[M-H]-	**140.6**	124.4	-13.0	123.5	-13.9	123.4	-14.0
2,4-diaminobutyric acid	[M-H]-	**140.7**	121.8	-15.6	120.9	-16.4	119.8	-17.5
N-(2-aminoethyl)glycine	[M-H]-	**141.9**	125.1	-13.4	122.8	-15.5	116.4	-21.9
Anatoxin-a	$[M+H]^+$	**136.1**	137.6	1.1	129.5	-5.1	132.9	-2.4
Anatoxin-a	$[M+H-H_2O]^+$	**132.6**	133.2	0.4		0.0	128.2	-3.5
Saxitoxin	$[M+H]^+$	**159.6**	166.2	3.9	170.9	6.6	173.5	8.0
Saxitoxin	$[M+H-H_2O]^+$	**157.2**	162.8	3.5			171.0	8.1
Cylindrospermopsin	$[M+H]^+$	**198.6**	194.8	-1.9	198.7	0.1	187.0	-6.2
Cylindrospermopsin	$[M-H]^-$	**198.9**	191.3	-4.0	194.9	-2.1	186.7	-6.6
Okadaic acid	$[M-H]^-$	**308.4**	286.10	-7.8	277.70	-11.1	280.5	-10.0
Okadaic acid	$[M+Na]^+$	**296.9**	272.90	-8.8	277.50	-7.0	308.1	3.6
Okadaic acid	$[M+K]^+$	**298.9**			284.80	-5.0		
Okadaic acid	$[M+H-H_2O]^+$	**275.5**	271.6	-1.4			304.6	9.6
Nodularin	$[M+H]^+$	**296.5**	284.3	-4.3	302.2	1.9	277.5	-6.8
Nodularin	$[M-H]^-$	**288.8**	268.2	-7.7	303.3	4.8	283.8	-1.8
Nodularin	$[M+Na]^+$	**274.9**	284.8	3.4	301.5	8.8	277.9	1.1
Nodularin	$[M+K]^+$	**277.3**			273.3	-1.4		
Anabaenopeptin B	$[M+H]^+$	**278.3**	283.7	1.9	304.3	8.6	251.5	-10.6
Anabaenopeptin B	$[M-H]^-$	**286.4**	263.1	-8.9	303.0	5.5	275.3	-4.0
Anabaenopeptin B	$[M+Na]^+$	**282.2**	283.5	0.5	302.5	6.7	252.7	-11.7
Anabaenopeptin B	$[M-H-H_2O]^-$	**280.5**	283.8	1.2				
Anabaenopeptin A	$[M+H]^+$	**279.2**	284.6	1.9	307.4	9.2	259.2	-7.7
Anabaenopeptin A	$[M-H]^-$	**278.3**	247.0	-12.7	305.7	9.0	276.7	-0.6
Anabaenopeptin A	$[M+Na]^+$	**285.5**	284.5	-0.4	306.6	6.9	260.8	-9.5

