UNIVERSIDAD DE GRANADA
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DEPARTAMENTO DE QUÍMICA ANALÍTICA

MICOTOXINAS: APROXIMACIONES ANALÍTICAS Y METABOLÓMICAS

TESIS DOCTORAL

Natalia Arroyo Manzanares

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MICOTOXINAS: APROXIMACIONES ANALÍTICAS Y METABOLÓMICAS

(Mycotoxins: Analytical and Metabolomics Approaches)

por

Natalia Arroyo Manzanares

DEPARTAMENTO DE QUÍMICA ANALÍTICA
UNIVERSIDAD DE GRANADA

MEMORIA presentada para aspirar al Grado de Doctor en Ciencias, Sección Químicas, con mención de "Doctor Internacional"

Fdo.: Natalia Arroyo Manzanares, Ingeniera Química

Las Directoras de la Memoria,

Fdo.: Dra. Laura Gámiz Gracia
Profesora Titular del Dpto. de Química Analítica de la Universidad de Granada

Fdo.: Dra. Ana Mª García Campaña
Catedrática del Dpto. de Química Analítica de la Universidad de Granada

VISADA en Granada en Julio 2013

Dña. Ana María 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” (FQM-302)

CERTIFICA:

Que el trabajo que se presenta en esta TESIS DOCTORAL, con el título de “MICOTOXINAS: APROXIMACIONES ANALÍTICAS Y METABOLÓMICAS”, ha sido realizado en los laboratorios del citado grupo bajo mi dirección y la de la Profesora Dª Laura Gámiz Gracia, 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 Grado de Doctor, con mención de “Doctor Internacional”, dentro del programa de doctorado “Química”

Granada a de de

Esta Tesis Doctoral ha sido realizada gracias a la concesión de una beca asociada a un proyecto de excelencia de la Junta de Andalucía, y a la financiación obtenida en los siguientes proyectos de investigación:

- *"Aplicación de la electroforesis capilar y cromatografía líquida de ultra-resolución con diversas detecciones para el control multirresiduo de fármacos veterinarios en alimentos"* (Ref.: P08-AGR-4268, Proyectos de Excelencia, Junta de Andalucía).

- *"Determinación de micotoxinas y plaguicidas en bebidas y derivados de cereales mediante técnicas miniaturizadas con detecciones UV-VIS, espectrometría de masas y fluorescencia inducida por láser*" (Ref: P07-AGR-03178, Proyectos de Excelencia, Junta de Andalucía).

La doctoranda Natalia Arroyo Manzanares y las directoras de la tesis Dª Laura Gámiz Gracia y Dª Ana María García Campaña garantizamos, al firmar esta tesis doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de las directoras de la tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Granada a de de

Directoras de la Tesis Doctoranda

Fdo.: Laura Gámiz Gracia Fdo.: Natalia Arroyo Manzanares

Fdo.: Ana Mª García Campaña

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OBJETO DE LA MEMORIA

Objeto de la memoria

Los objetivos de esta Tesis Doctoral se enmarcan dentro de diversos Proyectos de Investigación desarrollados a lo largo del transcurso de este trabajo y que forman parte de las líneas de investigación del grupo en el que se ha realizado.

Uno de los objetivos principales ha sido la puesta a punto de nuevos métodos de análisis de micotoxinas empleando técnicas de separación miniaturizadas, como la electroforesis capilar (CE) y la cromatografía de líquidos capilar (HPLC capilar), y de alta eficacia, como es la cromatografía de líquidos de ultra resolución (UHPLC), acopladas a diferentes técnicas de detección de gran sensibilidad y selectividad - fluorescencia inducida por láser (LIF) y espectrometría de masas en tándem (MS/MS)- con objeto de explorar las ventajas de estas técnicas. En esta Tesis se proponen métodos alternativos a los ya existentes para el tratamiento de muestras de diversos alimentos, más eficaces y respetuosos con el medioambiente, de acuerdo con las nuevas tendencias de la Química Verde.

Por otro lado, se ha empleado la espectrometría de masas de alta resolución (HRMS) y de múltiples etapas (MS^n) para el estudio de la metabolómica de algunos de los hongos implicados en la producción de micotoxinas y otros metabolitos secundarios.

Como objetivos concretos de esta Tesis Doctoral destacan los siguientes:

- Explorar el potencial del acoplamiento CE-LIF para la determinación de aflatoxinas.

- Emplear HPLC capilar-LIF para la determinación de ocratoxina A, como alternativa a la HPLC convencional.

- Demostrar el potencial de la UHPLC-MS/MS como potente herramienta para la identificación inequívoca y cuantificación de una amplia variedad de micotoxinas en muestras de diversa naturaleza.

- Proponer nuevos tratamientos de muestra para la determinación de las micotoxinas en estudio, como alternativa a los ya existentes, para conseguir la selectividad y sensibilidad requeridas en este tipo de análisis.

- Demostrar el potencial de la HRMS y MS^n en el estudio de alcaloides ergóticos y el perfil metabolómico del *Aspergillus flavus* (desarrollado en la Facultad de Ciencias Farmacéuticas de la Universidad de Gante, Bélgica).

Resumen

Resumen

Las micotoxinas son metabolitos secundarios tóxicos, de composición variada, producidos por diferentes hongos. Suelen contaminar alimentos, piensos, o las materias primas utilizadas para su elaboración, pudiendo afectar a la salud tanto de humanos como de animales. Debido a su gran variedad de efectos tóxicos, y sobre todo a su extrema resistencia al calor (termorresistencia), la presencia de micotoxinas en los alimentos es considerada de alto riesgo.

La contaminación de los alimentos con micotoxinas depende de las condiciones ambientales, que pueden propiciar el crecimiento del hongo y por ende la producción de las toxinas. Por tanto, la mayoría de los productos agrícolas pueden ser susceptibles de contaminación casi en cualquier momento, desde su producción en el campo, durante la cosecha, en el transporte y en el almacenamiento.

Como resultado, la legislación a nivel europeo sobre seguridad alimentaria es cada vez más restrictiva en cuanto a los niveles de micotoxinas en alimentos y ha establecido contenidos máximos de estos compuestos que no deben ser superados con objeto de garantizar la calidad del producto y permitir su distribución y consumo.

Considerando las recientes e importantes mejoras de las técnicas separativas en cuanto a miniaturización y aumento de la eficacia, y con objeto de explorar sus indudables ventajas (bajo consumo de disolventes, alta sensibilidad, elevada resolución, tiempos de análisis reducidos y baja cantidad de muestra), en esta Tesis Doctoral se proponen nuevos métodos analíticos para la determinación de micotoxinas empleando técnicas de separación miniaturizadas (CE y HPLC capilar) y de alta eficacia (UHPLC) con objeto de explorar las ventajas mencionadas. Estas técnicas, acopladas a sistemas de detección altamente sensibles y selectivos, como LIF o MS/MS, permiten la cuantificación e identificación inequívoca de estos compuestos a las bajas concentraciones esperadas en estas matrices.

Por otro lado, es de gran importancia disponer de técnicas que permitan el estudio del metaboloma de los hongos implicados en la producción de micotoxinas y otros

metabolitos secundarios, para conocer los genes productores o para determinar nuevos metabolitos, lo que podría ayudar a desarrollar estrategias para evitar la aparición de micotoxinas. En esta Tesis, se han empleado HRMS y MS^n para el estudio del metaboloma de varios hongos.

A continuación se describe brevemente el trabajo desarrollado:

- En el primer capítulo se describe el método desarrollado para la determinación de las aflatoxinas B_1, B_2, G_1 y G_2 en muestras de arroz utilizando la cromatografía capilar electrocinética micelar (MEKC) con detección LIF y preconcentración *online*.

- En el segundo capítulo se ha llevado a cabo una comparación de diversos tratamientos de muestra para la determinación de ocratoxina A en diferentes tipos de vino, como son la microextracción líquido-líquido dispersiva (DLLME) empleando disolventes orgánicos y líquidos iónicos como extractantes (IL-DLLME), y la metodología QuEChERS (acrónimo formado a partir de los términos en inglés: Rápido, Eficaz, Barato, Fácil, Robusto, Seguro) . En este caso, la técnica instrumental empleada ha sido la HPLC capilar-LIF, añadiendo a la fase móvil un medio micelar aniónico para aumentar la intensidad de la fluorescencia y mejorar la eficacia.

- En el capítulo tercero se ha llevado a cabo la determinación de 15 micotoxinas (incluyendo aflatoxinas, fumonisinas, tricotecenos, ocratoxina A, citrinina, esterigmatocistina y zearalenona) en cardo mariano (*Silybum marianum*). Para ello, se ha utilizado UHPLC-MS/MS para el análisis instrumental, mientras que el tratamiento de la muestra consistió en un primer paso basado en el procedimiento QuEChERS, que permitía la determinación de 5 micotoxinas (fumonisina B_1, fumonisina B_2, nivalenol, deoxinivalenol y fusarenona-X) y una posterior limpieza basada en la DLLME para la determinación del resto de las micotoxinas.

- Para demostrar el potencial de la UHPLC-MS/MS junto con el tratamiento de muestra QuEChERS, en el cuarto capítulo se han desarrollado y caracterizado

dos métodos sensibles, simples y rápidos para la determinación de 15 micotoxinas (aflatoxina B_1, aflatoxina B_2, aflatoxina G_1, aflatoxina G_2, ocratoxina A, fumonisina B_1, fumonisina B_2, nivalenol, deoxinivalenol, fusarenona-X, toxina T-2 y HT-2, citrinina, esterigmatocistina y zearalenona) en cereales (espelta, arroz blanco, arroz integral y arroz rojo) y pseudocereales (trigo sarraceno, quinoa y amaranto) y de 10 micotoxinas (ocratoxina A, fumonisina B_1, fumonisina B_2, deoxynivalenol, fusarenona-X, toxina T-2 y HT-2, citrinina, esterigmatocistina y zearalenona) en melazas (de arroz, trigo y cebada).

- El capítulo quinto describe un método de análisis para la determinación de 14 micotoxinas en muestras de frutos secos (almendras, cacahuetes, semillas de girasol, semillas de calabaza, nueces, nueces de macadamia, pistachos, avellanas y piñones). De forma similar al capítulo tercero, el tratamiento de muestra comprende una primera etapa basada en el procedimiento QuEChERS para la determinación de las fumonisinas B_1 y B_2, deoxinivalenol, fusarenona-X, toxinas T-2 y HT-2, citrinina, esterigmatocistina, zearalenona y ocratoxina A y una posterior etapa de limpieza basada en la DLLME para la determinación de las aflatoxinas (B_1, B_2, G_1 y G_2).

- En capítulo sexto, se ha desarrollado un nuevo enfoque basado en cromatografía de líquidos (LC) acoplada a HRMS y LC-MS^n, para la identificación de alcaloides ergóticos nuevos o poco explorados en muestras de cereales.

- Finalmente, en el último capítulo se han determinado los metabolitos producidos por el cluster 27 *pks* (*pks*27) en el *Aspergillus flavus* usando LC-HRMS y LC-MS^n. Asimismo, se ha estudiado por primera vez el patrón de fragmentación de estos compuestos usando ionización por electrospray en modo negativo. Este trabajo, junto con el correspondiente al capítulo sexto, han sido llevados a cabo en la Facultad de Ciencias Farmacéuticas de la Universidad de Gante (Bélgica).

SUMMARY

Summary

Mycotoxins are toxic secondary metabolites with different composition and produced by different fungi. Mycotoxins contaminated food, feed or raw materials used in their production, causing diseases and disorders in humans and animals. Because of its great variety of toxic effects, and especially its extreme heat resistance, the presence of mycotoxins in food is considered a high risk to human and animal health.

Mycotoxin contamination depends on the environmental conditions, which can lead to mould growth and thus the production of toxins. Therefore, most of the agricultural products can be susceptible to contamination in almost any time, from production in the field, during harvest, transport and storage.

European legislation is becoming more restrictive in terms of the levels of mycotoxins in foods and in order to ensure quality and health consumers, maximum contents of these compounds have been established in different matrixes.

In this Thesis, taking into account the last technical advances in terms of efficiency and miniaturization, different separation techniques have been assessed (capillary electrophoresis (CE), capillary liquid chromatography (capillary HPLC) and ultra-high performance liquid chromatography (UHPLC)), coupled to very sensitive and selective detection techniques such as laser induced fluorescence (LIF) and tandem mass spectrometry (MS/MS). In addition, alternative sample treatments to those previously reported have been proposed, making possible an increased efficiency and sample throughput as well as reducing contaminant waste.

On the other hand, the study of metabolome of the fungi involved in the production of mycotoxins in order to know mycotoxin-producing genes or to identify novel or less study metabolites (toxic and nontoxic) is of great importance. These studies could help to develop strategies to prevent the occurrence of mycotoxins. In this Thesis, high resolution mass spectrometry (HRMS) and multi-stage mass spectrometry (MS^n) were used to study the metabolome of several fungi.

As a summary, the Thesis compiles the following works:

- A method for the determination of aflatoxins B_1, B_2, G_1 and G_2 in rice samples, based on micellar electrokinetic chromatography (MEKC) with online preconcentration and LIF detection has been developed in the first chapter.

- In the second chapter, dispersive liquid–liquid microextraction (DLLME), dispersive liquid–liquid microextraction using ionic liquid as extractant solvent (IL-DLLME) and the QuEChERS procedure (Quick, Easy, Cheap, Efficient, Robust, Safe), have been evaluated and compared for the extraction of ochratoxin A in wine samples. Capillary HPLC-LIF has been used as an alternative to conventional HPLC and an anionic micellar medium was added to the mobile phase for increasing the fluorescence intensity and peak efficiency.

- In the third chapter, an UHPLC-MS/MS method has been developed for the determination of 15 mycotoxins in milk thistle (*Silybum marianum*), including aflatoxins, fumonisins, trichothecenes, ochratoxin A, citrinin, sterigmatocystin and zearalenone. Sample treatment consisted of a modified method based on a first step using a QuEChERS-based procedure which allowed the determination of fumonisin B_1, fumonisin B_2, nivalenol, deoxynivalenol and fusarenon-X, and a subsequent clean-up based on DLLME for the determination of the rest of mycotoxins.

- In order to show the potential of UHPLC-MS/MS combined with QuEChERS sample treatment, in the fourth chapter two sensitive, simple and rapid methods for the determination of 15 mycotoxins (aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, fumonisin B_1, fumonisin B_2, nivalenol, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, sterigmatocystin and zearalenone) in cereals (spelt, white rice, brown rice and red rice) and pseudocereals (buckwheat, quinoe and amaranth), and for the determination of 10 mycotoxins (ochratoxin A, fumonisin B_1, fumonisin B_2, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, sterigmatocystin and zearalenone) in

cereal syrups (rice, wheat and barley) have been developed and characterized.

- The determination of 14 mycotoxins in edible nuts and seed (almonds, peanuts, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) has been achieved in the fifth chapter. Similar to the third chapter, the sample treatment comprises a first step based on the QuEChERS procedure for the determination of fumonisin B_1, fumonisin B_2, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxins, citrinin, sterigmatocystin, zearalenone and ochratoxin A. A subsequent clean-up step based on DLLME was necessary for the determination of aflatoxins (B_1, B_2, G_1 and G_2).

- In chapter sixth a holistic approach based on LC-HRMS and LC-MS^n was developed for identification of less studied or novel ergot alkaloid derivatives in cereals. This work was carried out at the Faculty of Pharmaceutical Sciences, University of Ghent (Belgium).

- In the last chapter metabolites produced by the *Aspergillus flavus* cluster 27 *pks* gene (*pks27*) have been determined using LC-HRMS and LC-MS^n and the fragmentation pattern of these compounds in negative electrospray ionization mode was elucidated. This work was also carried out in the Faculty of Pharmaceutical Sciences, University of Ghent (Belgium).

INTRODUCCIÓN

I.1. Principios generales de la seguridad alimentaria

La legislación alimentaria está encaminada a asegurar un nivel elevado de protección de la vida y la salud de las personas, teniendo en cuenta el bienestar de los animales, los aspectos fitosanitarios y el medioambiente. Dicha legislación establece, tanto a escala nacional como comunitaria, el derecho de los consumidores a la seguridad de los alimentos y a disponer de una información precisa y veraz.

En España, la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) [1] es un Organismo Autónomo adscrito al Ministerio de Sanidad, Política Social e Igualdad que se creó en 2001 con la misión de garantizar el más alto grado de seguridad alimentaria, como aspecto fundamental de la salud pública, promover la salud de los ciudadanos y que éstos tengan confianza plena en los alimentos que consumen y dispongan de información adecuada para tener capacidad de elección.

A escala comunitaria, la legislación alimentaria tiene por objeto armonizar los requisitos nacionales a fin de garantizar la libre circulación de alimentos y piensos en la Unión Europea (EU). La legislación alimentaria reconoce la responsabilidad de la EU en el marco internacional y, por tanto, debe ser desarrollada y adaptada teniendo en cuenta las normas internacionales, salvo cuando ello pueda menoscabar el alto nivel de protección de los consumidores perseguido por la EU.

La Comisión Europea (EC) plasmó sus prioridades estratégicas en el "*Libro Blanco sobre Seguridad Alimentaria*", [2] presentado en el año 2000, donde se recoge como una de las principales, la de velar por conseguir los más elevados niveles de seguridad alimentaria en la EU. En el Libro Blanco se presentaron más de 80 acciones independientes que debían caracterizar la política sobre la seguridad alimentaria en Europa en los próximos años, así como las reformas legislativas y organizativas que se debían afrontar. De su plan de acción sobre seguridad alimentaria, ha derivado

[1] http://www.aesan.msc.es/

[2] Libro Blanco sobre Seguridad Alimentaria. Comisión de las Comunidades Europeas. Bruselas, 12-1-2000. COM (1999) 719 final.

el Reglamento 178/2002 del Parlamento Europeo y del Consejo [3], por el que se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la *Autoridad Europea de Seguridad Alimentaria* (*European Food Safety Authority*, EFSA) y se fijan procedimientos relativos a la seguridad alimentaria.

Así, el "*Libro Blanco sobre Seguridad Alimentaria*" es el punto de referencia que contribuye a alcanzar un elevado nivel de protección de la salud de los consumidores y a mantener la confianza de los mismos, velando por los más elevados niveles de seguridad alimentaria, todo ello bajo un enfoque integrado "*de la granja a la mesa*" que es considerado actualmente un principio general de la política de seguridad alimentaria de la UE. Dicho enfoque considera que la mejor garantía para evitar la aparición de peligros relacionados con la alimentación es considerar de manera integral la cadena alimentaria, siendo ésta un ciclo que se inicia en la producción primaria y finaliza en el consumo.

En este sentido, el Libro Blanco establece que el *Análisis del riesgo* debe ser la base política de la seguridad alimentaria, mediante sus tres componentes interrelacionados, también recogidos en el Reglamento 178/2002:

- *Evaluación del riesgo*: etapa consistente en el asesoramiento científico y análisis de datos, que debe llevarse a cabo de forma independiente, objetiva y transparente y estar basada en la mejor base científica disponible.

- *Gestión del riesgo*: es el proceso de ponderar las distintas opciones normativas a la luz de los resultados de una evaluación del riesgo y, si es necesario, la selección de las medidas apropiadas necesarias para prevenir, reducir o eliminar el riesgo y garantizar el alto nivel de protección de la salud que determina la EU. Es, por tanto, una etapa basada en la reglamentación y el control del riesgo.

- *Comunicación del riesgo*: la finalidad de esta etapa es proporcionar información apropiada, coherente, exacta y oportuna sobre seguridad

[3] Reglamento (CE) Nº 178/2002 por el que se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de Seguridad Alimentaria y se fijan procedimientos relativos a la seguridad alimentaria, DOCE L31 (2002) 91.

agroalimentaria a todas las partes interesadas conforme a las conclusiones científicas obtenidas.