Anabaenopeptin A	$[M+K]^+$	**286.7**			288.1	0.5		
Anabaenopeptin A	$[M+H-H_2O]^+$	**277.8**	284.7	2.4			257.8	-7.7
Microcystin-LA	$[M+H]^+$	**296.0**	304.0	2.6	313.7	5.6	276.5	-7.1
Microcystin-LA	$[M-H]^-$	**317.2**	274.6	-15.5	308.6	-2.8	280.6	-13.0
Microcystin-LA	$[M+Na]^+$	**301.3**	304.1	0.9	312.0	3.4	277.5	-8.6
Microcystin-LA	$[M+K]^+$	**303.2**			292.2	-3.8		
Microcystin-LA	$[M+H-H_2O]^+$	**296.0**	303.9	2.6			275.6	-7.4
[D-Asp3]Microcystin-LR	$[M+H]^+$	**305.9**	319.80	4.3	323.30	5.4	275.5	-11.0
[D-Asp3]Microcystin-LR	$[M-H]^-$	**324.8**	288.10	-12.7	317.40	-2.3	288.3	-12.7
[D-Asp3]Microcystin-LR	$[M+Na]^+$	**304.7**	319.60	4.7	320.10	4.8	275.8	-10.5
Microcystin-LF	$[M+H]^+$	**309.2**	327.3	5.5	324.2	4.6	286.3	-8.0
Microcystin-LF	$[M-H]^-$	**329.7**	269.1	-22.5	321.3	-2.6	287.8	-14.5
Microcystin-LF	$[M+Na]^+$	**316.3**	327.4	3.4	324.2	2.4	287.1	-10.2
Microcystin-LF	$[M+K]^+$	**319.2**			303.7	-5.1		
Microcystin-LF	$[M+H-H_2O]^+$	**310.2**	327.2	5.2			285.6	-8.6
Microcystin-LR	$[M+H]^+$	**309.3**	319.8	3.3	321.2	3.7	277.8	-11.3
Microcystin-LR	$[M-H]^-$	**326.9**	284.3	-15.0	315.5	-3.6	291.8	-12.0
Microcystin-LR	$[M+Na]^+$	**307.3**	319.8	3.9	318.2	3.4	277.9	-10.6
Microcystin-LR	$[M+K]^+$	**318.9**			294.4	-8.3		
Microcystin-LR	$[M+H-H_2O]^+$	**310.2**	319.8	3.0		0.0	277.7	-11.7
Microcystin-LY	$[M+H]^+$	**313.5**	327.4	4.3	324.9	3.5	280.6	-11.7
Microcystin-LY	$[M-H]^-$	**326.1**	271.6	-20.1	318.4	-2.4	287.4	-13.5
Microcystin-LY	$[M+Na]^+$	**320.0**	327.4	2.3	322.4	0.7	280.8	-14.0
Microcystin-LY	$[M+H-H_2O]^+$	**313.8**	327.3	4.2			280.3	-11.9
Microcystin-HilR	$[M+H]^+$	**314.7**	324.10	2.9	320.10	1.7	280.9	-12.0
Microcystin-HilR	$[M-H]^-$	**331.5**	287.90	-15.2	316.20	-4.8	293.2	-13.1
Microcystin-HilR	$[M+Na]^+$	**312.1**	324.10	3.7	316.90	1.5	281.2	-11.0
Microcystin-LW	$[M+H]^+$	**317.3**	333.7	4.9	327.0	3.0	285.3	-11.2
Microcystin-LW	$[M-H]^-$	**332.1**	265.7	-25.0	314.5	-5.6	308.4	-7.7

Microcystin-LW	$[M+Na]^+$	**320.7**	333.6	3.9	322.2	0.5	286.2	-12.1
Microcystin-LW	$[M+K]^+$	**322.0**			308.6	-4.3		
Microcystin-LW	$[M+H-H_2O]^+$	**317.2**	333.6	4.9			284.5	-11.5
Microcystin-RR	$[M+H]^+$	**316.4**	322.5	1.9	322.8	2.0	302.9	-4.5
Microcystin-RR	$[M-H]^-$	**327.4**	310.8	-5.3	316.2	-3.5	302.9	-8.1
Microcystin-RR	$[M+Na]^+$	**307.4**	322.3	4.6	318.1	3.4	303.1	-1.4
Microcystin-YR	$[M+H]^+$	**318.0**	328.7	3.3	328.0	3.1	291.8	-9.0
Microcystin-YR	$[M-H]^-$	**322.7**	282.7	-14.2	319.9	-0.9	310.5	-3.9
Microcystin-YR	$[M+Na]^+$	**316.6**	328.5	3.6	324.0	2.3	292.3	-8.3
Microcystin-HtyR	$[M+H]^+$	**316.4**	332.0	4.7	329.9	4.1	295.0	-7.3
Microcystin-HtyR	$[M-H]^-$	**331.2**	282.9	-17.1	321.7	-3.0	313.3	-5.7
Microcystin-HtyR	$[M+Na]^+$	**312.0**	331.9	6.0	325.7	4.2	295.4	-5.6
Microcystin-HtyR	$[M+K]^+$	**319.7**		0.0	303.7	-5.3		
Microcystin-WR	$[M+H]^+$	**320.0**	334.2	4.3	328.1	2.5	290.4	-10.2
Microcystin-WR	$[M-H]^-$	**328.2**	272.8	-20.3	314.2	-4.5	311.9	-5.2
Microcystin-WR	$[M+Na]^+$	**319.5**	334.1	4.3	322.0	0.8	290.8	-9.9

FINAL CONCLUSIONS

The different developed methods reported in this Thesis have contributed to increase the number of analytical methods available for the control of fipronil and its metabolites as well as for the control of cyanotoxins in the field of food and environmental safety. The potential of using miniaturized techniques such as CE and CLC and other very efficient techniques such as UHPLC and IMS coupled to different detection systems such as UV, MS and HRMS has been evaluated. In addition, the optimization of different sample treatments depending on the target analytes and matrix have been carried out. The methodologies have been validated and applied in real samples of waters, food and nutraceutical in order to show their usefulness.