De estas etapas, la que más atañe a esta Tesis es la *Gestión del riesgo*, dentro de los cuales podemos distinguir entre riesgos nutricionales, riesgos biológicos y riesgos químicos, siendo estos últimos los que más interesan en esta Tesis, por lo que se comentarán con más detalle en el siguiente apartado.

I.1.1. Gestión de riesgos químicos: contaminantes

Las sustancias químicas desempeñan un papel importante en la producción y la distribución de los alimentos. Cabe destacar la mejora en los rendimientos de las cosechas y de la producción ganadera debida al uso de los productos fitosanitarios y de los medicamentos de uso veterinario, respectivamente, consiguiéndose de esta forma abaratar los costes de las producciones y, por tanto, reducir el precio de venta al consumidor de los alimentos. Los aditivos alimentarios, cuya adición intencionada a los productos alimenticios tiene un propósito tecnológico, permiten mejorar la presencia de los alimentos en el momento de su puesta en el mercado. Por otro lado, se utilizan materiales, como plásticos, papel, cartón, etc., para mantener en condiciones higiénicas los alimentos y permitir su distribución, así como para mejorar su presentación. Sin embargo, la utilización de sustancias químicas puede conducir a su presencia en los alimentos, constituyendo un riesgo potencial que debe ser analizado de una manera eficaz, con el objeto de poder garantizar al consumidor la inocuidad de dichos alimentos.

En este contexto, se define contaminante como cualquier sustancia que no haya sido agregada intencionadamente al alimento en cuestión, pero que sin embargo se encuentra en el mismo como residuo de la producción (incluidos los tratamientos administrados a los cultivos y al ganado y en la práctica de la medicina veterinaria), de la fabricación, transformación, preparación, tratamiento, acondicionamiento, empaquetado, transporte o almacenamiento de dicho alimento o como consecuencia de la contaminación medioambiental. Esta definición no abarca las partículas extrañas tales como, por ejemplo, restos de insectos, pelos de animales y otras. La EU prohíbe

expresamente la comercialización de productos alimenticios en los que se haya comprobado la presencia de un contaminante en proporciones inaceptables desde el punto de vista toxicológico a través del Reglamento (CEE) Nº 315/93 [4]. Los contaminantes, por tanto, deben mantenerse al mínimo nivel posible.

Dentro de los contaminantes, podemos distinguir los siguientes grupos:

- ***Contaminantes industriales / medioambientales***: engloban aquellas sustancias que se derivan de la actividad industrial y/o se acumulan en el medioambiente.

- ***Contaminantes orgánicos persistentes***: son sustancias químicas extraordinariamente tóxicas y duraderas que, además, son solubles en grasas (y por consiguiente se acumulan en los tejidos vivos) y pueden viajar grandes distancias, lo que los hace sustancias consideradas de riesgo.

- ***Contaminantes agrícolas***: engloban aquellas sustancias indeseables que se derivan de la actividad agrícola, incluyendo el trasporte y almacenamiento de los alimentos. Se consideran contaminantes agrícolas a las **micotoxinas,** los nitratos y alcaloides de pirrolizidina.

I.2. Micotoxinas

El reino de los hongos comprende a un grupo de organismos que se pueden clasificar en levaduras y hongos filamentosos o mohos. Estos últimos, que son los productores de micotoxinas, son organismos eucariotas multicelulares y filamentosos, constituidos por micelios verdaderos. Además carecen de clorofila y están formados por una serie de células alineadas, llamadas hifas. El micelio es el conjunto de hifas ramificadas, y resulta visible sobre el alimento donde se desarrolla, bien en superficie o en el interior, mediante un aspecto y color característicos (Figura I.1). Los mohos utilizan para su crecimiento una serie de sustancias químicas denominadas metabolitos primarios, como pueden ser ácidos nucleicos, proteínas, carbohidratos y lípidos mayoritariamente.

[4] Reglamento (CEE) Nº 315/93 por el que se establecen procedimientos comunitarios en relación con los contaminantes presentes en los productos alimenticios, DOCE L37 (1993) 1.

El uso de estos metabolitos primarios se asocia con una fase de rápido crecimiento (fase exponencial) [5]. Por otra parte, los metabolitos secundarios son una serie de compuestos que no son esenciales para el crecimiento vegetativo en cultivo puro, y que incluyen a ciertos antibióticos y a las micotoxinas.

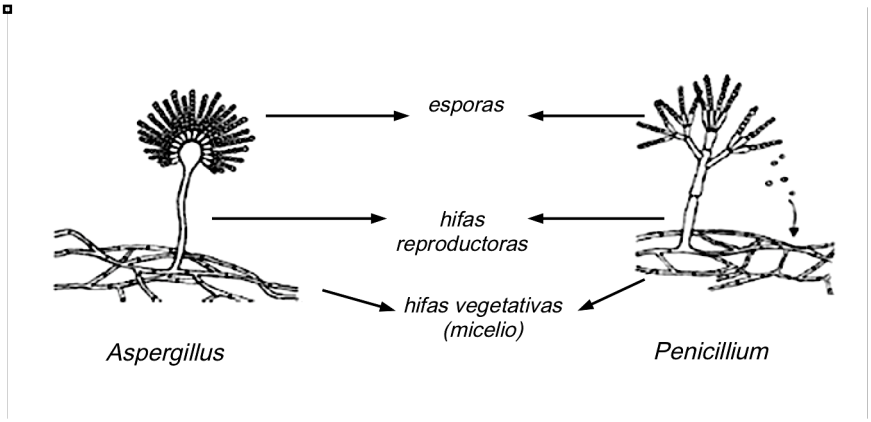

Figura I.1. Esquema de las partes del moho [6]

Las micotoxinas, término derivado de las palabras griegas *mikes* y *toxina,* que significan hongo y veneno respectivamente, son metabolitos secundarios que se producen durante la etapa final de la fase de crecimiento del moho o al principio de la fase estacionaria (Figura I.2), estando a menudo asociadas a la diferenciación y la esporulación [7]. Los metabolitos secundarios se originan mayoritariamente en la ruta policetónica, siendo la cadena general policetónica del tipo R-CO-CH_2-CO-CH_2-CO-CH_2-CO-CH_2-CO-SCoA de la cual se derivan la mayoría de las micotoxinas. Existen otras rutas biosintéticas pero son más complejas y esa complejidad se relaciona con un menor número de especies fúngicas capaces de elaborar micotoxinas [8].

En cuanto a la **toxicidad** de las micotoxinas, éstas pueden contaminar alimentos, piensos, o las materias primas utilizadas para su elaboración, originando un grupo de

[5] M. J. Carlile, S. C. Watkinson, G. W. Gooday, The Fungi, Academia Press, Londres, Reino Unido, 2nd Ed., 2001.

[6] Available on: http://biologiapuntocom.blogspot.com.es/2012/10/practico-estudio-de-un-moho.html

[7] L. A. Gotdblatt, Clin. Toxicol. 5 (1972) 453.

[8] M. O. Moss, en: J. E. Smith, R. S. Henderson (eds.), Mycotoxins and animal food. CRC Press. Boca Raton, EEUU, 1991, pp. 37.

enfermedades y trastornos, tanto en humanos como en animales, denominados *micotoxicosis*. La presencia de estas micotoxinas en los alimentos puede ser individual o simultánea con otras, lo que puede provocar efectos sinérgicos en su acción sobre el organismo, aumentando así su toxicidad.

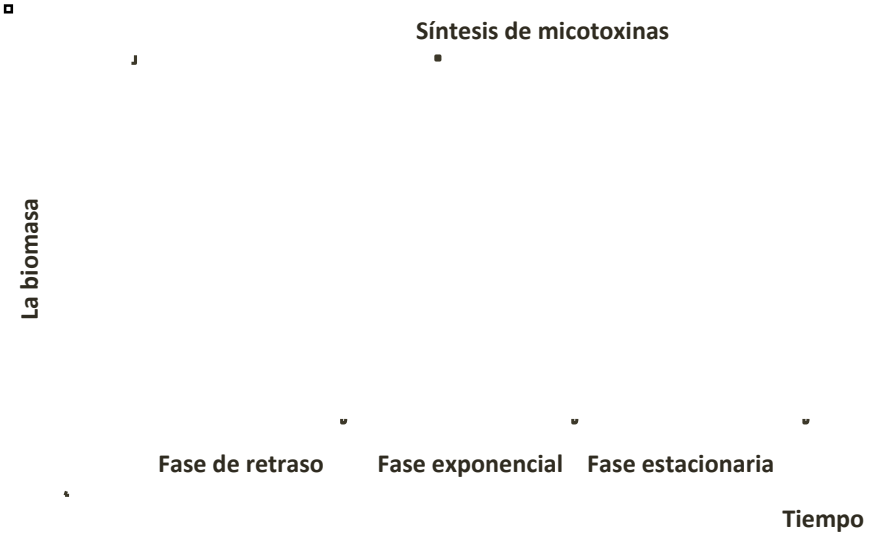

Figura I.2. Fases de crecimiento fúngico y localización de la síntesis de micotoxinas [9]

Elevados niveles de micotoxinas en la dieta pueden causar efectos adversos agudos o crónicos sobre la salud del hombre y una gran variedad de especies animales. Los efectos adversos pueden afectar a distintos órganos, aparatos o sistemas, especialmente al hígado, riñón, sistema nervioso, endocrino e inmunitario. Los síntomas causados por las micotoxinas suelen ser tan diferentes unos de otros como lo son las propias estructuras químicas de dichas toxinas [10-12].

[9] J. M. Soriano del Castillo, en: J. M. Soriano del Castillo (ed.), Micotoxinas en alimentos, Díaz Santos. Madrid, 2007, pp. 3.

[10] A. Cameán, M. Repetto, en: M. Repetto (ed.), Toxicología Avanzada. Díaz de Santos, Madrid, 1997, pp. 205.

[11] E. M. Faustman, G. S. Ommenn, en: C. D Klassen (ed.), Casarett y Doull, Fundamentos de Toxicología. McGraw Hill Interamericana, Madrid, 2005, pp. 50.

[12] R. M. Martínez-Larrañaga, A. Anadón, en: A. M. Caméan, M. Repetto (eds.), Toxicología Alimentaria, Díaz Santos. Madrid, 2006, pp. 289

En términos generales, el riesgo de intoxicación aguda por micotoxinas en el hombre es bajo o moderado en comparación con intoxicaciones de origen microbiológico o por contaminantes químicos. No obstante, según algunos autores [12], en la exposición crónica y teniendo en cuenta la severidad de las lesiones que pueden causar (especialmente el cáncer), las micotoxinas presentan mayor riesgo tóxico que los contaminantes de origen antropogénico, aditivos alimentarios y plaguicidas, tal y como se muestra en la Figura I.3.

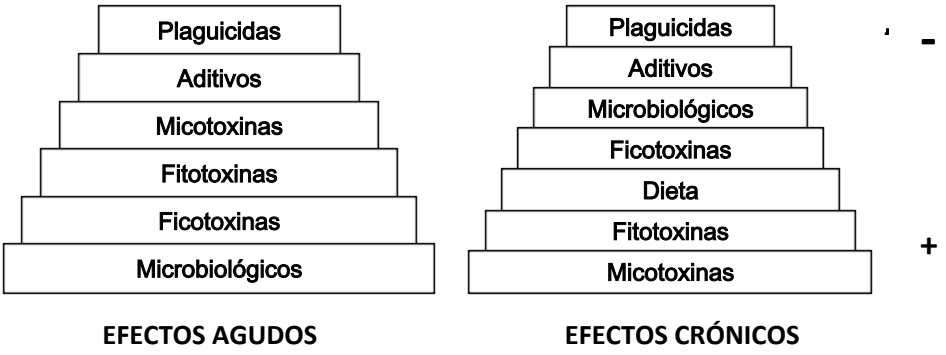

Figura I.3. Efectos sobre la salud humana de la exposición a distintos agentes [13]

Las micotoxinas abarcan un amplio espectro de efectos tóxicos fisiopatológicos, entre los que hay que destacar los producidos sobre el sistema inmunitario, ya que disminuyen las defensas en animales y en el hombre, aumentando la susceptibilidad a infecciones. El aumento de infecciones en el animal puede conllevar la transmisión de patógenos al hombre, como es el caso de la *Salmonella* y la *Listeria*, incrementando las infecciones en humanos.

Por último, hay que tener en cuenta la posible interrelación entre las distintas micotoxinas consumidas conjuntamente que pueden presentar efecto sinérgico, aditivo, antagónico o de potenciación sobre la salud humana.

[13] M. J. Ruíz, G. Font, en: J. M. Soriano del Castillo (ed.), Micotoxinas en alimentos, Díaz Santos. Madrid, 2007, pp. 15.

En cuanto a la *toxicidad crónica*, la Agencia Internacional de Investigación sobre el Cáncer (*International Agency for Research on Cancer,* IARC) [14] clasifica varias micotoxinas como carcinógenas o potencialmente carcinógenas para el hombre, de acuerdo a los siguientes grupos:

- Grupo 1: el agente es carcinógeno en humanos.
- Grupo 2A: agente probablemente carcinógeno en humanos; existe limitada evidencia sobre humanos pero suficiente con animales.
- Grupo 2B: agente posiblemente carcinógeno; la evidencia en humanos es limitada y tampoco hay suficiente evidencia con animales en experimentación.
- Grupo 3: el agente no es clasificable como carcinógeno para humanos, y no puede incluirse en otro grupo.
- Grupo 4: el agente probablemente no es carcinógeno en humanos; la evidencia disponible, tanto de humanos como de experimentación animal así lo sugiere.

En la Tabla I.1 se resumen la evaluación realizada por la IARC en relación al poder carcinógeno de las micotoxinas, cuyas características más relevantes pasan a comentarse en el siguiente apartado.

Tabla I.1. Clasificación de las micotoxinas según la IARC [5]

Micotoxinas	Grupo
Aflatoxinas de origen natural	1
Aflatoxina M_1	2B
Citrinina	3
Esterigmatocistina	2B
Fumonisina B_1	2B
Ocratoxina A	2B
Patulina	3
Toxinas derivadas de *Fusarium graminearum, F. culmorum, F. crookwellense* (zearalenona, deoxinivalenol, nivalenol y fusarenona X)	3

[14] International Agency for Research on Cancer (IARC) (2012). Available on: http://www.iarc.fr

Toxinas derivadas de *Fusarium sporotrichioides* (toxina T-2)	3

I.2.1. Clasificación de las micotoxinas

Las micotoxinas más importantes desde el punto de vista de la seguridad alimentaria son las toxinas producidas por mohos de los géneros *Aspergillus*, *Fusarium* y *Penicillium*, que pueden clasificarse en términos generales en los siguientes grupos [15]:

I.2.1.1. Aflatoxinas

Las aflatoxinas son un grupo de micotoxinas producidas por cepas de varias especies de mohos del género *Aspergillus* (*A. flavus, A. parasiticus, A. nomius y A. tamarii*) [16,17].

Figure I.4. *Aspergillus flavus* y maíz contaminado por el moho

Estos mohos pueden proliferar en muchos alimentos, principalmente en cacahuetes, maíz y semillas de algodón, aunque también se han encontrado aflatoxinas en todo tipo de frutos secos, copra, cereales, otras semillas oleaginosas como el girasol y la

[15] http://www.aesan.msc.es/AESAN/web/cadena_alimentaria/subdetalle/micotoxinas_categorias.shtml

[16] T. Goto, D. T. Wicklow, Y. Ito, Appl. Environ. Microbiol. 65 (1996) 4036.

[17] C. P. Kurtzman, B. W. Horn, C. W. Hesseltine, Antonie van Leewenhoek, 53 (1987) 147.

soja, aceites vegetales sin refinar, especias (como el pimentón, el chile, la pimienta, etc.), frutas desecadas (como los higos secos y las pasas), café, cacao y piensos [18].

La presencia de estos mohos es común en todo el mundo, pero resulta especialmente alta en climas tropicales, por la combinación de temperatura y humedad elevadas. *Aspergillus flavus* puede proliferar en alimentos con una actividad de agua superior a 0.85, estando la temperatura de producción máxima en torno a los 27 ºC. A una temperatura por debajo de 12 ºC prácticamente no se producen aflatoxinas.

Dentro de las aflatoxinas se han identificado unos 16 compuestos, pero sólo las aflatoxinas B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2) y M_1 (AFM_1) se analizan rutinariamente [19]. La letra B indica que estas aflatoxinas tienen fluorescencia azul (*blue*) frente a la luz ultravioleta (365 nm), mientras que la letra G indica la fluorescencia verde amarillenta (*green*) de las designadas así. En cuanto a la estructura, las AFB_1 y AFG_1 difieren de las AFB_2 y AFG_2 por la presencia de un doble enlace más en las primeras. A su vez, las aflatoxinas B difieren de las aflatoxinas G porque el anillo de furano que presentan las primeras se convierte en un anillo de lactona en las segundas.

Los animales que consumen alimentos contaminados por aflatoxinas B son capaces de metabolizarlas, hidroxilándolas en una posición determinada. Así, a partir de la AFB_1 se forma la AFM_1, y a partir de la AFB_2 se forma la aflatoxina M_2 (AFM_2) y estos metabolitos son secretados en la leche.

Actualmente se considera que las aflatoxinas son las micotoxinas de mayor riesgo para la salud, en especial por su potencial hepatocarcinógeno. Entre ellas, la AFB_1 es la considerada como la de mayor riesgo. Así, el Comité Científico de la Alimentación Humana (*Scientific Committee on Food*, EU) ha establecido que las aflatoxinas son cancerígenos genotóxicos [20], siguiendo el grado de toxicidad y carcinogenicidad el

[18] C. Juan, J. M. Soriano, P. Burdaspal, en: J. M. Soriano del Castillo (ed.), Micotoxinas en alimentos, Díaz Santos. Madrid, 2007, pp. 167.

[19] G. S. Shephard, Anal. Bioanal. Chem. 395 (2009) 1215.

[20] Scientific Committee on Food (1996). “Opinion on Aflatoxins, Ochratoxin A and Patulin” expressed on 23 september 1994. Reports of the Scientific Committee on Food-35º Series (European Commision, DG Industry).

siguiente orden: $AFB_1 > AFG_1 > AFB_2 > AFG_2$. Por tanto, la más tóxica es la AFB_1, que ha demostrado ser carcinógena en todos los animales de experimentación [14] y está clasificada desde 1988 por la Organización Mundial de la Salud (*World Health Organization*, WHO) como un carcinógeno para el hombre, y tal y como se ha expuesto anteriormente, la IARC también ha clasificado a la AFB_1 dentro de la categoría de sustancias del grupo 1 en base a la existencia de suficientes evidencias acerca de su carácter carcinogénico para el hombre, tanto aisladamente como en mezclas naturales con las otras aflatoxinas [21,22].

En los humanos, las aflatoxinas son probablemente responsables de múltiples episodios de intoxicaciones masivas con producción de hepatitis aguda, en distintas zonas de la India, Sudeste Asiático y África tropical y ecuatorial, y un factor de agravamiento de enfermedades producidas por la malnutrición. También son responsables muy probablemente, combinadas con otros factores, de la elevada tasa de cáncer hepático observado en algunas de esas zonas.

Las aflatoxinas resisten los tratamientos habituales de los alimentos. Sin embargo, en el caso de determinados productos, como los cacahuetes, los frutos de cáscara, los frutos secos y el maíz, está demostrado que los métodos de selección u otros tratamientos físicos permiten reducir el contenido de aflatoxinas.

I.2.1.2. Ocratoxina A

Las ocratoxinas son micotoxinas producidas por determinados hongos, entre ellos *Aspergillus ochraceus* y *Penicillium verrucosum*, entre las que se encuentran las ocratoxinas A, B y C, y los metil y etil ésteres derivados de las ocratoxinas A y B. También se han identificado otros metabolitos derivados de la ocratoxina A, tanto en cultivos de células como en animales: los compuestos hidroxilados en la posición 4 o 10, los productos resultantes de la hidrólisis de la ocratoxina A y B, la ocratoxina α y

[21] IARC (1996). IARC Monographs on the evaluation of carcinogenic risk to human: Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. Lyon, Francia. Vol. 56.

[22] IARC (2002). IARC Monographs on the evaluation of carcinogenic risk to human: Some traditionally herbal medicines, some mycotoxins, naphthalene and styrene. Lyon, Francia. Vol. 82.

ocratoxina β, así como la forma abierta de la lactona [23]. Sin embargo, la ocratoxina más importante, debido a su toxicidad e incidencia en los alimentos es la ocratoxina A y es la que será objeto de estudio en esta Tesis.

La ocratoxina A (OTA) estructuralmente, tiene la particularidad de contener un átomo de cloro. Se encuentra presente de manera natural en numerosos productos vegetales de todo el mundo, como los cereales, los granos de café, el cacao y los frutos secos. Se ha detectado, asimismo, en productos elaborados a base de cereales, el café, el vino, la cerveza y el zumo de uva, pero también en productos de origen animal, como los riñones de cerdo [24].

La ocratoxina A presenta propiedades carcinógenas, nefrotóxicas, teratógenas, inmunotóxicas y, posiblemente, neurotóxicas. Se la ha asociado también a la nefropatía en los seres humanos.

I.2.1.3. Toxinas de *Fusarium*

Estos mohos son los principales contaminantes de los alimentos en las regiones templadas, siendo el maíz el principal producto contaminado, aunque aparecen en otros cereales y en la malta de cervecería [25-27].

[23] R. Remiro-Íñigo, Tesis doctoral: Incidencia y niveles de ocratoxina A y cinco análogos en vino tinto. Universidad de Navarra, Pamplona, 2011.

[24] A. López de Cerain, J. M. Soriano, en: J. M. Soriano del Castillo (ed.), Micotoxinas en alimentos, Díaz Santos. Madrid, 2007, pp. 201.

[25] O. Vendl, F. Berthiller, C. Crews, R. Krska. Anal. Bioanal. Chem. 395 (2009) 1347.

[26] M. Zachariasova, O. Lacina, A. Malachova, M. Kostelanska, J. Poustka, M. Godula, J. Hajslova, Anal. Chim. Acta 662 (2010) 51.

[27] G. D'Arco, M. Fernández-Franzón, G. Font, P. Damiani, J. Mañes. J. Chromatogr. A 1209 (2008) 188.

Figura I.5. Mohos del género *Fusarium* y ajo contaminado por el moho

Las toxinas de *Fusarium* se pueden agrupar de la siguiente forma:

- **Toxinas estrogénicas**, con estructura de lactona, como zearalenona y zearalenol. A pesar de su escasa semejanza estructural aparente con los estrógenos fisiológicos; la estructura tridimensional del anillo de lactona sitúa un grupo -OH en una posición tal que puede interactuar con los receptores de estrógenos.

- **Toxinas no estrogénicas**, con estructuras sesquiterpénicas, conocidas bajo el nombre común de tricotecenos. Incluyen al deoxinivalenol, nivalenol, toxinas T-2 y HT-2 y diacetoxiscirpenol. Dentro de los tricotecenos, la toxina más importante y frecuente es el deoxinivalenol (también conocida como "vomitoxina") que frente a los tratamientos tecnológicos es muy estable. Aunque la intoxicación en humanos es relativamente poco frecuente, se conocen episodios en la India y en Japón. El deoxinivalenol no parece ser cancerígeno en animales de experimentación.

  La toxina T-2 se produce a una temperatura óptima notablemente más baja que la de las otras micotoxinas, lo que explicaría su presencia en alimentos producidos en zonas frías.

  En cuanto a otros tricotecenos, tales como el 3-acetildeoxinivalenol, 15-acetildeoxinivalenol, fusarenona-X, T2-triol, diacetoxiscirpenol, neosolaniol, monoacetoxiscirpenol y verrucol, la limitada información disponible indica que no están muy extendidos y los niveles descubiertos suelen ser reducidos, por

lo que no se tiene constancia de que representen un riesgo para la salud humana.

- **Fumonisinas**: constituyen otro grupo de toxinas principalmente producidas por mohos del género *Fusarium*. Se conocen al menos 15 tipos de fumonisinas, de las cuales la más importante es la fumonisina B_1, producida por el hongo *Fusarium verticilloides* (conocido anteriormente por *Fusarium moniliforme*) y también por *Fusarium proliferatum*. Este tipo de hongos son contaminantes frecuentes del maíz, y estas tóxinas están relacionadas con la aparición de cáncer de esófago e hígado, defectos neuronales e intoxicación aguda.

I.2.1.4. Patulina

La patulina es una micotoxina producida por diversas especies de *Penicillium, Aspergillus* y *Byssochylamys*. Aparece en la fruta, incluyendo pera y uva, aunque también se ha detectado en vegetales, granos de cereales y ensilados. Sin embargo, su mayor incidencia se da en la manzana y productos derivados de la manzana, especialmente en zumos y en sidra. Las frutas almacenadas bajo condiciones que promuevan el maltrato y el pudrimiento aumentan la probabilidad de formación de patulina [28-31].

La patulina posee características de antibiótico de amplio espectro y se ha probado para tratar el resfriado común. Sin embargo, nunca se ha demostrado su efectividad y, a la luz de su toxicidad, no se ha insistido en su uso para tratamiento médico debido a que es irritante estomacal y produce nauseas y vómitos.

Entre los síntomas de intoxicación por patulina encontramos hemorragia del tracto digestivo en ganado. En 1954 se acusó a la patulina de la muerte de 100 vacas en Japón que consumieron alimento contaminado.

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[28] M. Murillo, E. González-Peñas, S. Amézqueta, Food Chem. Toxicol. 46 (2008) 57.

[29] D. R. Katerere, S. Stockenström, G. S. Shephard, Food Control 19 (2008) 389.

[30] M. Murillo-Arbizu, E. González-Peñas, S. Amézqueta. Food Chem. Toxicol. 48 (2010) 2429.

[31] M. D. Víctor-Ortega, F. J. Lara, A. M. García-Campaña, M. del Olmo-Iruela. Food Control 31 (2013) 353.

I.2.1.5. Citrinina

La critrinina es una micotoxina producida mayoritariamente por algunas especies de los géneros *Aspergillus*, *Penicilium* y *Monascus* y aislada por primera vez en 1931. Aunque se caracterizó como antibiótico, demostrando su capacidad antifúngica y bacteriostática, posteriormente se la hizo responsable de neuropatía porcina en varios países de Europa.

Su presencia ha sido detectada en diferentes alimentos como cereales, frutas y quesos, aunque también ha sido aislada en bellotas, nueces, zanahoria, tomate, carne, etc. [32,33]. Es muy importante su presencia en alimentos coloreados, como el arroz rojo, que es fermentado con diversas especies del género *Monascus* [34].

La citrinina tiene efectos nefrotóxicos y mutagénicos. Su ingestión provoca diarrea y pérdida peso por degeneración renal. Es importante el efecto sinérgico observado cuando su presenta de forma combinada con la ocratoxina A [32,35].

I.2.1.6. Esterigmatocistina

Es una micotoxina producida por *A. versicolor*, aislada por primera vez en 1954, precursora de las AFB_1, AFG_1 y AFG_2.

La IARC clasifica a esta micotoxina como agente posiblemente carcinógeno (grupo 2B), debido a que la evidencia en humanos es limitada y tampoco hay suficiente evidencia con animales en experimentación. Sin embargo, está relacionada con los carcinomas gástricos, hepáticos y esofágicos [36].

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[32] H. Barrada, J. M. Soriano, en: J. M. Soriano del Castillo (ed.), Micotoxinas en alimentos, Díaz Santos. Madrid, 2007, pp. 313.

[33] B. Andersen, J. Smedsgaard, J. C. Frisvad, Agric. Food Chem. 52 (2004) 2421.

[34] N. I. Samsudin, N. Abdullah, Mycotoxin Res. 29 (2013) 89.

[35] B. Xu, X. Jia, L. Gu, Ch. Sung, Food Control 17 (2006) 271.

[36] D. Yao, H. Cao, S. Wen, D. Liu, Y. Bai, W. Zheng, Bioelectrochemistry 68 (2005) 131.

Su presencia ha sido detectada en cereales, café, jamón, pimienta y queso entre otros alimentos [37].

I.2.1.7. Alcaloides ergóticos

Los alcaloides ergóticos, conocidos comúnmente como alcaloides del cornezuelo del centeno, son producidos en el esclerocio de diferentes hongos de los órdenes *Hypocreales* y *Eurotiales*. Dentro del primero, *Claviceps purpúrea* es el hongo más extendido en Europa e infecta a granos de centeno, trigo, cebada, mijo y avena, entre otros cereales.

La mayoría de los alcaloides ergóticos de origen natural muestran un sistema de anillos tetracíclico derivado de la ergolina (Figura I.6).

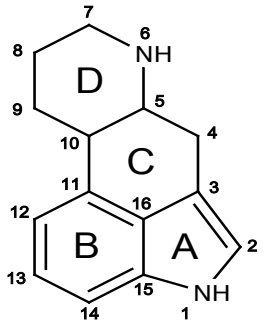

Figura I.6. Sistema de anillo tetracíclico derivado de la ergolina

En el pasado, se han identificado más de 50 alcaloides ergóticos diferentes [38] cuyas cantidades y tipos varían entre cepas de hongos, regiones geográficas y plantas hospederas [39].

Los alcaloides ergóticos se clasifican en tres grupos principales:

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[37] P. Catalá, J. M. Soriano, en: J. M. Soriano del Castillo (ed.), Micotoxinas en alimentos, Díaz Santos. Madrid, 2007, pp. 358.

[38] M. Flieger, M. Wurst, R. Shelby, Folia Microbiol (Praha) 42 (1997) 3.

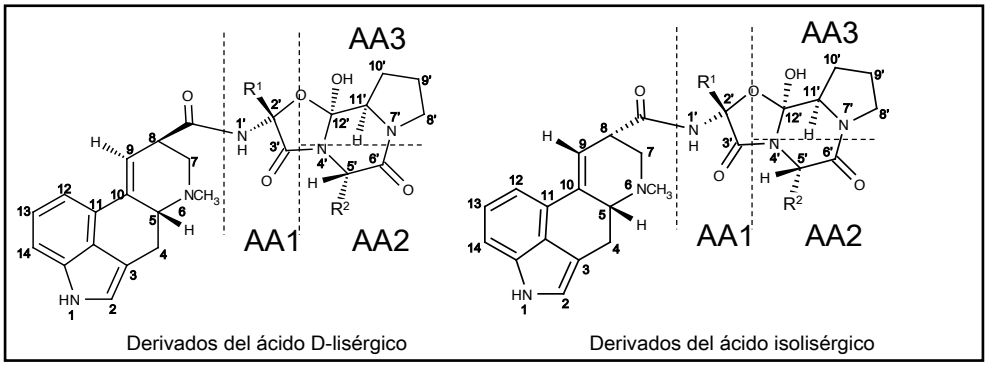
[39] R. Krska, C. Crews, Food Addit. Contam. 25 (2008) 722.

- **Clavinas y 6,7-secoergolinas**. Los miembros de esta clase de alcaloides ergóticos se derivan a partir de precursores del ácido lisérgico (por ejemplo, agroclavina o elimoclavina). Son hidroxilo- o deshidroderivados del 6,8-dimetilergolina (por ejemplo agroclavina), e incluyen el grupo de 6,7-secoergolinas, que se caracterizan por la apertura del anillo D (Figura I.6).

- **Derivados simples del ácido lisérgico.** Son hidrosolubles, siendo el alcaloide mayoritario la ergometrina (derivado amida), también conocida como ergonovina.

- **Alcaloides péptidos.** Son insolubles en agua y se clasifican en:

    - *Ergopeptinas*: se componen de dos partes, una es el ácido lisérgico y el resto es un tripéptido formando un sistema tricíclico (Figura I.7). Dependiendo del aminoácido (AA) implicado se pueden identificar cuatro subclases de ergopeptinas: ergotaminas (AA1: alanina), ergoxinas (AA1: ácido α-aminobutírico), ergotoxinas (AA1: valina) y ergoanninas (AA1: isoleucina). Además, los isómeros correspondientes derivados del ácido isolisérgico (caracterizados por el sufijo-*inina*) también se incluyen en las subclases correspondientes.



Derivados del ácido D-lisérgico | Derivados del ácido isolisérgico

Figura I.7. Estructura de las ergopeptinas, incluyendo la asignación de los anillos y la asignación de los distintos aminoácidos (AA) del sistema tricíclico péptido. R^1 y R^2 corresponden a la cadena lateral de los AA implicados

- *Ergopeptamas*: se asemejan a las ergopeptinas, con la excepción de que AA3 es D-prolina y AA1 no es α-hidroxilado (Posición: 2') (Figura I.8). Las ergopeptamas se dividen en: ergotamams (AA1: alanina), ergoxams (AA1: ácido α-aminobutírico), ergotaxams (AA1: valina) y ergoannams (AA1: isoleucina). Además, los isómeros correspondientes derivados del ácido isolisérgico (caracterizados por el sufijo-*inam*) también se incluyen en las subclases correspondientes.

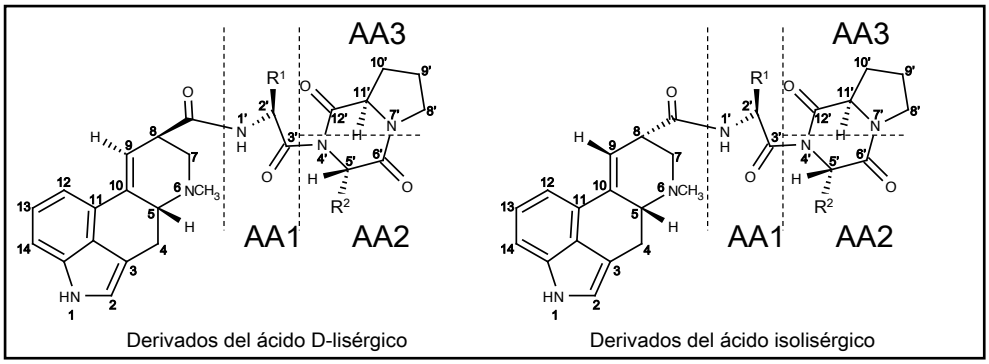


Figura I.8. Estructuras de las ergopeptamas, incluyendo la asignación de los anillos y la asignación de los distintos aminoácidos (AA) del sistema tricíclico péptido. R^1 y R^2 corresponden a la cadena lateral de los AA implicados

Sus efectos se conocen desde tiempos inmemorables, de hecho, en la Edad Media causaron epidemias severas conocidas como "el fuego de San Antonio" por consumo de cereales, harina o pan contaminados con estos compuestos. Hoy en día, a esta intoxicación por *Claviceps purpurea* presente en los cereales se le denomina "ergotismo", aunque su incidencia se ha visto disminuida en gran medida en las últimas décadas por el conocimiento científico sobre su origen y la mejora en las prácticas agronómicas.

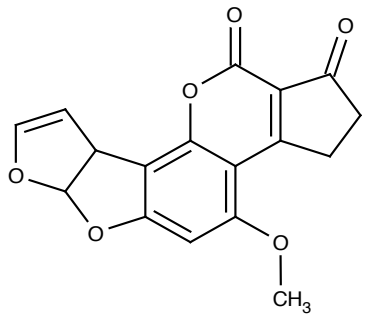
I.2.1.8. Otras micotoxinas

La citreoviridina es una toxina producida por *Penicillium citreonigrum*. Fue considerada responsable de episodios de "beriberi cardiaco agudo" producidos en Japón. Otras

toxinas fúngicas son la rubratoxina, ciclocloratina, maltorricina y rugulosina. En algunos casos se ha relacionado su presencia con enfermedades de animales de granja, pero su efecto en salud animal y humana no está todavía bien establecido. Recientemente está apareciendo todo un conjunto de micotoxinas , consideradas como micotoxinas emergentes, entre las que se encuentran las enniatinas, la beauvericina, fusaproliferina o la moniliformina [40,41].

I.2.2. Propiedades físico químicas

A continuación se muestran algunas de las propiedades físico-químicas de las micotoxinas que han sido objeto de estudio en la presente Tesis: aflatoxinas (AFB_1, AFB_2, AFG_1 y AFG_2), ocratoxina A, fumonisinas (fumonisisna B_1 y fumonisina B_2), tricotecenos (toxina T-2, toxina HT-2, nivalenol, fusarenona X, deoxinivalenol), citrinina, esterigmatoscistina, zearalenona y ergoalcaloides (ergometrina, metilergometrina y metisergida, ergotamina, ergocristina, ergocriptina, ergocornina, ergosina, dihidroergotamina). Los datos han sido obtenidos de las base de datosdatod Scifinder y Chemspider.

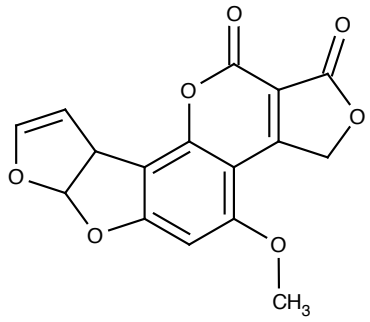
Aflatoxina B_1 (AFB_1)	
	**Nombre sistemático:** (6aR,9aS)-4-Metoxi-2,3,6a,9a-tetrahidrociclopenta[c]furo[3',2':4,5]furo[2,3-h]cromeno-1,11-diona
	Fórmula: $C_{17}H_{12}O_6$
	Masa monoisotópica: 312.0634 Da
	Log P: 2.039
	pKa: ---
	Solubilidad: 0.087 g/L
	Punto de fusión: 528.151 °C a 760 mmHg

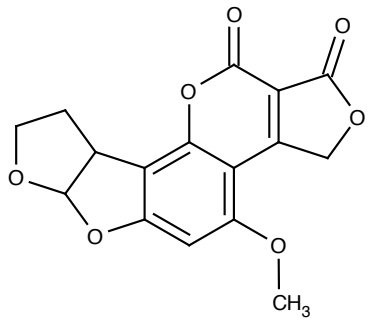
Aflatoxina B_2 (AFB_2)	
	**Nombre sistemático:** 4-Metoxi-

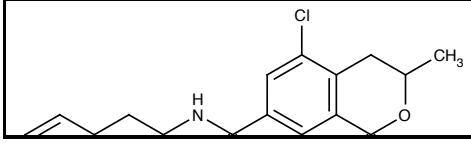
[40] A. Santini, R. Ferracane, G. Meca, A. Ritieni, Anal Bioanal Chem 395 (2009) 1253.

[41] M. Carvajal, C. E. Peralta, en: J. M. Soriano del Castillo (ed.), Micotoxinas en alimentos, Díaz Santos. Madrid, 2007, pp. 323.