The conclusions obtained in this Thesis are as follows:

- It has been demonstrated for the first time that fipronil and its main metabolite, fipronil-sulfone can be satisfactory determined by MEKC-UV employing APFO as a volatile surfactant, which acts as both BGE and micellar medium. The use of APFO makes the separation compatible with MS, which would be an improvement in further studies, while other non-volatile surfactants would contaminate the ion source. Results showed the importance of the sample solvent in peak shape.

- CE and CLC methods for the monitoring of insecticides involve lower solvent consumption, especially of organic solvents, in comparison with traditional LC-

methods, while maintaining a good efficiency. This is reflected in a more economically affordable and environmentally friendly analysis.

- Among the considered techniques to determine fipronil and its metabolites, CE is the greenest technique, since no organic solvents are needed for the separation due to the use of a small amount of aqueous BGE, and few waste is generated. In addition, the use of economical silica capillaries instead of more expensive chromatographic columns along with the short analysis times obtained are positive aspect in relation to the CE technique.

- A simple sample treatment based on SALLE proved to be suitable for the determination of fipronil and its metabolites in egg samples. MeCN as extraction solvent along with $(NH_4)_2SO_4$ as salting-out reagent resulted in great extraction performance, with recoveries > 79.5% in both MEKC-UV and CLC-UV methods. However, while there was no inconvenient during CLC-UV analysis, with the proposed MEKC method coupled to a low-selective detection system such as UV, a subsequent cleaning step would be necessary to determine fipronil-sulfide.

- UV detection is the most widely used detection method primarily due to its availability and simplicity. UV detector is easy to operate and maintain and economically affordable, which makes it suitable for its use in routine analysis and even for a previous screening and quantification in laboratories from different economical backgrounds. In addition, DAD offers spectral molecules information. However, in terms of sensitivity, on-column sample concentration, sample treatments allowing extra preconcentration or more sensitive detection, such as MS, would be required in most of the cases to fulfill the low regulation limits set by the legislation, also with the aim to ensure an unequivocal confirmation of the residues.

- A new method for the determination of eight cyanotoxins from different families, including cyclic peptides, alkaloids and non-protein amino acids has been developed for application in natural water samples. The separation was carried out using HILIC-MS/MS, employing a SeQuant ZIC-HILIC column composed by zwitterionic functional groups which allowed baseline separation of three cyanotoxin isomers. It is the first time that this complex mixture of cyanotoxins with very different properties has been separated in a single run.

- The very different nature and physico-chemical properties of the cyanotoxins studied, from small polar amino acids to large hydrophobic cyclic peptides, is the key point that makes a challenge also to develop a sample treatment that encompasses simultaneous extraction of every cyanotoxin with good performance. In this sense, a tandem-SPE procedure was developed using two cartridges. A Strata-X and a MCX cartridges were connected in series, being the Strata-X cartridge at the top and the MCX cartridge at the bottom of the dual system. After loading the sample, and before the elution step, the order of the cartridges was reversed to fully elute MC-RR cyanotoxin.

- This tandem-SPE procedure allowed the extraction of the target cyanotoxins from reservoir water samples by means of a single sample loading step and the use of a single eluent in the elution step, making the process much more efficient than in previous studies, where different extraction procedures were employed based on each family of cyanotoxins.

- The high analyte preconcentration factor (×100) obtained with the proposed tandem-SPE sample treatment led to very low LOQ values (between $7 \cdot 10^{-3}$ and 0.1 μg/L) for cyanotoxins in water samples. These values were significantly lower than those proposed in the guideline by WHO for MC-LR in drinking water (1 μg/L).

- Despite the good performance of the tandem-SPE method, the optimization procedure highlighted the importance of water ion content for the extraction of BMAA and DAB. It was observed that recoveries were reduced when reservoir water samples with high content of ions were submitted to the tandem-SPE procedure.