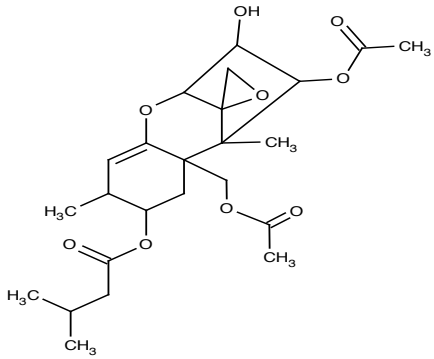
	2,3,6a,8,9,9a-hexahidrociclopenta[c]furo[3',2':4,5]furo[2,3-h]cromeno-1,11-diona
	Fórmula: $C_{17}H_{14}O_6$
	Masa monoisotópica: 314.0790 Da
	Log P: 1.802
	pKa: ---
	Solubilidad: 0.012 g/L
	Punto de fusión: 520.999 °C a 760 mmHg

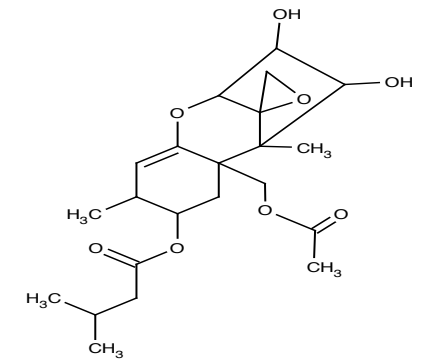
Aflatoxina G_1 (AFG_1)	
	**Nombre sistemático:** 5-Metoxi-3,4,7a,10a-tetrahidro-1H,12H-furo[3',2':4,5]furo[2,3-h]pirano[3,4-c]cromeno-1,12-diona
	Fórmula: $C_{17}H_{12}O_7$
	Masa monoisotópica: 328.0583 Da
	Log P: 0.443
	pKa: ---
	Solubilidad: 0.28 g/L
	Punto de fusión: 602.505 °C a 760 mmHg

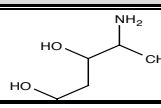
Aflatoxina G_2 (AFG_2)	
	**Nombre sistemático:** 5-Metoxi-3,4,7a,9,10,10a-hexahidro-1H,12H-furo[3',2':4,5]furo[2,3-h]pirano[3,4-c]cromeno-1,12-diona
	Fórmula: $C_{17}H_{14}O_7$
	Masa monoisotópica: 330.0739 Da
	Log P: 0.443
	pKa: ---
	Solubilidad: 0.28 g/L
	Punto de fusión: 602.505 °C a 760 mmHg

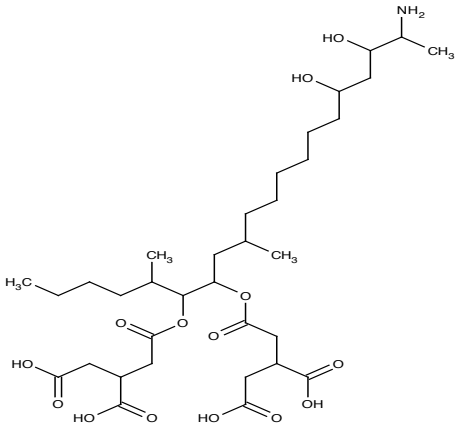
Ocratoxina A (OTA)	
	**Nombre sistemático:** N-{[(3R)-5-Cloro-8-hidroxi-3-methyl-1-oxo-3,4-dihidro-1H-isocromen-7-yl]carbonil}-L-fenilalanina
	Fórmula: $C_{20}H_{18}ClNO_6$

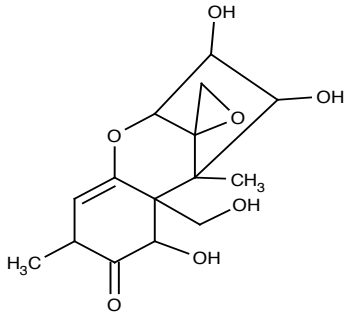
	Masa monoisotópica: 403.082275 Da
	Log P: 4.370
	pKa: 3.29 y -2.20
	Solubilidad: 370 g/L a pH 10
	Punto de fusión: 632.416 °C a 760 mmHg

Toxina T-2 (T-2)	
	**Nombre sistemático:** (3β,4α,8α)-4,15-Diacetoxi-3-hidroxi-12,13-epoxitricotec-9-en-8-il 3-metilbutanoato
	Fórmula: $C_{24}H_{34}O_9$
	Masa monoisotópica: 466.220276 Da
	Log P: 1.965
	pKa: 13.24
	Solubilidad: 0.38 g/L
	Punto de fusión: 544.877 °C a 760 mmHg

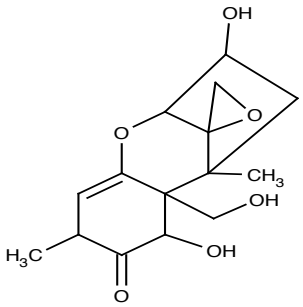
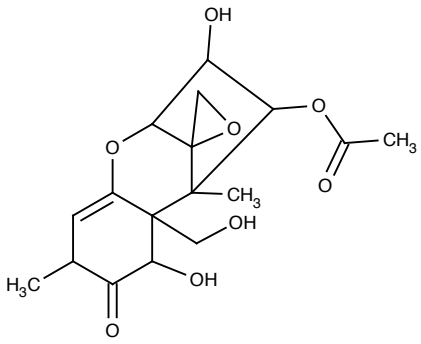
Toxina HT-2 (T-2)	
	**Nombre sistemático:** (3β,4α,8α,12R)-15-Acetoxy-3,4-dihydroxy-12,13-epoxytrichothec-9-en-8-yl 3-methylbutanoate
	Formula: $C_{22}H_{32}O_8$
	Masa monoisotópica: 424.209717 Da
	LogP: 0.964
	pKa: 13.56
	Solubilidad: 1.1 g/L
	Punto de fusión: 537.081 °C at 760 mmHg

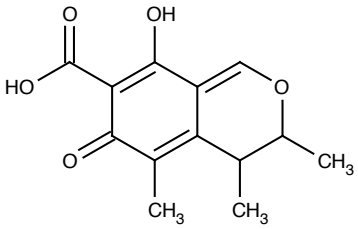
Fumonisina B_1 (FB_1)	
	**Nombre sistemático:** 2,2'-{(19-Amino-11,16,18 -trihidroxi-5,9-dimetil-6,7-icosanediyl)bis[oxi(2-oxo-2,1-etanediilo)]}ácido

	disuccínico
	Fórmula: $C_{34}H_{59}NO_{15}$
	Masa monoisotópica: 721.388489 Da
	Log P: 2.240
	pKa: 3.24 y 9.24
	Solubilidad: 1000 g/L a pH 10
	Punto de fusión: 924.914 °C a 760 mmHg
Fumonisina B_2 (FB_2)	
	**Nombre sistemático:** (2R,2'R)-2,2'-{[(5R,6R,7S,9S,16R,18S,19S)-19-Amino-16,18-dihidroxi-5,9-dimetil-6,7-icosanediyl]bis[oxy(2-oxo-2,1-ethanediyl)]} ácido disuccínico
	Fórmula: $C_{34}H_{59}NO_{14}$
	Masa monoisotópica: 705.393555 Da
	Log P: 4.302
	pKa: 3.64 y 9.25
	Solubilidad: 1000 g/L a pH 10
	Punto de fusión: 864.399 °C a 760 mmHg

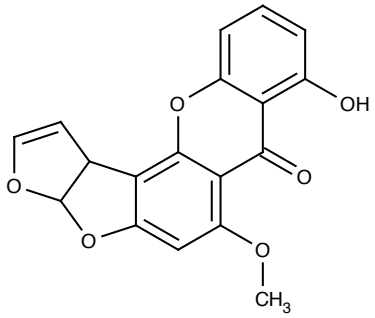
Nivalenol (NIV)	
	**Nombre sistemático:** (3β,4α,7α,12ξ)-3,4,7,15-Tetrahidroxi-12,13-epoxitricotec-9-en-8-uno
	Fórmula: $C_{22}H_{32}O_8$
	Masa monoisotópica: 424.209717 Da
	Log P: 0.964
	pKa: 13.56
	Solubilidad: 1.1 g/L
	Punto de fusión: 537.081 °C a 760 mmHg

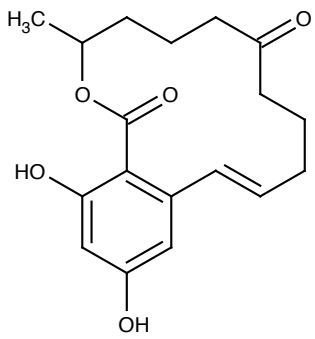
Deoxinivalenol (DON)

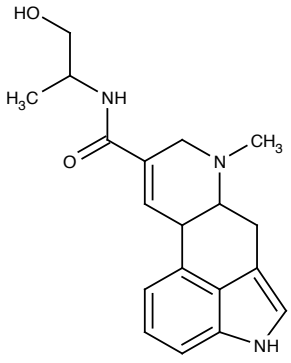
	**Nombre sistemático:** (3β,7α)-3,7,15-Trihidroxi-12,13-epoxitricotec-9-en-8-uno
	Fórmula: $C_{15}H_{20}O_6$
	Masa monoisotópica: 296.125977 Da
	Log P: -1.250
	pKa: 11.91
	Solubilidad: 19 g/L
	Punto de fusión: 543.875 °C a 760 mmHg
Fusarenona X (F-X)	
	**Nombre sistemático:** (3β,4α,7α)-3,7,15-Trihidroxi-8-oxo-12,13-epoxitricotec-9-en-4-il acetato
	Fórmula: $C_{17}H_{22}O_8$
	Masa monoisotópica: 354.13147 Da
	Log P: -0.663
	pKa: 11.70
	Solubilidad: 8.9 g/L a pH 10
	Punto de fusión: 569.219 °C at 760 mmHg

Citrinina (CIT)	
	**Nombre sistemático:** (3R,4S)-8-Hidroxi-3,4,5-trimetil-6-oxo-4,6-dihidro-3H-isocromeno-7-ácido carboxílico
	Fórmula: $C_{13}H_{14}O_5$
	Masa monoisotópica: 250.084122 Da
	Log P: 4.543
	pKa: 1.52
	Solubilidad: 1000 g/L a pH 10
	Punto de fusión: 409.826 °C a 760 mmHg

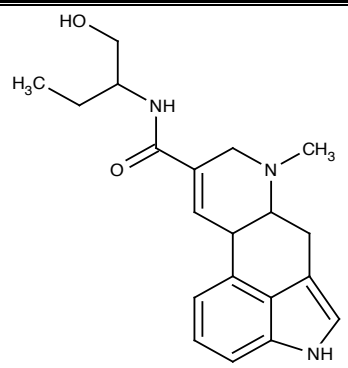
Esterigmatocistina (STE)

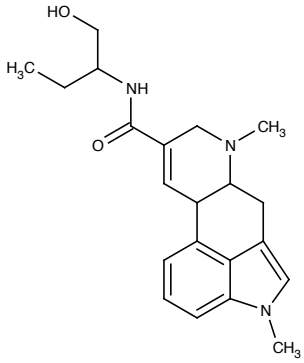
	**Nombre sistemático:** (3aR,12cS)-8-Hidroxi-6-metoxi-3a,12c-dihidro-7H-furo[3',2':4,5]furo[2,3-c]xanten-7-uno
	Fórmula: $C_{18}H_{12}O_6$
	Masa monoisotópica: 324.063385 Da
	Log P: 3.169
	pKa: 6.92
	Solubilidad: 0.39 g/L a pH 10
	Punto de fusión: 569.745 °C a 760 mmHg

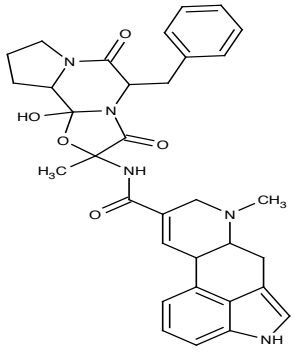
Zearalenona (ZEN)	
	**Nombre sistemático:** (3S,11E)-14,16-Dihidroxi-3-metil-3,4,5,6,9,10-hexahidro-1H-2-benzoxaciclotetradecino-1,7(8H)-diona
	Fórmula: $C_{18}H_{22}O_5$
	Masa monoisotópica: 318.146729 Da
	Log P: 2.765
	pKa: 7.58
	Solubilidad: 1000 g/L a pH 10
	Punto de fusión: 600.396 °C a 760 mmHg

Ergometrina (Em)	
	**Nombre sistemático:** (8β)-N-[(2S)-1-Hidroxi-2-propanil]-6-metil-9,10-didehidroergolina-8-carboxamida
	Fórmula: $C_{19}H_{23}N_3O_2$
	Masa monoisotópica: 325.179016 Da
	Log P: 2.483
	pKa: 7.14 y 14.46
	Solubilidad: 830 g/L a pH 1
	Punto de fusión: 635.167 °C a 760 mmHg

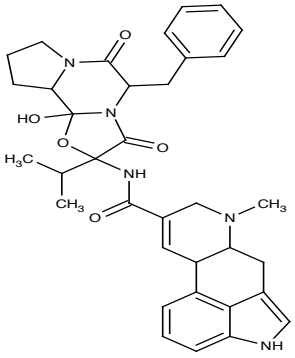
Metilergometrina (MeEm)

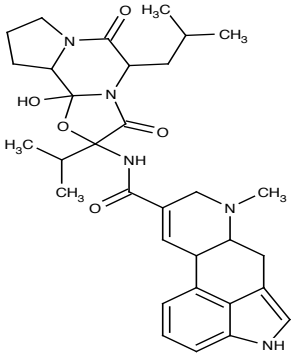
	**Nombre sistemático:** (8β)-N-[(2S)-1-Hidroxi-2-butanil]-6-metil-9,10-didehidroergolina-8-carboxamida
	Fórmula: $C_{20}H_{25}N_3O_2$
	Masa monoisotópica: 339.194672 Da
	Log P: 2.993
	pKa: 7.14 y 14.46
	Solubilidad: 390 g/L a pH 1
	Punto de fusión: 638.36 °C a 760 mmHg

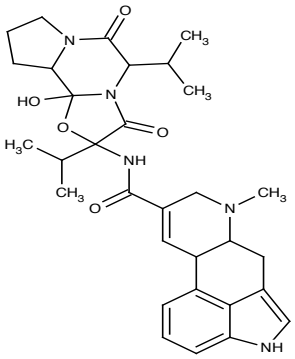
Metisergida (MeErgi)	
	**Nombre sistemático:** (8β)-N-[(2S)-1-Hidroxi-2-butanil]-1,6-dimetil-9,10-didehidroergolina-8-carboxamida
	Fórmula: $C_{21}H_{27}N_3O_2$
	Masa monoisotópica: 353.210327 Da
	Log P: 1.806
	pKa: 7.15 y 14.46
	Solubilidad: 788 g/L a pH 1
	Punto de fusión: 634.447 °C a 760 mmHg

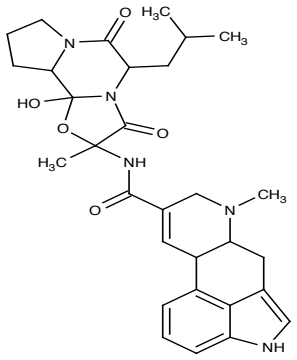
Ergotamina (Et)	
	**Nombre sistemático:** (5'α)-5'-Benzil-12'-hidroxi-2'-metil-3',6',18-trioxoergotaman
	Fórmula: $C_{33}H_{35}N_5O_5$
	Masa monoisotópica: 581.263794 Da
	Log P: 7.373
	pKa: 6.94 y 9.62
	Solubilidad: 0.93 g/L a pH 1
	Punto de fusión: 914.485 °C a 760 mmHg

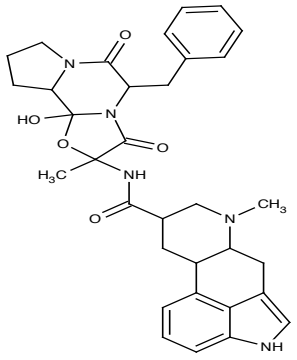
Ergocristina (Ecr)

	**Nombre sistemático:** (5'α)-5'-Benzyl-12'-hidroxi-2'-isopropil-3',6',18-trioxoergotaman
	Fórmula: $C_{35}H_{39}N_5O_5$
	Masa monoisotópica: 609.295105 Da
	Log P: 8.236
	pKa: 6.94 y 9.59
	Solubilidad: 0.27 g/L a pH 1
	Punto de fusión: 912.791 °C a 760 mmHg

Ergocriptina (Ekr)	
	**Nombre sistemático:** (5'α)-12'-Hidroxi-5'-isobutil-2'-isopropil-3',6',18-trioxoergotaman
	Fórmula: $C_{32}H_{41}N_5O_5$
	Masa monoisotópica: 575.310791 Da
	Log P: 8.054
	pKa: 6.94 y 9.61
	Solubilidad: 0.92 g/L a pH 1
	Punto de fusión: 861.746 °C a 760 mmHg

Ergocornina (Eco)	
	**Nombre sistemático:** (5'α)-12'-Hidroxi-2',5'-diisopropil-3',6',18-trioxoergotaman
	Fórmula: $C_{31}H_{39}N_5O_5$
	Masa monoisotópica: 561.295105 Da
	Log P: 7.545
	pKa: 6.94 y 9.58
	Solubilidad: 1.9 g/L pH 1
	Punto de fusión: 858.285 °C a 760 mmHg

Ergosina (Es)	
	**Nombre sistemático:** (5'α)-12'-Hidroxi-5'-isobutil-2'-metil-3',6',18-trioxoergotaman
	Fórmula: $C_{30}H_{37}N_5O_5$
	Masa monoisotópica: 547.27948 Da
	Log P: 7.191
	pKa: 6.94 y 9.64
	Solubilidad: 3.3 g/L a pH 1
	Punto de fusión: 861.371 °C a 760 mmHg

Dihidroergotamina (DHEt)	
	**Nombre sistemático:** (5'α,10α)-5'-Benzil-12'-hidroxi-2'-metil-3',6',18-trioxo-9,10-dihidroergotaman
	Fórmula: $C_{33}H_{37}N_5O_5$
	Masa monoisotópica: 583.27948 Da
	Log P: 5.691
	pKa: 7.22 y 9.64
	Solubilidad: 5.4 g/L a pH 1
	Punto de fusión: 899.534 °C a 760 mmHg

I.3. Legislación vigente sobre contaminación por micotoxinas

Actualmente, la EU tiene establecidos unos contenidos máximos permitidos de determinados contaminantes en diversos alimentos a través del Reglamento (CE) Nº 1881/2006 [42]. Concretamente están incluidos en ese reglamento los contenidos máximos para aflatoxinas (AFB_1, AFB_2, AFG_1, AFG_2 y AFM_1) en frutos secos, cereales, leche y alimentos infantiles; OTA en cereales, uvas pasas, cafés, vino, zumo de uva, alimentos a base de cereales, alimentos dietéticos, para lactantes y niños de corta

[42] Reglamento (CE) Nº 1881/2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. DOUE L364 (2006) 5.

edad; patulina en zumos de frutas, bebidas espirituosas elaboradas con manzana, alimentos infantiles y productos sólidos elaborados con manzana; deoxinivalenol en cereales, pasta, pan y alimentos infantiles a base de cereales; zearalenona en cereales, aperitivos y alimentos infantiles a base de cereales; y fumonisinas en cereales y alimentos elaborados a base de maíz. Generalmente los contenidos máximos se encuentran entre 2-200 μg kg^{-1}, siendo en algunos casos inferiores, como para las aflatoxinas o la OTA, o superiores, como para deoxinivalenol, zearalenona o fumonisinas.

Este Reglamento ha sido modificado en lo que respecta al contenido en toxinas de Fusarium por el Reglamento (CE) Nº 1126/2007 [43], aflatoxinas por el Reglamento (UE) Nº 165/2010 [44] (en el que se aumenta el contenido máximo permitido de aflatoxinas en algunos productos alimenticios con objeto de armonizar la legislación de la UE con el Codex Alimentarius) y por el Reglamento (EU) Nº 1058/2012 [45] (en el que se modifica el contenido máximo permitido en higos secos y otros frutos secos diferentes de éstos y OTA por el Reglamento (UE) Nº 105/2010 [46], que fija el contenido máximo en especias y regaliz, y por el Reglamento (UE) Nº 594/2012 [47] (que modifican el contenido máximo en especias desecadas, cereales y fija el contenido máximo permitido en el gluten de trigo).

Además, y dado que las estimaciones de la ingesta han indicado que la presencia de toxinas T-2 y HT-2 y alcaloides del corzuelo puede ser preocupante para la salud

[43] Reglamento (CE) Nº 1126/2007 que modifica el Reglamento (CE) nº 1881/2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios por lo que se refiere a las toxinas de Fusarium en el maíz y los productos del maíz. DOUE L255 (2007) 14.