- As a result of the increase in the consumption of BGA dietary supplements, the risk of exposure to cyanotoxins is also increasing. Therefore, analytical methods are required to monitor these substances as precaution measures for the safe consumption of these products. In this sense, a method based on HILIC-MS/MS has been developed to determine the presence of cyanotoxins from different families in BGA dietary supplements. Cyanotoxin extraction was based on SLE followed by a tandem-SPE. From a critical point of view, the main challenge posed in this work has been to perform the extraction of multiclass cyanotoxins in such a complicate matrix as it is BGA and being able to quantify concentrations of each toxin at very low levels.

- In matrices such as the BGA-based dietary supplements (*spirulina*, *aphanizomenon-flos aquae* etc.) which contains a relatively high number of amino acids, special attention should be paid to the determination and quantification of ANA since it may be impaired by the presence of the essential amino acid Phe, which is an isobaric compound of ANA and presents a similar fragmentation pattern and LC retention.

- One of the main downsides of the proposed SLE-tandem-SPE resides in the fact that the whole procedure is rather time-consuming and complex, thus it may not be the most suitable procedure for routine analysis. Multitoxin analysis is a challenge that is still under development and in which there is still much to

investigate, especially in relation to sample treatment when the aim is to cover compounds with very different characteristics.

- Positive samples found for MCs and NPAs set a point of attention toward the need of a satisfactory quality control in the production of BGA dietary supplements and envisaged the importance of international legislation to ensure product safety and to protect consumers from cyanotoxin contamination. In this sense, further toxicity studies covering a wider number or cyanotoxins should be carried out to assess the risk of toxins other than the main studied and legislated MC-LR.

- The collaboration with the University of Parma (Parma, Italy) allowed the use of the analytical platform UHPLC-TWIMS-QTOF to characterize for the first time 20 cyanotoxins and 1 marine biotoxin in terms of CCS. Reproducibility and robustness of $^{TW}CCS_{N2}$ measurements were assessed to confirm the reliability of the database.

- The developed $^{TW}CCS_{N2}$ database has extended the current IMS knowledge about natural toxins leading to the use of $^{TW}CCS_{N2}$ values as an additional separation dimension in cyanotoxin workflows alongside with the traditional parameters for identification purpose, such as retention time, accurate *m*/*z* or tandem mass spectra.

- Further studies would be highly recommended to extend the IMS investigation and verify $^{TW}CCS_{N2}$ measurements in an interlaboratory study and among different IMS systems, such as DTIMS, TIMS or DMS analyzers. In addition, the characterization of a larger number of compounds for each group would be very useful to identify the structural family distribution trends more clearly. Moreover, it would improve the reliability of identification of unknown substances in IMS-MS by determining the chemical categories in complex samples.

- Suspect screening analysis of commercial BGA dietary supplements, adding the newly determined $^{TW}CCS_{N2}$ values as identification parameter, confirmed the presence of cyanotoxins from different families, as it was previously observed, reaffirming the need for regulation in this type of products.

CONCLUSIONES FINALES

Los diferentes métodos descritos en esta Tesis han contribuido a incrementar el número de métodos analíticos disponibles para el control de fipronil y sus metabolitos, así como para el control de cianotoxinas, útiles en el ámbito de la seguridad alimentaria y calidad medioambiental. Se ha evaluado el potencial de técnicas miniaturizadas como CE y CLC, y de otras técnicas muy eficaces como UHPLC e IMS acopladas a diferentes sistemas de detección, como UV, MS y HRMS. Además, se ha llevado a cabo el desarrollo de diferentes tratamientos de muestra en función de los analitos de estudio y de la matriz. Las metodologías han sido validadas y aplicadas en muestras reales de aguas, alimentos y nutracéuticos para demostrar su aplicabilidad.

Las conclusiones obtenidas de esta Tesis son las siguientes:

- Se ha demostrado por primera vez que fipronil y su principal, metabolito, fipronil-sulfona pueden ser determinados satisfactoriamente mediante MEKC-UV empleando APFO como surfactante volátil, el cual actúa como BGE y como medio micelar. El uso de APFO hace que la separación sea compatible con MS, lo que sería un avance en futuros estudios, mientras que otros surfactantes no volátiles podrían contaminar la fuente de ionización. Los resultados mostraron la importancia del disolvente de muestra en la forma de pico.

- Los métodos de CE y CLC para el control de residuos de insecticidas involucran menor consumo de disolventes, especialmente de disolventes orgánicos, en comparación con métodos tradicionales de LC a la vez que mantienen una buena

eficiencia y una baja generación de residuos. Esto se refleja en la propuesta de análisis más asequibles económicamente y más respetuosos con el medio ambiente.