[44] Reglamento (UE) Nº 165/2010 que modifica, en lo que respecta a las aflatoxinas, el Reglamento (CE) nº 1881/2006 por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. DOUE L50 (2010) 8.

[45] Reglamento (UE) Nº 1058/2012 por el que se modifica el Reglamento (CE) nº 1881/2006 en lo que respecta al contenido máximo de aflatoxinas en los higos secos. DOUE L313 (2012) 14.

[46] Reglamento (UE) Nº 105/2010 que modifica el Reglamento (CE) nº 1881/2006, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios por lo que se refiere a la ocratoxina A. DOUE L35 (2010) 7.

[47] Reglamento (UE) Nº 594/2012 por el que se modifica el Reglamento (CE) nº 1881/2006 de la Comisión, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios, en lo concerniente a los contenidos máximos de los contaminantes ocratoxina A, PCBs no similares a las dioxinas y melamina en los productos alimenticios. DOUE L176 (2012) 43.

pública, se está estudiando la pertinencia de establecer un nivel máximo para estas micotoxinas, y actualmente existen dos recomendaciones europeas para el control de alcaloides del corzuelo (al menos de ergocristina / ergocristinina, ergotamina / ergotaminina, ergocriptina / ergocriptinina, ergometrina / ergometrinina, ergosina / ergosinina y ergocornina / ergocorninina) en piensos y alimentos [48], y de las toxinas T-2 y HT-2 en cereales y productos a base de cereales [49].

En cuanto a los requisitos de los métodos de muestreo y análisis para su aplicación en el control de micotoxinas en alimentos, éstos están establecidos en el Reglamento (CE) Nº 401/2006 [50], modificado para algunos alimentos por el Reglamento (UE) 178/2010 [51]. De este modo, cuando la legislación comunitaria no exija ningún método específico para la determinación del contenido de micotoxinas en los productos alimenticios, los laboratorios podrán aplicar cualquier método de su elección, siempre que se ajuste a los criterios establecidos por este Reglamento.

En la página web de la AESAN puede encontrarse una relación completa de la legislación vigente relativa a las micotoxinas [52].

Cabe destacar que en ninguno de los Reglamentos anteriores aparece especificado el contenido máximo permitido de micotoxinas en los complementos alimenticios. En lo que respecta a la contaminación de micotoxinas en principios activos para la fabricación de medicamentos, la Farmacopea Europea únicamente establece límite para la AFB_1 en derivados de productos botánicos, donde queda establecido que, a no ser que la monografía correspondiente especifique lo contrario, el contenido máximo de AFB_1 no debe superar los 2 μg kg^{-1}. Asimismo, establece que la autoridad

[48] Recomendación 2012/154/UE sobre el control de la presencia de alcaloides de cornezuelo en los piensos y los alimentos. DOUE L77 (2012) 20.

[49] Recomendación 2013/165/UE sobre la presencia de las toxinas T-2 y HT-2 en los cereales y los productos a base de cereales. DOUE L91 (2013) 12.

[50] Reglamento (CE) Nº 401/2006 por el que se establecen los métodos de muestreo y de análisis para el control oficial del contenido de micotoxinas en los productos alimenticios. DOUE nº L70 (2006) 12.

[51] Reglamento (UE) Nº 178/2010 por el que se modifica el Reglamento (CE) nº 401/2006 en lo que respecta a los cacahuetes y otras semillas oleaginosas, a los frutos de cáscara arbóreos, a los huesos de albaricoque, al regaliz y al aceite vegetal. DOUE L52 (2010) 32.

[52] http://www.aesan.msps.es/AESAN/web/legislacion/subdetalle/micotoxinas.shtml

competente podrá requerir la especificación de que el producto no supera un contenido máximo de 4 μg kg^{-1} en la suma de AFB_1, AFB_2, AFG_1 y AFG_2 [53]. Para el resto de micotoxinas, la Farmacopea Europea no hace ningún tipo de recomendación ni establece ningún contenido máximo permitido.

I.4. Métodos analíticos para la determinación de micotoxinas

Se considera que alrededor del 20 % de los alimentos de origen vegetal están contaminados por micotoxinas, por lo tanto el análisis continuo de los alimentos en busca de estos contaminantes es importante, no solamente para la protección del consumidor sino también para los productores de alimentos. Esto implica el empleo de métodos de análisis contrastados que cumplan con unos parámetros de calidad establecidos y la verificación de los sistemas de control de seguridad alimentaria con el fin de preservar la salud de la población, desde la toma de muestra hasta el análisis del producto [54,55]. Por ello, la UE ha regulado los requerimientos mínimos que debe cumplir un método analítico para su aplicación en el control de estos contaminantes en alimentos [50,51].

Además, existen diversos métodos de análisis recomendados para la determinación de micotoxinas en alimentos, como los Métodos Oficiales de Análisis de la Asociación de Químicos Analíticos Oficiales (*Association of Official Analytical Chemists*, AOAC) [56] donde se pueden encontrar más de cincuenta métodos validados para la determinación de diversas micotoxinas en gran variedad de alimentos, o los métodos normalizados propuestos por la Organización Internacional para la Normalización (*International Standard Organization*, ISO) y el Comité Europeo de Normalización (CEN) .

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[53] European Pharmacopoeia 6.0. “01/2008:20818. Determination of Aflatoxin B_1 in herbal drugs”. Pag. 256-257.

[54] D. L. Park, H. Njapau, R. D. Coker, R. D. en: M. Miraglia, H.P. van Edmond, C. Brera, J. Gilbert (eds.). Mycotoxins and phycotoxins-developments in chemistry, toxicology and food safety. Alaken, Inc. Colorado, EEUU, 1998, pp. 53-64.

[55] Comité Europeo de Normalización. Food analysis- Biotoxins-criteria of analytical method of mycotoxins. CEN Report CR 13505, 1999, Bruselas, Bélgica.

[56] Official Methods of Analysis of AOAC International. 17th Edition, W. Horwitz (ed). AOAC International, 2002, Gaithersburg, MD, EEUU.

Los análisis requieren un alto grado de exactitud (precisión y veracidad), y por esta razón se recomienda el uso de métodos recomendados o la validación de los nuevos métodos propuestos a través de procedimientos que garanticen la calidad de los resultados, incluido el uso de materiales de referencia certificados, o de comparaciones interlaboratorio que ayudan a validar los métodos analíticos empleados y mejoran la competitividad entre laboratorios [57-59].

Los métodos analíticos publicados en la última década para la determinación de micotoxinas en alimentos y sus principales aplicaciones, han sido recopilados en diversos artículos de revisión [60-71].

Los métodos más numerosos son los que emplean cromatografía de líquidos (HPLC) con detección UV [72-76], o fluorescencia (FL) [75-79], ya que ciertas micotoxinas

[57] P. E. Key, A. L. Patey, S. Rowling, S. Wilbourne, F. W. Worner, J. AOAC Int. 80 (1997) 895.
[58] M. Thompson, R. Wood, J. AOAC Int. 76 (1993) 926.
[59] A. C. Williams, K. Jorgensen, C. Donnelly, A. Boenke, The second interlaboratory study of methods for the analysis of ochratoxin A in pig kidney. Report EUR 18458 EN. Office for Official Publications of European Communities, 1998, Luxemburgo.
[60] M. W. Trucksess, A. E. Pohland, Molecular Biotechnology 22 (2002) 287.
[61] R. Krska, E. Welzing, F. Berthiller, A. Molinelli, B. Mizaikoff, Food Addit. Contam. 22 (2005) 345.
[62] P. Zöllner, B. Mayer-Helm, J. Chromatogr. A 1136 (2006) 123.
[63] G. S. Shephard, Chem. Soc. Rev. 37 (2008) 2468.
[64] G. S. Shephard, F. Berthiller, J. Dorner, R. Krska, G. A. Lombaert, B. Malone, C. Maragos, M. Sabino, M. Solfrizzo, M. W. Trucksess, H. P. van Egmond, T. B. Whitaker, World Mycotoxin J. 2 (2009) 3.
[65] N. W. Turner, S. Subrahmanyam, S. A. Piletsky, Anal. Chim. Acta 632 (2009) 168.
[66] V. M. T. Lattanzio, M. Pascale, A. Visconti, TrAC-Trends Anal. Chem. 28 (2009) 758.
[67] E. Reiter, J. Zentek, E. Razzazi, Mol. Nutr. Food Res. 53 (2009) 508.
[68] R. Köppen, M. Koch, D. Siegel, S. Merkel, R. Maul, I. Nehls, Appl. Microbiol. Biotechnol. 86 (2010) 1595.
[69] G. S. Shephard, F. Berthiller, J. Dorner, R. Krska, G. A. Lombaert, B. Malone, C. Maragos, M. Sabino, M. Solfrizzo, M. W. Trucksess, H. P. van Egmond, T. B. Whitaker, World Mycotoxin J. 3 (2010) 3.
[70] J. P. Meneely, F. Ricci, H. P. van Egmond, C. T. Elliott, TrAC-Trends Anal. Chem. 30 (2011) 192.
[71] G. S. Shephard, F. Berthiller, P. A. Burdaspal, C. Crews, M. A. Jonker, R. Krska, S. MacDonald, R. J. Malone, C. Maragos, M. Sabino, M. Solfrizzo, H. P. Van Egmond, T. B. Whitaker, World Mycotoxin J., 5 (2012) 30.
[72] G. J. Soleas, J. Yan, D. M. Goldberg, J. Agric. Food Chem. 49 (2001) 2733.
[73] M. W. Trucksess, L. Bao, C. M. Weaver, K. D. White, J. AOAC Int. 93 (2010) 1236.
[74] A. Curticapean, F. Toma, M. Tarcea, M. Curticapean, V. Samarghitan, I. A. Pop, A. Gulea, Croat. Chem. Acta 84 (2011) 413.

(OTA, aflatoxinas) presentan fluorescencia nativa y este tipo de detección ofrece ciertas ventajas, como mayor sensibilidad y selectividad [80]. Existen numerosos métodos HPLC-FL para la determinación de OTA sin derivatización previa en distintas matrices, principalmente en vinos, café o cerveza [61]. En el caso de las aflatoxinas, su fluorescencia se muestra atenuada en presencia de ciertos disolventes, siendo necesaria su derivatización bien precolumna con ácido trifluoroacético, o poscolumna mediante una disolución de yodo o bromo, o mediante hidrólisis por fotodegradación usando una lámpara UV y un reactor [81,82,83,84,85,86]. Otras micotoxinas que no presentan fluorescencia nativa también se han determinado usando HPLC-FL con derivatización, como es el caso de las fumonisinas [87,88] o T-2 and HT-2 [75].

El acoplamiento de HPLC y la espectrometría de masas (*Mass Spectrometry*, MS) mediante técnicas de ionización a presión atmosférica (*Atmospheric Pressure Ionization*, API), como la ionización por electrospray (*Electrospray Ionization*, ESI) o la ionización química a presión atmosférica (*Atmospheric-Pressure Chemical Ionization*, APCI), ha permitido el desarrollo de nuevas metodologías para la determinación de micotoxinas [89 - 91]. Algunos artículos de revisión recogen los métodos de

[75] G. Barros, M. S. Alaniz Zanon, J. M. Palazzini, M. Haidukowski, M. Pascale, S. Chulze, Food Addit. Contam. 29 (2013) 1436.
[76] M. E. H. Müller, U. Korn, Food Control 34 (2013) 191.
[77] J. L. Urraca, E. Benito-Peña, C. Pérez-Conde, M. C. Moreno-Bondi, J. J. Pestka, J. Agric. Food Chem. 53 (2005) 3338.
[78] C. A. F. Oliveira, J. C. O. Ferraz, Food Control 18 (2007) 375.
[79] X. –M. Li, X. –H. Shen, L. Xue, Z. W. Duan, S. –R. Guo, E-J. Chem. 9 (2012) 260.
[80] J. Jaimez, C. A. Fente, B. I. Vázquez, C. M. Franco, A. Cepeda, G. Mahuzier, P. Prognon, J. Chromatogr. A 882 (2000) 1.
[81] G. Tavcar-Kalcher, K. Vrtac, U. Pestevsek, A. Vengust, Food Control 18 (2007) 333.
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[83] A. E. Waltking, D. Wilson, J. AOAC Int. 89 (2006) 678.
[84] M. J. O' Riordan, M. G. Wilkinson, Food Chem. 20 (2009) 700.
[85] P. Afshar, M. Shokrzadeh, S. Kalhori, Z. Babaee, S. S. Saeedi Saravi, Food Control 31 (2013) 525.
[86] A. M. Turcotte, P. M. Scott, B. Tague, Mycotoxin Res. (2013) DOI 10.1007/s12550-013-0167-x
[87] L. Ma, W. Xu, X. He, K. Huang, Y. Wang, Y. Luo, J. Sci. Food Agr. 93 (2013) 1128.
[88] W. Kong, T. Xie, J. Li, J. Wei, F. Qiu, A. Qi, Y. Zheng, M. Yang, Analyst 137 (2012) 3166.
[89] G. S. Shephard, N. L. Leggott, J. Chromatogr. A 882 (2000) 17.
[90] G. S. Shephard, A. Fabiani, S. Stockenström, N. Mshicileli, V. Sewram, J. Agric. Food Chem. 51 (2003) 1102.
[91] A. K. Malik, C. Blasco, Y. Picó, J. Chromatogr. A 1217 (2010) 4018.

determinación de micotoxinas mediante HPLC-MS [62, 92]. Además, mediante el empleo de MS en tándem (MS/MS), usando detectores como la trampa iónica (*Ion Trap*, IT) y el triple cuadrupolo (QqQ), es posible la identificación y cuantificación de los analitos en mezclas complejas, siendo imprescindibles para la confirmación de resultados positivos obtenidos con otras detecciones. Como ejemplos recientes, podemos mencionar los métodos para la determinación de OTA en queso [93], fumonisinas en alimentos infantiles [27], deoxinivalenol, zearalenona y sus metabolitos en productos derivados de cereales [25], toxinas de Fusarium en cereales para el desayuno y alimentos infantiles [94], y trigo y galletas [95], toxinas T-2 y HT-2 en cereales [96], zearalenona y sus metabolitos en medicinas chinas tradicionales [97] y las micotoxinas emergentes enniatinas (A, A_1, B and B_1) y beauvericina en productos a bases de cereales [98].

HPLC-MS/MS ha permitido también el establecimiento de métodos multirresiduo, que abarquen micotoxinas de diferentes familias. En este contexto, recientemente se han propuesto métodos HPLC-MS/MS para, por ejemplo, la determinación simultánea de OTA, acido micofenólico y fumonisina B_2 en productos cárnicos [99], 27 micotoxinas y otros metabolitos secundarios en maíz [100], 39 micotoxinas en maíz y trigo [101], 49 micotoxinas diferentes en diversos alimentos [102], 18 micotoxinas en cerveza [103] y 21 micotoxinas en alimentos infantiles [104]. Considerando que, debido a la sensibilidad y selectividad de esta técnica sería posible inyectar directamente un

[92] S. Sforza, C. Dall'asta, R. Marchelli, Mass Spectrom. Rev. 25 (2006) 54.
[93] X. Zhang, E. Cudjoe, D. Vuckovic, J. Pawliszyn, J. Chromatogr. A 1216 (2009) 7505.
[94] B. Romagnoli, M. Ferrari, C. Bergamini, J. Mass. Spectrom. 45 (2010) 1075.
[95] H. Tanaka, M. Takino, Y. Sugita-Konishi, T. Tanaka, D. Leeman, A. Toriba, K. Hayakawa, Rapid Commun. Mass Spectrom. 24 (2010) 2445.
[96] Á. Tölgyesi, Z. Kunsági, Microchem. J. 106 (2013) 300.
[97] Z. Han, Y. Ren, H. Zhou, L. Luan, Z. Cai, Y. Wu, J. Chromatogr. B 879 (2011) 411.
[98] J. Blesa, R. Marín, C. M. Lino, J. Mañes, Food Addit. Contam. 29 (2012) 1727.
[99] L. M. Sørensen, J. Mogensen, K. F. Nielsen, Anal. Bioanal. Chem. 398 (2010) 1535.
[100] R. R. Rasmussen, I. M. L. D. Storm, P. H. Rasmussen, J. Smedsgaard, K. F. Nielsen, Anal. Bioanal. Chem. 397 (2010) 765.
[101] M. Sulyok, F. Berthiller, R. Krska, R. Schuhmacher, Anal. Bioanal. Chem. 389 (2007) 1505.
[102] M. Sulyok, R. Krska, R. Schuhmacher, Food Chemistry 119 (2010) 408.
[103] J. Rubert, C. Soler, R. Marín, K. J. James, J. Mañes, Food Control 30 (2013) 122.
[104] J. Rubert, C. Soler, J. Mañes, Food Chem. 133 (2012) 176.

extracto diluido en el equipo, hay que resaltar que es adecuado realizar un *clean-up* previo con objeto de que las posibles impurezas no supriman la ionización de los compuestos de interés.

I.4.1. Aplicación de técnicas miniaturizadas en la determinación de micotoxinas

En los últimos años las técnicas de separación miniaturizadas han cobrado gran interés debido a las numerosas ventajas que presentan, tales como la reducción del consumo de disolventes, bajo volumen de muestras requerido, incremento en la resolución e incluso una mejorada sensibilidad [105]. Dentro de los sistemas miniaturizados se incluyen los que utilizan columnas de bajo diámetro interno o capilares de sílice fundida, y aquellos en los que la separación se realiza en microchips. En la presente Tesis Doctoral se han utilizado dos técnicas pertenecientes al primer grupo: la electroforesis capilar (*Capillary Electrophoresis*, CE) y la cromatografía de líquidos capilar (HPLC capilar), que pasan a comentarse brevemente a continuación.

I.4.1.1. Electroforesis Capilar

Son numerosas las referencias bibliográficas relacionadas con la CE, donde se pueden consultar sus fundamentos, instrumentación, diferentes modalidades y aplicaciones [106-111]. En esta introducción, se dará sólo una visión general de la CE, así como de las diferentes vías para mejorar la sensibilidad de esta técnica miniaturizada.

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[105] M. Szumski, B. Buszewski, Crit. Rev. Anal. Chem. 32 (2002) 1.
[106] C. Cruces Blanco, Electroforesis Capilar, Universidad de Almería, Almería, 1ª Ed., 1998.
[107] W. T. Kok, Chromatographia Supplement 51 (2000) 1.
[108] H. H. Lauer, G. P. Rozing, High Performance Capillary Electrophoresis, Agilent Tecnhologies 2ª Ed., Alemania, 2010.
[109] J. L. Felhofer, L. Blanes, C. D. García, Electrophoresis 31 (2010) 2469.
[110] P. Camilleri, Capillary Electrophoresis: Theory and Practice, CRC Press INC, Boca Raton, 2ª Ed., 1998.
[111] K. D. Altria, Capillary Electrophoresis Guidebook: Principles, Operation, and Applications, Humana Press, New Jersey, 2ª Ed., 2010.

La CE presenta como principales ventajas una elevada eficacia, tiempos de separación cortos y volúmenes de muestras y disolventes muy pequeños, ya que se emplean capilares muy estrechos (25-150 µm de diámetro interno), por lo que el coste y la contaminación ambiental disminuyen considerablemente en comparación con la HPLC convencional (flujos de ml min^{-1}).

CE es altamente versátil, pudiendo emplearse en la determinación de analitos polares, apolares, iónicos, neutros y de alto peso molecular, gracias a sus diferentes modos de separación, entre los que se encuentran la electroforesis capilar en zona (*capillary zone electrophoresis*, CZE), la cromatografía capilar electrocinética micelar (*micellar electrokinetic chromatography*, MEKC), electroforesis capilar en gel (*capillary gel electrophoresis,* CGE), isotacoforesis capilar (*capillary isotacophoresis,* CITP), enfoque isoeléctrico capilar, (*capillary isoelectrofocusing*, CIEF), y electrocromatografía capilar (*capillary electrochromatography*, CEC). En la Figura I.9 se muestra un esquema de las diferentes modalidades en CE.