- De entre las técnicas consideradas para determinar fipronil y sus metabolitos, CE es la técnica más "verde", ya que no necesita disolventes orgánicos para la separación debido al uso de pequeñas cantidades de BGE acuoso, y a que no genera apenas residuos. Además, el uso de económicos capilares de sílice en lugar de caras columnas cromatográficas junto con los cortos tiempos de análisis obtenidos son aspectos positivos en relación a la técnica de CE.

- Se ha propuesto un tratamiento de muestra sencillo basado en SALLE que demostró su eficacia para la determinación de fipronil y sus metabolitos en muestras de huevo. El uso de MeCN como disolvente de extracción junto con $(NH_4)_2SO_4$ como agente inductor de la separación de fases proporcionaron una buena extracción, con recuperaciones > 79.5% en ambos MEKC-UV y CLC-UV métodos. Sin embargo, mientras que no hubo inconvenientes en el análisis por CLC-UV, con el método MEKC propuesto acoplado a un detector de baja selectividad como es el UV, sería necesaria una posterior etapa de limpieza para poder determinar fipronil-sulfuro.

- La detección UV es el método de detección más usado debido principalmente a su versatilidad y asequibilidad. Este detector es fácil de operar y mantener, y es relativamente económico, lo que hace esta detección adecuada para su uso análisis de rutina e incluso como un método de cribado o cuantificación previo en laboratorios de distinta capacidad adquisitiva. Además, DAD ofrece información espectral de las moléculas. Sin embargo, para cumplir con los bajos límites que establece la legislación vigente y también para confirmar de manera inequívoca la

presencia de residuos, se requieren, en la mayoría de los casos, sistemas de preconcentración de la muestra en la columna, tratamientos de muestra que permitan una preconcentración extra, o sistemas de detección más sensibles y capaces de identificar como MS.

- Se ha desarrollado un método novedoso para la determinación de ocho cianotoxinas de diferentes familias, incluyendo péptidos cíclicos, alcaloides y aminoácidos no proteicos, aplicado en muestras de aguas naturales. La separación se ha llevado a cabo mediante HILIC-MS/MS, empleando una columna SeQuant ZIC-HILIC compuesta de grupos funcionales zwiteriónicos que permitió la separación a línea base de tres cianotoxinas isómeros. Es la primera vez que esta compleja mezcla de cianotoxinas con tan distintas propiedades físico-químicas se ha separada en un solo análisis.

- La gran diferencia en la naturaleza y propiedades físico-químicas de las cianotoxinas estudiadas, desde los pequeños y polares aminoácidos hasta los grandes e hidrófobos péptidos cíclicos, es un punto clave que hace complejo además el desarrollo de un tratamiento de muestra que aúne simultáneamente la extracción todas las cianotoxinas con buenos resultados. En este sentido, se ha desarrollado un procedimiento basado en SPE en tándem usando dos cartuchos de SPE. Un cartucho Strata-X y un cartucho MCX se conectaron en serie, estando el cartucho Strata-X en la parte superior y el cartucho MCX en la parte inferior del sistema dual de cartuchos. Tras cargar la muestra, y antes de la elución, el orden de los cartuchos se intercambió para conseguir la elución completa de la cianotoxina MC-RR.

- Este procedimiento de SPE en tándem permitió la extracción de las cianotoxinas de interés de muestras de agua de pantano empleando una única etapa de carga

de muestra y una única etapa de elución, haciendo el proceso mucho más eficiente que en estudios previos, donde se empleaban diferentes procedimientos de extracción en función de la familia de cianotoxinas.

- El gran factor de preconcentración (x100) obtenido con el tratamiento de muestra de SPE en tándem propuesto dio lugar a valores de LOQ muy bajos (entre $7 \cdot 10^{-3}$ and 0.1 μg/L) para las cianotoxinas en muestras de agua. Estos valores fueron significativamente más bajos que los establecidos por la OMS para la presencia de MC-LR en aguas de consumo (1 μg/L).

- A pesar del buen rendimiento del método de extracción basado en SPE en tándem, el proceso de optimización reveló la importancia del contenido de iones del agua para la extracción de BMAA y DAB. Se observó que las recuperaciones disminuían cuando muestras de agua de pantano con un alto un alto contenido en iones se sometían al procedimiento de SPE en tándem.

- Como resultado del incremento a nivel mundial del consumo de suplementos dietéticos basados en BGA, también está aumentando el riesgo de exposición a cianotoxinas. Por tanto, se requieren métodos analíticos para monitorizar estas sustancias como medida de precaución para un consumo seguro de estos productos. En este sentido, se ha desarrollado un método basado en HILIC-MS/MS para determinar la presencia de cianotoxinas de diferentes familias en suplementos dietéticos basados en BGA. La extracción de las cianotoxinas se hizo mediante SLE seguida de SPE en tándem. Desde un punto de vista crítico, el principal reto de este trabajo ha sido el desarrollo de una extracción de cianotoxinas de diferentes clases en una matriz compleja como BGA y la cuantificación a de las toxinas a niveles muy bajos.

- En matrices como suplementos dietéticos basados en BGA (*spirulina*, *aphanizomenon-flos aquae* etc.) que contienen cantidades relativamente altas de aminoácidos, se debe poner especial atención en la determinación y cuantificación de ANA ya que puede ser alterada por la presencia del aminoácido esencial Phe, el cual es un compuesto isobárico de ANA que presenta patrones de fragmentación y retención en LC similares a los de ANA.

- Uno de los principales puntos débiles de la extracción SLE-SPE en tándem propuesta reside en el hecho de que el procedimiento es algo largo y complejo para análisis de rutina. El análisis de multitoxina es un reto que está aún en pleno desarrollo y en que hay mucho que investigar, sobre todo con relación al tratamiento de muestra cuando el objetivo es abarcar compuestos con características muy distintas.

- Las muestras positivas en MCs y NPAs encontradas suponen un punto de atención hacia la necesidad de controles de calidad satisfactorios en la producción de suplementos dietéticos a base de BGA y pone de manifiesto la importancia de una legislación internacional que garantice la seguridad de los productos y proteja al consumidor de contaminaciones por cianotoxinas. En este sentido, deberían llevarse a cabo más estudios implicando un mayor número de cianotoxinas para determinar el riesgo de toxinas más allá de la principal estudiada y legislada, la MC-LR.

- La colaboración con la Universidad de Parma (Parma, Italia) hizo posible el uso de la plataforma analítica UHPLC-TWIMS-QTOF para caracterizar por primera vez 20 cianotoxinas y una biotoxina marina en términos de CCS. Se evaluó la reproducibilidad y la robustez de las medidas de $^{TW}CCS_{N2}$ para confirmar la confiabilidad de la base de datos.

- La base de datos de $^{TW}CCS_{N2}$ desarrollada ha ampliado los conocimientos actuales de IMS sobre toxinas naturales dando lugar al uso de los valores de $^{TW}CCS_{N2}$ como una dimensión de separación adicional en los flujos de trabajo con cianotoxinas con propósitos de identificación, junto con los parámetros tradicionales como el tiempo de retención, la relación *m*/*z* exacta o los espectros de masas en tándem.

- Sería muy recomendable realizar más estudios para ampliar la investigación IMS y verificar las mediciones de $^{TW}CCS_{N2}$ en un estudio interlaboratorio y entre diferentes sistemas de IMS, como DTIMS, TIMS o analizadores DMS. Además, la caracterización de un mayor número de compuestos para cada grupo sería muy útil para identificar con mayor claridad las tendencias de distribución de las familias estructurales. Esto mejoraría la fiabilidad de la identificación de sustancias desconocidas en IMS-MS mediante la determinación de familias químicas en muestras complejas.

- El análisis de sospechosos en suplementos dietéticos basados en BGA comerciales mediante UHPLC-TWIMS-QTOF añadiendo los valores de $^{TW}CCS_{N2}$ recientemente determinados como un parámetro de identificación confirmó la presencia de cianotoxinas de diferentes familias, tal y como se había visto en estudios previos, reafirmando la necesidad de regulación en este tipo de productos.