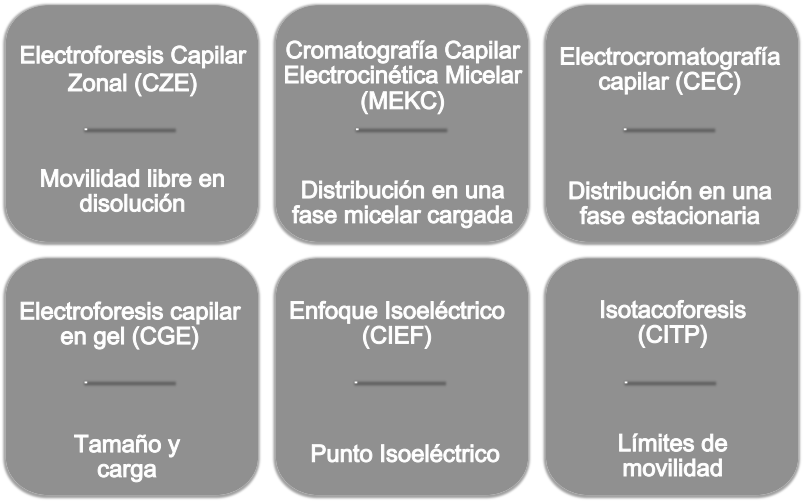

Figura I.9. Modos de operación en CE

En esta Tesis Doctoral, la modalidad utilizada ha sido la MEKC. En MEKC, existen dos fases, la acuosa y la micelar (fase pseudoestacionaria), ambas en movimiento; la separación se basa en las diferencias en la interacción de cada uno de los analitos con

las micelas presentes en la disolución tampón. Se trata de un modo de separación que combina el mecanismo de separación de la cromatografía (las micelas actúan como fase pseudoestacionaria) con los movimientos electroforéticos y electroosmóticos de los solutos y las disoluciones.

El principal inconveniente que presenta la aplicación de la CE en la determinación de micotoxinas radica en la necesidad de una elevada sensibilidad, lo cual contrasta con la limitación de la técnica de CE acoplada a la detección UV/Vis, debido a los bajos volúmenes de inyección introducidos y a la baja sensibilidad de esta detección. Por ello, existen escasas aplicaciones en el análisis de micotoxinas por CE-UV, generalmente empleando el modo CZE o MEKC. Algunos ejemplos son la determinación de moliniformina en maíz [112], patulina en manzana y zumos [113,114,115,31] y OTA en vino [116,117].

No obstante, el desarrollo comercial de otros detectores más sensibles, como la fluorescencia inducida por láser (*Laser Induced Fluorescence*, LIF), empleada en esta Tesis, ha contribuido a subsanar este problema. La LIF tiene como particularidad el poder incidir en una zona muy reducida del capilar, alcanzando límites de detección del orden de los 10^{-18} - 10^{-21} moles utilizando detección "*on-column*", es decir, en el mismo capilar [118]. Existen distintos tipos de láseres, siendo los más utilizados los de He-Cd (emisión a 325 y 442 nm) e ión Ar (de varios tipos, emitiendo entre 350-360, 476, 488 y 514 nm). Para su selección es necesario considerar que la longitud de onda que emiten sea próxima a la longitud de onda de excitación del analito.

[112] C. M. Maragos, Food Addit. Contam. 21 (2004) 803.

[113] R. Tsao, T. Zhou, J. Agric. Food Chem. 48 (2000) 5231.

[114] J. L. McCallum, R. Tsao, T. Zhou, J. Food Protection 65 (2002) 1937.

[115] M. Murillo-Arbizu, E. González-Peñas, S. H. Hansen, S. Amézqueta, J. Østergaard, Food and Chem. Toxicol. 46 (2008) 2251.

[116] E. González-Peñas, C. Leache, A. López de Cerain, E. Lizarraga, Food Chem. 97 (2006) 349.

[117] S. Almeda, L. Arce, M. Valcárcel, Electrophoresis 29 (2008) 1573.

[118] C. Huan-Tsung, C. Tai-Chia, H. Chih-Ching, en: J. Cazes, (ed.), Encyclopedia of Chromatography (3rd Edition), Taylor & Francis, Londres, Reino Unido, 2010, pp. 1325.

De forma general dos son los mayores beneficios de la detección fluorescente acoplada con la CE o con HPLC: la sensibilidad y la selectividad, que se comentan a continuación:

- Sensibilidad: esta detección puede dar lugar a una mayor sensibilidad que la medida de la absorbancia porque la detección fluorescente tiene una señal de fondo muy baja. Normalmente este detector puede proporcionar límites de detección 2 o 3 órdenes de magnitud mayores que los detectores de absorbancia. Cuando se utiliza la detección fluorescente, la señal de una muestra se compara con una muestra no fluorescente (el electrolito de fondo), por ello la señal de fondo es cero o muy próxima a cero. En cambio, cuando se utiliza un detector de absorbancia, la transmitancia de la muestra se compara con la de un blanco por lo que a bajas concentraciones la diferencia entre ambas es pequeña y el error en la medida puede ser significativo. Además, la sensibilidad del detector está íntimamente ligada a la intensidad de la fuente de radiación utilizada para excitar al compuesto, y en el caso de LIF es una fuente de excitación extremadamente intensa, con un haz colimado con una potencia del orden de los miliwatios.

- Selectividad: La selectividad va a depender en este caso de lo estrecha que sea la banda de longitud de onda utilizada para la excitación. Este hecho hace posible la excitación a 325 nm (longitud de onda del laser empleado en esta Tesis) y la reducción de las posibles interferencias en la matriz. Además, cuando se emplea un detector de absorbancia, las medidas se realizan a una sola longitud de onda al mismo tiempo, mientras que en el caso de la fluorescencia se usan dos. En el caso de dos analitos que eluyan al mismo tiempo y absorban radiación a la misma longitud de onda pero diferentes longitudes de onda de emisión (o uno de ellos no emite fluorescencia), un detector fluorescente puede diferenciar las señales analíticas mientras que uno de absorbancia no.

A continuación (Figura I.10) se incluye un esquema del sistema óptico del detector LIF (ZETALIF Evolution, Picometrics) acoplable con CE y con HPLC capilar, empleado en el desarrollo de esta Tesis.

En el caso de las micotoxinas, los bajos límites de detección requeridos para su determinación en alimentos hacen que la LIF sea una técnica ideal para su detección, teniendo en cuenta que algunas de las más importantes desde el punto de vista toxicológico presentan fluorescencia nativa (como las aflatoxinas, la OTA o la zearalenona) o pueden ser derivatizadas (como las fumonisinas o algunos tricotecenos) para obtener compuestos fluorescentes. La detección fluorescente se ha empleado para la determinación semicuantitativa (*screening*) de aflatoxinas [119] y, empleando CE-LIF se han determinado OTA en varios alimentos [120], zearalenona [121], AFB_1 [122] y toxina T-2 en maíz [123]. En esta Tesis se ha desarrollado un método de determinación de aflatoxinas mediante MEKC-LIF, siendo posible su determinación en arroz con límites de detección comprendidos entre 0.04-0.52 μg Kg^{-1} [124].

[119] R. Peña, M.C. Alcaraz, L. Arce, A. Ríos, M. Valcárcel, J. Chromatog. A 967 (2002) 303.

[120] S. Corneli, C. M. Maragos, J. Agric. Food Chem. 46 (1998) 3162.

[121] C. M. Maragos, M. Appell, J. Chromatogr. A 1143 (2007) 252.

[122] C. M. Maragos, J. I. Greer, J. Agric. Food Chem. 45 (1997) 4337.

[123] C. M. Maragos, M. Appell, V. Lippolis, A. Visconti, L. Catucci, M. Pascale, Food Add. Contam. 25 (2008) 164.

[124] N. Arroyo-Manzanares, L. Gámiz-Gracia, A. M. García-Campaña, J. J. Soto-Chinchilla, L. E. García-Ayuso, Electrophoresis 31 (2010) 2180.

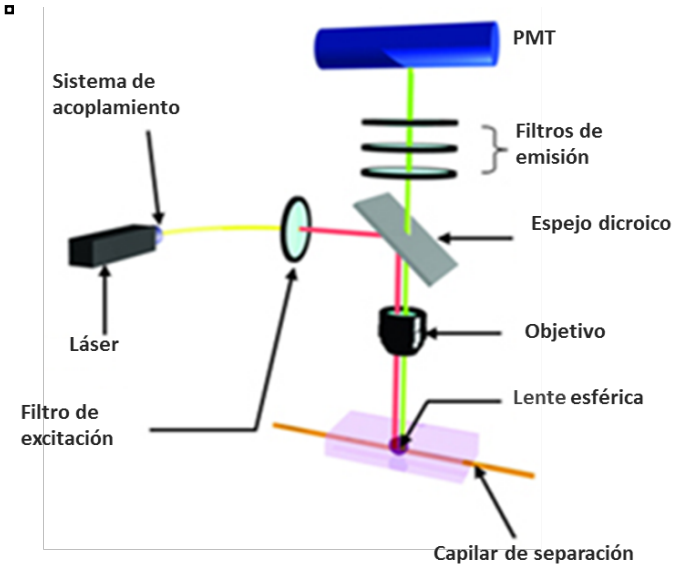

Figure I.10. Esquema del sistema óptico del detector LIF (Figura adaptada de Picometrics)

❖ **Técnicas de preconcentración *on-line* para mejorar la sensibilidad en CE**

El mayor inconveniente de la CE es su capacidad de carga limitada (1-2 µL), lo que conlleva límites de detección altos. Para disminuir estos límites de detección, se han desarrollado técnicas para introducción de muestra que proporcionan un efecto de concentración en el capilar, permitiendo aumentar el volumen de muestra inyectada sin disminuir la eficacia de la electroforesis.

La técnica más simple y conocida de preconcentración *on-line* es el "apilamiento" o *stacking* [125,126] que, básicamente, consiste en focalizar la muestra eléctricamente dentro del capilar, aumentando su concentración en una determinada zona del mismo. El inconveniente que presenta es que permite preconcentrar aniones o cationes, pero no ambos a la vez.

[125] Z. Malá, L. Krivánková, P. Gebauer, P. Bocek, Electrophoresis 28 (2007) 243.
[126] Z. Malá, A. Šlampová, P. Gebauer, P. Boček, Electrophoresis 30 (2009) 215.

Otra modalidad de preconcentración *on-line* es la denominada *"barrido" o sweeping*, que se realiza en modo MEKC [127]. Aquí la muestra se concentra en una estrecha banda dentro del capilar gracias a su interacción con el aditivo presente en el tampón electroforético (ausente en la disolución de muestra), que actúa "barriendo" a los analitos cuando estos pasan de la zona de muestra a entrar en contacto con el tampón electroforético (Figura I.11). Mediante *sweeping* se puede conseguir la preconcentración tanto de moléculas neutras como cargadas.

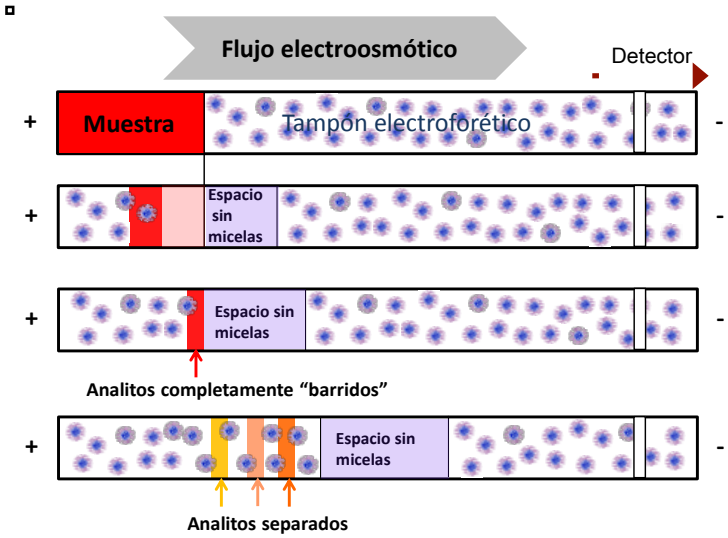

Figure I.11. Principio básico de la preconcentración mediante *sweeping*

Esta metodología se ha aplicado en la determinación de aflatoxinas en arroz mediante MEKC-LIF [124], desarrollado en esta Tesis.

De este modo, combinando técnicas de detección sensibles con sistemas de preconcentración *on-line*, la CE puede conseguir límites de detección satisfactorios para el análisis de micotoxinas en alimentos.

I.4.1.2. Cromatografía de líquidos capilar

[127] A. T. Aranas, A. M. Guidote Jr., J. P. Quirino, Anal. Bioanal. Chem. 394 (2009) 175.

En las dos últimas décadas, la automatización y la miniaturización han sido muy importantes en el desarrollo de la cromatografía de líquidos. Los sistemas de cromatografía de líquidos de alta resolución miniaturizados y basados en el empleo de columnas micro, capilares y nano ofrecen considerables ventajas respecto a la HPLC convencional (con columnas de diámetro interno 4.6 mm), mejorando la detección y obteniendo eficacias de separación elevadas. Debido al diámetro interno reducido, los caudales de fase móvil utilizados con columnas capilares son muy pequeños, disminuyendo considerablemente el consumo de reactivos y disolventes, y por tanto, los desechos generados y el impacto medioambiental.

En función del diámetro interno de la columna y de los caudales utilizados, las técnicas de HPLC se han clasificado en diversas categorías [105]. La terminología actualmente usada se muestra en la siguiente Tabla, donde aparecen clasificados los distintos tipos de cromatografía en función de los distintos parámetros que se ven afectados por la miniaturización de la técnica.

Tabla I.2. Terminología usada en cromatografía de líquidos

Nombre	Diámetro interno de la columna	Velocidad de flujo	Tamaño de partícula (µm)	Longitud (cm)
HPLC convencional	3.2-4.6 mm	0.5-2.0 mL/min	3-10	3-25
HPLC microbore	1.5-3.2 mm	100-500 µL/min	3-8	15-25
Micro-LC	0.5-1.5 mm	10-100 µL/min	3-5	5-15
LC capilar	150-500 µm	1-10 µL/min	3-5	5-15
Nano-LC	10-150 µm	10-1000 nL/min	3-5	5-15

En comparación con los sistemas convencionales de HPLC, este tipo de técnicas mejoran la detección y permiten obtener eficacias de separación elevadas. Teóricamente, el uso de micro-columnas aumenta la velocidad de la separación por la mayor velocidad linear de la fase móvil. Estas mejoras cinéticas, junto con el pequeño caudal de fase móvil, permiten reducir la sobrepresión, conectar columnas en serie y conseguir eficacias de hasta 105-107 platos teóricos, que permiten la resolución de mezclas complejas. Por otra parte, el pequeño volumen en el que se eluyen los analitos proporciona mayor sensibilidad y mejoras de hasta 20 órdenes de magnitud

en la respuesta del detector [128]. De acuerdo con el factor teórico de disminución de escala, las sensibilidades se pueden incrementar del orden de 200 veces en detectores másicos, al reducir el diámetro interno de la columna de 4.6 mm a 320 µm, de forma que las micro-columnas han sido muy utilizadas para aumentar la sensibilidad de la detección y la resolución cuando el volumen de muestra disponible para la inyección es limitado o los compuestos a determinar se encuentran presentes a bajas concentraciones en las distintas muestras [129].

A pesar de las ventajas de la HPLC capilar respecto a la HPLC convencional (disminución en consumo de disolventes y fase estacionaria, uso de pequeños volúmenes de muestra, mayor sensibilidad másica y mejor resolución), la única aplicación de este tipo de cromatografía en el análisis de micotoxinas de la que tenemos conocimiento es el método desarrollado en esta Tesis basado en HPLC capilar con detección LIF para la determinación de OTA en vinos [130,131].

I.4.2. Empleo de la cromatografía de líquidos de ultra resolución en la determinación de micotoxinas

Además de la miniaturización, otro paso importante dentro del avance de la HPLC ha sido el desarrollo de fases estacionarias de partícula híbrida, con un componente inorgánico (sílice) y un componente orgánico (organosiloxanos) [132,133] y con un tamaño de partícula inferior a los 2 µm [134], originando lo que se ha denominado "Cromatografía de Líquidos de Ultra Resolución (UHPLC)" [133]. Esta reducción del tamaño de partícula, disminuye significativamente la altura equivalente de plato teórico (*Height Equivalent Theoretical Plate*, HETP), lo que hace posible mejorar la eficacia del

[128] A. Braithwaite, F. J. Smith, "Chromatographic Methods", 5th Edition. Kluwer Academic Publishers, 1999, The Netherlands.
[129] J. P. C. Vissers, H. A. Claessens, C. A. Cramers, J. Chromatogr. A 779 (1997) 1.
[130] N. Arroyo-Manzanares, L. Gámiz Gracia, A. M. García Campaña, Food Chemistry 135 (2012) 368.
[131] N. Arroyo-Manzanares, A. M. García-Campaña, L. Gámiz-Gracia, Anal. Bioanal. Chem. 401 (2011) 2987.
[132] E. S. Grumbach, T. E. Wheat, M. Kele, J. R. Mazzeo, LC-GC N. Am. 23 (2005) 40.
[133] M. E. Swartz, J. Liq. Chromatogr. Rel. Tech. 28 (2005) 1253.
[134] D. T. T. Nguyen, D. Guillarme, S. Rudaz, J. L. Veuthey, J. Sep. Sci. 29 (2006) 1836.

proceso cromatográfico, permitiendo trabajar a elevados caudales de fase móvil sin pérdida de la calidad en la separación cromatográfica [135].

En la Figura I.12 se representa una gráfica de Van Deemter en función de los tamaños de partícula desarrollados en las últimas décadas. Como se puede observar, la disminución del tamaño de partícula por debajo de 2.5 µm ha dado lugar a un gran aumento de la eficiencia de la separación ya que se produce una disminución notable de la HETP, manteniéndose ésta prácticamente constante al aumentar el caudal o la velocidad lineal [133]. Esta posibilidad de aumentar el caudal sin disminuir la resolución conlleva una reducción del tiempo de análisis, llegándose a determinar más de 100 compuestos en menos de diez minutos [133,136].

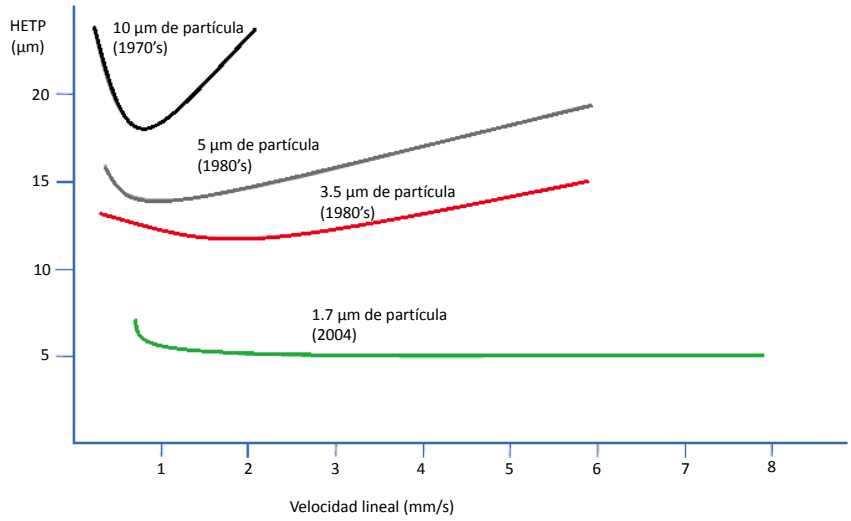

Figura I.12. Gráfica de van Deemter ilustrando la evolución del tamaño de partícula en las últimas décadas [133].