ABBREVIATIONS AND ACRONYMS

A.D. – *Anno Domini*

ADDA - (all-S,all-E)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid

APFO – Ammonium perfluorooctanoate

B.C. – Before Christ

BGA – Blue-green algae

BGE – Background electrolyte

Bw -Bodywheight

CA – Cellulose acetate

CAD – Collisionally activated dissociation

CCD – Colony collapse disorder

CCS – Collision cross section

CD – circular dichroism

CD – Cyclodextrin

CE – Capillary electrophoresis

CE – Collision energy

CEP – Collision entrance potential

CI-ATPS – Cold-induced aqueous two-phase system

CLC – Capillary liquid chromatography

CNS – Central nervous system

CUR – Curtain gas

CXP – Collision cell exit potential

CZE – Capillary zone electrophoresis

DAD – Diode array detector

DART - Direct analysis in real time

DDT – Dichloro-diphenyl-trichloroethane

DLLME – Dispersive liquid-liquid microextraction

DMS – Differential mobility spectrometry

DP – Declustering potential

d-SPE – Dispersive solid phase extraction

DTIMS – Drift tube ion mobility spectrometry

EC – European Commission

ECD – Electron capture detector

EFSA – European food safety authority

ELISA – Inmmunosorbent assay

EOF – Electroosmotic flow

EP – Entrance potential

EPA – Environmental protection agency

ESI – Electrospray ionization

FAO – Food and Agriculture Organization

FaPEx – Fast pesticides extraction

FD – Fluorescence detection

FDA – Food and Drug Administration

GABA – γ-aminobutyric acid

GC – Gas chromatography

GCB – Graphitized carbon black

GF – Glass microfiber

GMOs – Genetically modified organisms

GS1 – Nebulizing gas

GS2 – Drying gas

HAB – Harmful algae bloom

HDMS^{E} – Enhanced high definition mass spectrometry

HILIC – Hydrophilic interaction liquid chromatogrpahy

HLB – Hydrophilic-lipophilic balance

HMDB – Human metabolome database

HPLC – High performance liquid chromatogrpahy

HRMS – High resolution mass spectrometry

i.d. – Inner diameter

I$_{ion}$ – Identification ion

IL – Ionic liquid

IL – TGO - Ionic liquid-thiol-graphene oxide

IMS – Ion mobility spectrometry

IS- Ion sprapy

ISPE – Integrated solid-phase extraction

LC – Liquid chromatography

LLE – Liquid-liquid extraction

LOD – Limit of detection

LOQ – Limit of quantification

LRMS – Low resolution mass spectrometry

m*/*z – Mass-to-charge ratio

MCs – Microcystins

MCX – Mixed-mode cation exchange

ME – Matrix effect

MeCN – Acetonitrile

MEKC – Micellar electrokinetic capillary chromatography

MeOH – Methanol

MOF – metal organic framework

MRL – maximum residue level

MRM – Multiple reaction monitoring

MS – Mass spectrometry

MS/MS – Tandem mass spectrometry

NACE – Non-aqueous capillary electrophoresis

NPA – Non-protein amino acid

OCLLE – On-column liquid-liquid extraction

OHA – Oregon Health Authority

PFOA – Perfluorooctanoic acid

PGC – Polygraphitized carbon

Phe - Phenylalanine

pK_a – Acid dissociation constant

PPPs – Plant protection products

PSA – Primary secondary amine

PTFE – Polytetrafluoroethylene

PT-SPE - Pipette-tip solid phase extraction

PVDF – Polyvinylidene fluoride

Q_{ion} – Quantificaation ion

QqQ – Triple quadrupole

QTOF – Quadrupole time-of-flight

QuEChERS – Quick, Easy, Cheap, Effective, Rugged, Safe

R – Pearson coefficient

R^2 – Coefficient of determination

RASFF – Rapid alert system for food and feed

RP – Reversed phase

RPLC – Reversed phase liquid chromatography

Rpm – revolution per minute

RSD – Relative standard deviation

S/N – Signal-to-noise ratio

SALLE – Salting-out assisted liquid-liquid extraction

SDS – Sodium dodecyl sulfate

SIM – Single ion monitoring

SLE – Solid-liquid extraction

SPE – Solid phase extraction

TCA – Trichloroacetic acid

TDI – Tolerable daily intake

TEM – source temperature

TFA – Trifluoroacetic acid

TGO – Thiol-graphene oxide

TIMS -Trapped ion mobility spectrometry

tSIM – target single ion monitoring

$^{TW}CCS_{N2}$ – Travelling wave derived collision cross section

TWIMS – Travelling wave ion mobility spectrometry

UHPLC – Ultra-high-performance liquid chromatography

UPLC – Ultra performance liquid chromatography

UPLC- Ultra-high performance liquid chromatography

UV – Ultraviolet

UV-Vis – Ultraviolet-Visible

VA-DLLME – Vortex-assisted dispersive liquid-liquid microextraction

WHO – World health organization