En resumen, los beneficios de la UHPLC son la mejora de la resolución entre picos cromatográficos, la disminución de los tiempos de análisis, además de la mejora de la sensibilidad. No obstante, el uso de un tamaño de partícula menor (< 2 µm) ha

[135] A. D. Jerkovich, J. S. Mellors, J. W. Thompson, J. W. Jorgenson, Anal. Chem. 77 (2005) 6292.
[136] I. D. Wilson, R. Plumb, J. Granger, H. Major, R. Williams, E. M. Lenz, J. Chromatogr. B 817 (2005) 67.

obligado a desarrollar tanto bombas que puedan ejercer la presión suficiente para trabajar a caudales adecuados, soportando presiones superiores a 10000 psi [137], inyectores capaces de inyectar cantidades cada vez menores y de soportar presiones mucho mayores, y detectores mucho más rápidos que sean capaces de proporcionar suficientes puntos por pico cromatográfico y con celdas de detección mucho más pequeñas para evitar el ensanchamiento de pico.

En cuanto a los sistemas de detección, la mayoría de las aplicaciones de UHPLC desarrolladas hasta ahora han implicado la utilización de MS como detector [138]. El acoplamiento de ambas técnicas mejora considerablemente la detección, puesto que la reducción de la anchura de pico aumenta la altura del mismo, lo que conlleva una mejora en la sensibilidad del método analítico. Sin embargo, debido a los picos considerablemente más estrechos obtenidos en UHPLC (normalmente entre 1–3 s), el acoplamiento con sistemas de detección de MS es crítico. Por esta razón se han comercializado instrumentos específicos con tiempos de adquisición suficientemente rápidos que pueden garantizar un número de puntos por pico adecuado para una correcta detección [138]. El acoplamiento UHPLC-MS se ha convertido así en una poderosa alternativa a la HPLC convencional en el ámbito del análisis multirresiduo en alimentos, reduciendo los tiempos de análisis y permitiendo una confirmación inequívoca de los analitos. Los analizadores QqQ, que permiten el modo de trabajo en MS/MS, son los que claramente se han impuesto para llevar a cabo la determinación de un número elevado de analitos con una buena sensibilidad y selectividad, gracias a la monitorización de transiciones MS/MS operando en el modo de detección múltiple de fragmentos (*Multiple Reaction Monitoring*, MRM).

En cuanto a la aplicación de la UHPLC para la determinación de micotoxinas, las aportaciones en los últimos años han sido numerosas, entre las que se encuentran un método que emplea UHPLC-UV para la determinación de aflatoxinas en maíz y cacahuete [139], y diversos métodos basados en UHPLC-MS para la determinación

[137] M. I. Churchwell, N. C. Twaddle, L. R. Meeker, D. R. Doerge, J. Chromatogr. B 825 (2005) 134.
[138] D. Guillaume, J. Schappler, S. Rudaz, J. L. Veuthey, TrAC-Trends Anal. Chem. 29 (2010) 15.
[139] Z. Fu, X. Huang, S. Min, J. Chromatogr. A 1209 (2008) 271.

multiclase de micotoxinas [26,140-142] o de micotoxinas junto con otros contaminantes [143-146].

I.4.3. Acoplamiento LC-MS

La cromatografía acoplada con MS es probablemente la técnica analítica instrumental más extendida hoy en día para el análisis en los campos de la salud, alimentación y medioambiente. El principio de la MS es la producción de iones en estado gaseoso a partir de compuestos neutros o cargados, y la detección de los iones o los fragmentos de esos iones producidos en el proceso de ionización o en el espectrómetro antes de llegar a la zona de alto vacío. Cuando se trata de un acoplamiento LC-MS, como es el caso de los métodos descritos en esta Tesis, los analitos han de pasar a estado gaseoso antes de producirse la ionización. Estos iones descompuestos (fragmentos que también poseen carga) o no descompuestos se dirigen rápidamente a través del espectrómetro para ser "clasificados" de acuerdo a su relación *m/z*. El espectrómetro de masas no sólo clasifica los fragmentos, sino que además mide la cantidad de ellos que se forman. Finalmente se produce una amplificación de la señal producida por cada molécula medida y se genera el espectro de masas. Los espectrómetros de masas constan, básicamente, de cuatro partes [147]:

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[140] A. Garrido-Frenich, R. Romero-González, M. L. Gómez-Pérez, J. L. Martínez-Vidal, J. Chromatogr. A 1218 (2011) 4349.

[141] E. Beltrán, M. Ibáñez, J. V. Sancho, F. Hernández, Rapid Commun. Mass Spectrom. 23 (2009) 1801.

[142] E. Varga, T. Glauner, F. Berthiller, R. Krska, R. Schuhmacher, M. Sulyok, Anal. Bioanal. Chem. (2013) DOI 10.1007/s00216-013-6831-3

[143] M. M. Aguilera-Luiz, P. Plaza-Bolaños, R. Romero-González, J. L. Martínez-Vidal, A. Garrido-Frenich, Anal. Bioanal. Chem. 399 (2011) 2863.

[144] R. Romero-González, A. Garrido-Frenich, J. L. Martínez-Vidal, O. D. Prestes, S. L. Grio, J. Chromatogr. A 1218 (2011) 1477.

[145] H. G. J. Mol, P. Plaza-Bolaños, P. Zomer, T. C. de Rijk, A. A. M. Stolker, P. P. J. Mulder, Anal. Chem. 80 (2008) 9450.

[146] P. Pérez-Ortega, B. Gilbert-López, J.F. García-Reyes, N. Ramos-Martos, A. Molina-Díaz, J. Chromatogr. A 1249 (2012) 32.

[147] Agencia Estatal Antidopaje. Disponible en noviembre 2012 en: http://www.aea.gob.es/media/126618/espectrometr%C3%ADa%20de%20masas-nuevo.pdf.

1. Un sistema de introducción de muestra, que puede ser entre otros un cromatógrafo de líquidos (empleado en esta Tesis Doctoral) o un cromatógrafo de gases. No es más que la herramienta que conduce las moléculas previamente separadas hasta la entrada del espectrómetro de masas.

2. Una fuente de ionización, que es donde se produce el paso al estado gaseoso de la fase móvil y de los analitos transportados por ésta y donde las moléculas neutras pasan al estado ionizado. Las fuentes de ionización desarrolladas para el acoplamiento LC-MS han tenido la doble función de eliminar el disolvente (normalmente presente en órdenes de 0.1-2 ml min^{-1}) y vaporizar la muestra e ionizarla. En este acoplamiento, la ionización se produce a presión atmosférica. Aunque existen otros modos de ionización como la ionización por nebulización térmica (*Thermospray*, TSP) o la fotoionización a presión atmosférica (*Atmospheric Pressure Photoionization*, APPI), las técnicas de ionización más frecuentemente empleadas hoy en día en los acoplamientos LC-MS son la ESI y APCI. En la Tabla I.3 se recogen las características más importantes de estas fuentes y en la Figura I.13 la aplicabilidad de estos modos de ionización.

Tabla I.3. Técnicas de ionización más comunes en el acoplamiento LC-MS [147]

Tipo	Principales características	Limitaciones
ESI	• Permite obtener iones cargados de moléculas de muy diferente peso molecular. • Funciona bien con ácidos y bases en disolución y con compuestos a los que se les pueda inducir carga. • Puede utilizar temperaturas elevadas para evaporar gran cantidad de fase móvil (permite análisis de moléculas lábiles). • Se pueden obtener espectros de masas de iones positivos y negativos. • Es una ionización muy suave. Genera pocos fragmentos por lo que permite estudiar moléculas sin fragmentar.	• Suelen formarse aductos con los tampones utilizados en la fase móvil de LC. • La sensibilidad es muy dependiente del flujo de fase móvil de HPLC y del pH de ésta.
APCI	• Es una ionización química mediante reacciones ion-molécula a presión atmosférica. • Permite análisis de moléculas con bajo peso molecular no volátiles. • Su sensibilidad no depende del pH ni del flujo de fase móvil. • Más sensible que ESI. • Permite obtener espectros de masas de iones positivos y negativos.	• Es una ionización suave. Genera pocos fragmentos. • Mayor facilidad de que se descompongan moléculas lábiles.

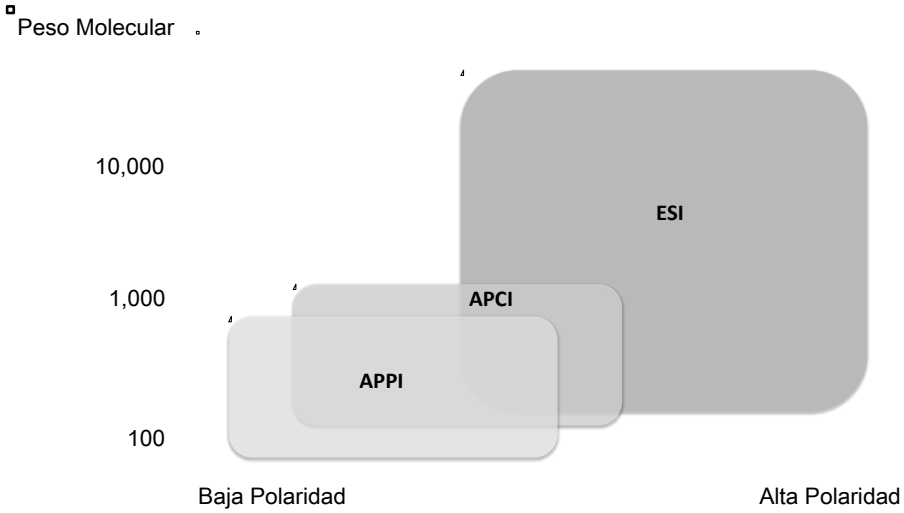

Figura I.13. Relación entre polaridad,peso molecular y técnicas de ionización (ESI, APCI y APPI) (Figura adaptada de http://notijenck.com.ar/?p=775)

3. Un analizador o filtro de masas que separa y/o selecciona las moléculas ionizadas así como los fragmentos iónicos generados en función de su relación *m/z*.

4. Un detector que recoge y caracteriza los fragmentos iónicos que salen del analizador. Los iones procedentes del analizador llegan al detector el cual generalmente está constituido por un cátodo emisor que, al recibir el impacto producido por las partículas cargadas, emite electrones. Estos electrones son acelerados hacia un dínodo el cual emite varios electrones más al recibir el impacto de cada electrón. Este proceso se repite hasta obtenerse una cascada de electrones que llega al colector lográndose una corriente amplificada, por un procedimiento similar al que se utiliza en los tubos fotomultiplicadores, que se lleva a un sistema registrador.

En general, los distintos tipos de espectrómetros de masas se clasifican en función del analizador que posean: cuadrupolo simple (Q), QqQ, IT, Orbitrap, resonancia de ion ciclotrón (*Ion Cyclotron Resonance,* ICR), sector magnético y tiempo de vuelo (*Time of flight*, TOF). Cada analizador utiliza una tecnología diferente, lo cual le confiere unas características que lo hacen diferir en cuanto a sus aplicaciones. Se caracterizan por el

rango de masas que pueden medir, resolución de masa (que puede ser de una unidad, como es el caso de los cuadrupolos, en torno a 15000 para TOF o de hasta 100000 en el caso del Orbitrap), exactitud de masa (la cual varía enormemente de un instrumento a otro siendo los equipos TOF y Orbitrap los que mayor exactitud pueden proporcionar llegando hasta valores inferiores a las 5 ppm), rango dinámico lineal, velocidad de barrido y sensibilidad. En la Tabla I.4 se puede ver un resumen de estas características [148]. Además, en los últimos años se han comercializado espectrómetros de masas que combinan algunos de los analizadores nombrados, como es el caso de los espectrómetros de tipo Q-TOF, donde se combina el analizador de cuadrupolo con el de TOF, o el Q-Trap, el cual combina el cuadrupolo con la IT.

Tabla I.4. Especificaciones de espectrómetros de masas usados en LC–MS [148]

Tipo de MS[a]	Resolución [×10³]	Exactitud de masa (ppm)	Rango de *m/z* (hasta) [×10³]	Velocidad de adquisición (Hz)	Rango dinámico lineal
Cuadrupolo	3–5	Baja[b]	2–3	2–10	10^5–10^6
IT	4–20	Baja	4–6	2–10	10^4–10^5
TOF	10–60	1–5	10–20	10–50	10^4–10^5
Orbitrap	100–240	1–3	4	1–5	5×10^3
ICR	750–2500	0.3–1	4–10	0.5–2	10^4

a) TOF, Orbitrap e ICR también incluyen configuraciones híbridas con cuadrupolos o IT lineales.

b) Los cuadrupolos hiperbólicos proporcionan exactitud de masas inferior a las 5 ppm.

En esta Tesis Doctoral se han propuesto métodos de análisis basados en LC-MS utilizando tres modalidades de MS: un analizador QqQ para la determinación de micotoxinas en diferentes muestras de alimentos, y una IT y un analizador Orbitrap para los estudios de metabolómica. A continuación se comentan brevemente las características principales de estos analizadores.

❖ Analizador de triple cuadrupolo:

En el analizador QqQ, se emplean campos eléctricos para separar los iones de acuerdo con su relación *m/z* cuando pasan a través del espacio central entre 4 barras

[148] M. Holcapek, R. Jirásko, M. Lísa, J. Chromatogr. A 1259 (2012) 3.

longitudinales (o polos) dispuestas en torno a un eje central y equidistantes sobre las que se aplica una corriente continua y un voltaje de radiofrecuencia que permite que sólo las moléculas con una relación *m/z* determinada atraviesen el espacio cuadrupolar hasta llegar al detector. Todo el sistema se encuentra sometido a alto vacío para evitar colisiones entre los fragmentos iónicos generados o con otras moléculas, que podrían dar lugar a la fragmentación de la molécula o los fragmentos ya generados, algo que es totalmente indeseable.

En MS/MS con analizadores QqQ se acoplan dos cuadrupolos y entre ellos se coloca una celda de colisión (Figura I.14). En esta celda se puede producir, si se desea, la fragmentación de los iones conducidos a través del primer cuadrupolo por colisión del ion seleccionado con moléculas de un gas inerte, y tanto las no fragmentadas como los fragmentos producidos se pueden conducir a través del segundo cuadrupolo hasta el detector.

Así, trabajando en modo MRM, se pueden medir los fragmentos generados en la celda de colisión. La adquisición en esta modalidad de MS/MS ofrece así una gran selectividad, ya que ofrece la posibilidad de aislar un ion en la celda de colisión, eliminando otros iones o fragmentos que puedan interferir. Asimismo, gracias a la selectividad, se mejora mucho la sensibilidad ya que se puede tener una mejor relación señal/ruido (S/N). Por todo ello, la información proporcionada por los sistemas de MS/MS aumenta la selectividad y la información generada para la elucidación estructural.

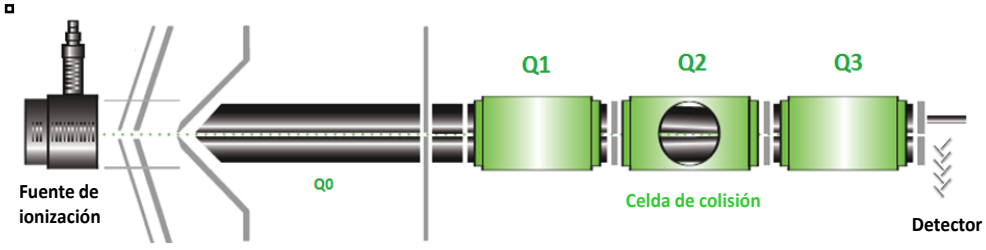

Figura I.14. Esquema de un espectrómetro de masas de triple cuadrupolo. (Figura adaptada de http://www.absciex.com/, AB Sciex)

La HPLC acoplada con el QqQ ha sido ampliamente empleada en la determinación de micotoxinas [62,103,149,150], gracias a que es un instrumento con una excelente sensibilidad y permite llevar a cabo la detección y cuantificación con muy buena exactitud. Por todo ello es muy utilizado como herramienta de confirmación.

No obstante, cuando esta técnica se emplea en la determinación multirresiduo de un gran número de analitos, presenta algunas limitaciones:

- el tiempo que requiere es elevado puesto que hay que caracterizar cada analito (tanto iones precursores como iones fragmento) individualmente y hay que construir ventanas de tiempo para la monitorización de cada analito, ya que las medidas en *full scan* (modo de barrido continuo) no permiten medir tan elevado numero de compuestos al mismo tiempo;

- cuando el número de analitos estudiados es muy elevado el *dweell time* (periodo de tiempo que el instrumento dedica a la medida de cada ion) ha de ser menor y esto dificulta el análisis ya que se reduce el tiempo dedicado por el detector a la medida de cada analito;

- las ventanas de tiempo para la adquisición de cada analito tienen que ser actualizadas cada cierto tiempo ya que los tiempos de retención pueden variar incluso dentro de la misma serie de análisis;

- la resolución alcanzada con equipos de cuadrupolo es de tan sólo la unidad, lo cual no es suficiente en muchos casos en los que la matriz es compleja. Además cuanto más compleja es la matriz estudiada, mayor es la posibilidad de encontrar compuestos isobaros que dificultan la identificación.

[149] C. D. Liao, J. W. Wong, K. Zhang, D. G. Hayward, N. S. Lee, M. W. Trucksess, J. Agr. Food Chem. 61 (2013) 4771.

[150] E. Beltrán, M. Ibáñez, T. Portolés, C. Ripollés, J. V. Sancho, V. Yusà, S. Marín, F. Hernández, Anal. Chim. Acta 783 (2013) 39.

❖ **Trampa de iones (IT):**

El analizador IT (empleado en esta Tesis) consiste fundamentalmente en un electrodo anular y dos electrodos laterales de geometría hiperbólica, que poseen una perforación que permite la entrada y la salida de los iones. Cuando los iones están dentro, se aplican diferentes voltajes generando un campo eléctrico tridimensional en la cavidad de la trampa. Este campo atrapa y concentra los iones dada su trayectoria de oscilación estable. La naturaleza de la trayectoria depende del potencial y de la relación *m/z* de los iones. Para llevar a cabo la determinación de las especies que entran o se forman en la trampa, los potenciales de los electrodos se alteran, sometiendo a los iones confinados a una rampa lineal de radiofrecuencia, de manera que son expulsados progresivamente en la dirección axial en función de su relación *m/z*, como resultado de desestabilizaciones de las trayectorias que mantienen dentro de la trampa. Una vez que estos iones llegan al detector, la señal se procesa y da lugar al espectro de masas.

En la Figura I.15 puede verse el esquema del equipo empleado para llevar a cabo parte del desarrollo experimental de la presente memoria, con sus distintas partes. El equipo está constituido por la interfase, la zona de "transporte y convergencia" de iones formada por *skimmers*, cuadrupolos, octopolo y lentes, el analizador (IT) y el detector.

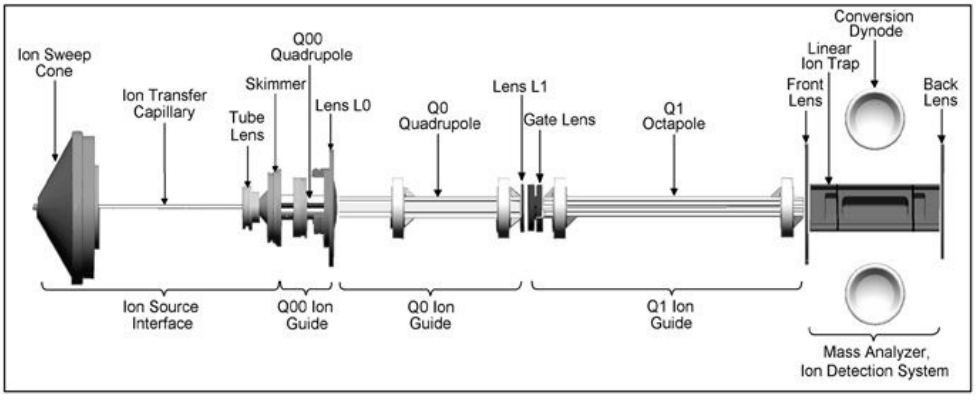

Figura I.15. Esquema de un espectrómetro de masas IT con interfase ESI. (Figura adaptada de Thermo Fisher)

En la primera zona se forma el espray (interfase). La zona de transporte y focalización de iones posee cuatro zonas a alto vacío, originado por una serie de bombas que posee el sistema. Los iones pasan a esta zona a través de un capilar: el *skimmer* elimina el volumen del gas de secado; a continuación pasan por los cuadrupolos y octopolo que los transportan y guían desde justo detrás del *skimmer* hasta el detector, atravesando una serie de lentes. Por último los iones entran en el analizador de IT, que los colecta y libera en función de su relación *m/z*. Después del analizador, los iones pasan a la zona de detección, que tiene también una serie de lentes que dirigen los iones hasta el propio detector, y un dínodo, que permite cambiar el modo de detección de positivo a negativo.

Cuando los iones se encuentran dentro de la trampa puede llevarse a cabo tanto el análisis de sus masas (espectro de MS) como el aislamiento de uno o varios iones precursores y su posterior fragmentación (dando lugar a lo que se denomina espectros de MS/MS). Otra característica de estos analizadores es que permiten obtener espectros de masas de iones productos (MS^n) lo que tiene mucho interés en la elucidación estructural, y ha sido la aplicación en esta Tesis Doctoral.

La IT se ha utilizado para la determinación de micotoxinas [62,151], así como para obtener información estructural de ciertos metabolitos y/o micotoxinas [152-156].

❖ **Orbitrap:**

Estas limitaciones que se acaban de comentar y que surgen cuando se quiere estudiar un gran número de compuestos simultáneamente, se pueden solventar, en parte, a través del empleo de técnicas de espectrometría de masas de alta resolución (*High-Resolution Mass Spectrometry*, HRMS) trabajando en modo *full scan* [157,158]. La

[151] M. Suman, E. Bergamini, D. Catellani, A. Manzitti, Food Chem. 136 (32013)1568.
[152] U. Berger, M. Oehme, F. Kuhn, J. Agr. Food Chem. 47 (1999) 4240.
[153] S. Uhlig, D. Petersen, Toxicon 52 (2008) 175.
[154] R. Mohamed, E. Gremaud, J. C. Tabet, P. A. Guy. Rapid Commun. Mass Spectrom. 20 (2006) 2787.
[155] A. F. Lehner, M. Craig, N. Fannin, L. Bush, T. Tobin, J. Mass Spectrom. 39 (2004) 1275.
[156] A. F. Lehner, J. M. Duringer, C. T. Estill, T. Tobin, A. M. Craig, Toxicol. Mech. Method. 21 (2011) 606.
[157] J. Wang, D. Leung, J. Agric. Food Chem. 57 (2009) 2162.
[158] M. Taylor, G. Keenan, K. Reid, D. Fernández, Rapid Commun. Mass Sp. 22 (2008) 2731.

HRMS aporta algunas ventajas importantes como son la precisión de masas, la posibilidad de hacer medidas de la relación isotópica de las moléculas, no siendo necesaria la optimización de los parámetros instrumentales para cada analito, además de proporcionar información de cualquier compuesto ya que realiza análisis de desconocidos (*untargeted*), siendo posible estudiar compuestos de los que no se dispone de patrones. Asimismo, maximiza el número de compuestos que pueden determinarse en cada análisis, lo cual puede suponer un ahorro económico y de tiempo muy importante.

Uno de estos analizadores de alta resolución es el Orbitrap. Este es un tipo de IT ideado por Alexander Makarov, que logra mejorar ampliamente algunas de las características de este tipo de analizadores. Así, aunque los espectrómetros de masas de tipo IT son ampliamente utilizados, suelen tener algunas desventajas como su baja exactitud de masas, un rango dinámico lineal no muy grande o capacidad de carga reducida.

El Orbitrap (Figura I.16) consiste fundamentalmente en una serie de multipolos, similares a los cuadrupolos descritos para el sistema QqQ, que son utilizados para conducir los iones producidos en una fuente de ionización previa, hasta un último cuadrupolo que actúa como trampa de iones (conocido como C-Trap por su forma curvada y no lineal como es habitual), desde donde pueden ser transferidos directamente al analizador o a una celda de colisión para producir su fragmentación. Esta celda de colisión es conocida como HCD (*higher energy collision dissociation*) y consiste en un multipolo conectado con la C-Trap. Este analizador está formado por un electrodo exterior con forma de barril y un electrodo interior coaxial con forma de huso que forma un campo electrostático. Ambos electrodos están conectados a fuentes de voltaje independientes. El electrodo exterior se divide en dos partes, una de ellas se utiliza para la excitación de los iones y otra para su detección. El espacio libre entre el electrodo interno y el externo forma la cámara de medida. Esta cámara se encuentra conectada a una bomba de vacío que permite alcanzar presiones de vacío de aproximadamente de 10^{-8} torr e incluso menores. En el analizador, los iones son inyectados tangencialmente entre los electrodos y quedan atrapados girando alrededor del electrodo central. Al mismo tiempo los iones también oscilan a lo largo del eje del

electrodo central. Así, los iones con una relación *m/z* específica se mueven en anillos que oscilan alrededor del electrodo central; la frecuencia de estas oscilaciones armónicas es independiente de la velocidad del ion y es inversamente proporcional a la raíz cuadrada de la relación *m/z*. La frecuencia de las oscilaciones axiales de cada molécula girando alrededor del electrodo central induce una corriente en los electrodos externos. En realidad pueden ser millones de iones los que originen una corriente en los electrodos y sus frecuencias son determinadas usando la Transformada de Fourier.

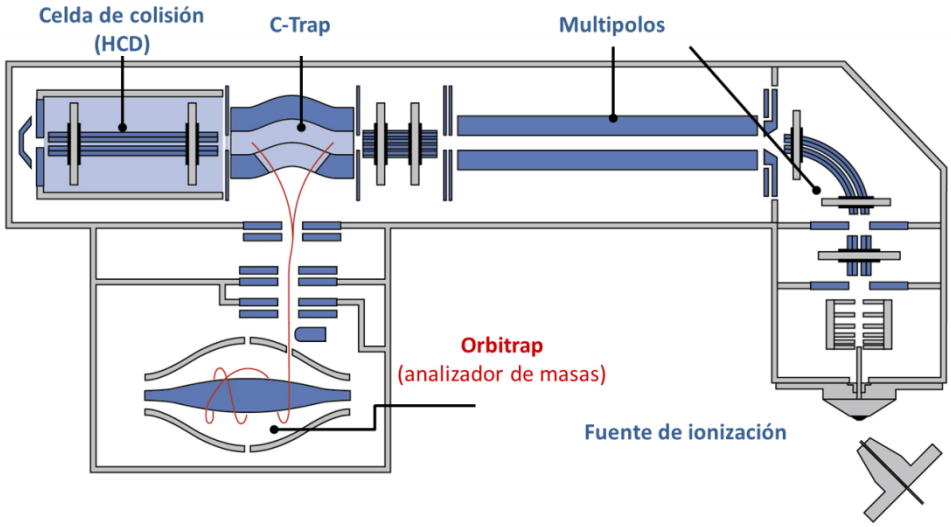

Figura I.16. Esquema del analizador de masas Orbitrap (Figura adaptada de http://planetorbitrap.com/q-exactive#tab:schematic, Thermo Fisher)

Aunque el uso del Orbitrap está menos extendido que el resto de espectrómetros de masas debido a su más reciente comercialización y mayor coste, el número de trabajos publicados en los que se utiliza este tipo de analizador ha crecido rápidamente en los últimos años. Así, se ha empleado para la determinación multiclase de micotoxinas [159-163] y su determinación junto a otros compuestos de interés

[159] B. Škrbić, S. Koprivica, M. Godula, Food Control 31 (2013) 461.

[160] L. Vaclavik, M. Vaclavikova, T. H. Begley, A. J. Krynitsky, J. I. Rader, J. Agr. Food Chem. 61 (2013) 4822.

[161] M. Zachariasova, T. Cajka, M. Godula, A. Malachova, Z. Veprikova, J. Hajslova, Rapid Commun. Mass Sp. 24 (2010) 3357

[145]. También se ha propuesto para la identificación de metabolitos de hongos en alimentos con moho y muestras de pienso [164].

I.5. Extracción y purificación de micotoxinas

Las micotoxinas en los alimentos presentan una distribución poco uniforme por lo que se requiere una cuidadosa homogeneización de la muestra previa a la extracción de los residuos, que se suelen encontrar en concentraciones muy bajas. Por otra parte, la complejidad de los alimentos, donde se encuentran presentes cantidades importantes de proteínas, lípidos, hidratos de carbono, agua y otros componentes minoritarios, requiere generalmente una purificación para eliminar las sustancias interferentes, antes de proceder a la medida analítica.

Las técnicas de extracción y limpieza que han sido empleadas para la determinación de micotoxinas, junto con algunas referencias de sus aplicaciones en alimentos, son las siguientes [165]:

- Extracción en fase sólida:
  - Extracción en fase sólida convencional (*Solid Phase Extraction*, SPE) [103,166].
  - Extracción con columnas Mycosep® [167-169].
  - Extracción con columnas de intercambio iónico (*Ion Exchange Column*) [170,171].

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[162] S. M. Lehner, N. K. N. Neumann, M. Sulyok, M. Lemmens, R. Krska, R. Schuhmacher, Food Addit. Contam. 28 (2011) 1457.

[163] V. M. T. Lattanzio, S. D. Gatta, M. Godula, A. Visconti, Food Addit. Contam. 28 (2011) 1424.

[164] K. F. Nielsen, J. M. Mogensen, M. Johansen, T. O. Larsen, J. C. Frisvad, Anal. Bioanal. Chem. 395 (2009) 1225.

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[166] J. Rubert, Z. Dzuman, M. Vaclavikova, M. Zachariasova, C. Soler, J. Hajslova Talanta 99 (2012) 712.

[167] L. Monaci, E. De Angelis, A. Visconti, J. Chromatogr. A 1218 (2011) 8646.

[168] J. Barthel, C. Gottschalk, M. Rapp, M. Berger, J. Bauer, K. Meyer, Mycotoxin Research 28 (2012) 97.

[169] T. Varzakas, V. Demopoulos, E. Manolopoulou, Acta Horticulturae 963 (2013) 119.

[170] J. M. Mogensen, T. O. Larsen, K. F. Nielsen, J. Agr. Food Chem. 58 (2010) 4853.

- Extracción con columnas de inmunoafinidad (*InmunoAffinity Column*, IAC) [172-174].
- Microextracción en fase sólida (*Solid Phase Micro-Extraction*, SPME) [175].
- Dispersión de matriz en fase sólida (*Matrix Solid Phase Dispersion*, MSPD) [104,166,176,177].

❖ Extracción por fluidos supercríticos (*Supercritical Fluid Extraction*, SFE) [178,179].

❖ Extracción asistida por microondas (*Microwave Assisted Extraction*, MAE) [180].

❖ Extracción líquida presurizada (*Pressurized liquid extraction, PLE*) [181,182].

El empleo de estas técnicas para la extracción de micotoxinas en alimentos se recoge en varios artículo de revisión de reciente publicación [62,65,67,68,70,183,184]. Un resumen de sus principales ventajas e inconvenientes se muestra en la Tabla I.5.

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[171] K. W. Von Bargen, L. Lohrey, B. Cramer, H.-U. Humpf, J. Agr. Food Chem. 60 (2013) 3586.
[172] R. Wei, F. Qiu, W. Kong, J. Wei, M. Yang, Z. Luo, J. Qin, X. Ma, Food Control 32 (2013) 216.
[173] A. Ennouari, V. Sanchis,S. Marín, M. Rahouti, A. Zinedine, Food Control 32 (2013) 115-118.
[174] J. Rubert, J. M. Soriano, J. Mañes, C. Soler, Food Chem. Toxicology 56 (2013) 387.
[175] E. F. Moazami, S. Jinap, Food Addit. Contam. 26 (2009) 1290.
[176] A. B. Serrano, G. Font, M. J. Ruiz, E. Ferrer, Food Chemistry 135 (2012) 423.
[177] J. Blesa, J. M. Soriano, J. C. Moltó, R. Marin, J. Mañes, Food Addit. Contam. 21 (2003) 165.
[178] A. S. Arribas, E. Bermejo, A. Zapardiel, H. Téllez, J. Rodríguez-Flores, M. Zougagh, A. Ríos, M. Chicharro, Electrophoresis 30 (2009) 499.
[179] M. Zougagh, H. Téllez, A. Sánchez, M. Chicharro, A. Ríos, Anal. Bioanal. Chem. 391 (2008) 709.
[180] L. Pallaroni, C. Von Holst, Anal. Bional. Chem. 376 (2003) 908.
[181] A. Desmarchelier, J. M. Oberson, P. Tella, E. Gremaud, W. Seefelder, P. Mottier, J. Agr. Food Chem. 58 (2010) 7510.
[182] L. González, C. Juan, J. M. Soriano, J. C. Moltó, J. Mañes, Int. J. Food Microbiol. 107 (2006) 223.
[183] A. L. Capriotti, G. Caruso, C. Cavaliere, P. Foglia, R. Samperi, A. Lagana, Mass Spectrom. Rev. 31 (2012) 466.
[184] A. Veršilovskis, S. de Saeger, Mol. Nutr. Food Res. 54 (2010) 136.

Tabla I.5. Ventajas e inconvenientes de algunas técnicas de extracción y purificación

Extracción	Ventajas	Inconvenientes
Extracción en fase sólida	• Ideal para muestras líquidas • Menor gasto de disolvente	• A veces extractos sucios • Poco selectivo
Extracción en columnas de inmunoafinidad	• Ideal para muestras líquidas • Menor gasto de disolvente • Extractos limpios • Elevada selectividad	• Elevado coste • No reutilizables
Extracción con fluidos supercríticos	• Rápido (30-60 min) • Se puede conseguir alta selectividad modificando algunos parámetros • Baja cantidad de disolventes (5-10 ml) • No es necesaria la filtración posterior del extracto	• Tamaño de muestra limitado (<10 g) • Necesidad de modificadores para mejorar la eficiencia de la extracción • Coste elevado del equipo
Extracción con microondas	• Rápido (15 min) • Bajo consumo de disolventes (15-40 ml) • Sencillo	• El extracto obtenido debe ser filtrado • Es necesaria la adicción de un disolvente polar • Es necesaria la limpieza posterior del extracto • Coste moderado del equipo
Extracción liquida presurizada	• Rápido y de alta eficacia • Baja cantidad de disolventes (15-40 ml) • Control absoluto de los parámetros de extracción (temperatura y presión, etc.)	• Elevado coste del equipo • Dependiente del tipo de matriz • Extractos sucios que requieren de un proceso de limpieza

En esta Tesis se proponen tratamientos de muestra alternativos para mejorar la eficacia de los procedimientos de extracción de micotoxinas y limpieza de los extractos, considerando las tendencias actuales en cuanto a simplificación y miniaturización de los sistemas de tratamiento de muestra, introduciendo disolventes menos contaminantes y disminuyendo en lo posible las cantidades de disolventes orgánicos utilizados, en consonancia con los principios de la llamada Química Verde

[185,186,187], o Química beneficiosa para el medioambiente, que se ocupa del diseño de productos y procesos químicos que reducen o eliminan el uso y producción de sustancias peligrosas, implicando una mayor seguridad y un menor coste en relación a los procesos convencionales. Además, se propondrán procedimientos de extracción genérica multirresiduo que permitan su posible automatización y/o aumentar la rapidez de tratamiento.

A continuación se comentarán los aspectos más destacados de cada uno de los métodos de extracción y limpieza que se han explorado y utilizado durante la realización de esta Tesis.

I.5.1. Columnas de inmunoafinidad (IACs)

Las IACs [188] constituyen el método de extracción más común hoy en día en los laboratorios de rutina para la extracción y purificación de micotoxinas [189], siendo además el seleccionado por la mayoría de los métodos recomendados, incluido el de la Farmacopea para la determinación de AFB_1 [53].

En esta técnica se inmoviliza en el relleno de la columna un anticuerpo específico frente al analito que se quiere determinar. La introducción de la muestra en la columna da lugar a la captura selectiva del analito gracias a la especificidad del reconocimiento antígeno-anticuerpo, mientras que el resto de los componentes de la muestra eluyen sin retenerse en la columna.

Las micotoxinas tienen, por lo general, un tamaño molecular bajo, comportándose como haptenos. Los anticuerpos producidos (monoclonales) requieren una unión a

[185] M. Farré, S. Pérez, C. Gonçalves, M.F. Alpendurada, D. Barceló, TrAC-Trends Anal. Chem. 29 (2010) 1347.

[186] M. de la Guardía, S. Garrigues, Challenges in Green Analytical Chemistry, RSC Publishing, Cambridge, 1ª Ed., 2011.

[187] D. L. Rocha, A. D. Batista, F. R. P. Rocha, G. L. Donati, J. A. Nóbrega, TrAC - Trends Anal. Chem. 45 (2013) 79.

[188] H. Z. Senyuva, J. Gilbert, J. Chromatogr. B 878 (2010) 115.

[189] P. M. Scott, M. W. Trucksess, J. AOAC Int. 80 (1997) 941.

distintos transportadores tales como la agarosa, sefarosa o dextrano (soporte), para fijarlo en la fase estacionaria (Figura I.17).

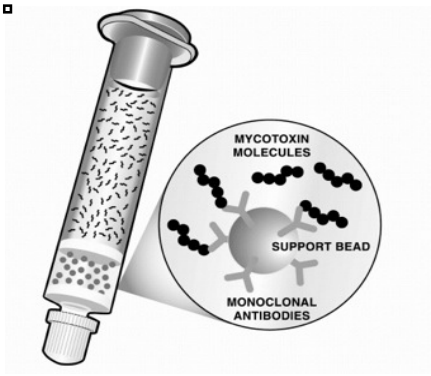

Figura I.17. Columna de inmunoafinidad

En la Figura I.18 se muestra el procedimiento de extracción mediante IACs. La micotoxina problema se unirá a los anticuerpos monoclonales fijados en la IAC y mediante un líquido de lavado podrán eliminarse los restos de extracto. Por último, la elución de la micotoxina permitirá obtener el analito aislado para su determinación.

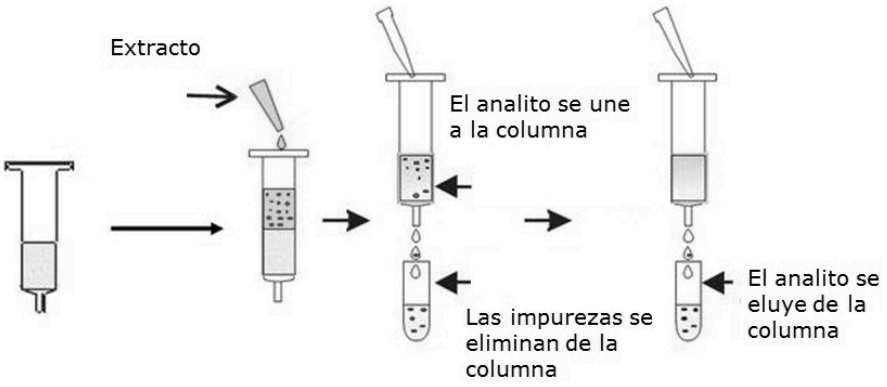

Figura I.18. Procedimiento de extracción con IAC

Actualmente se comercializan IACs para aflatoxinas del grupo B, G y M, OTA, deoxinivalenol, fumonisinas, toxina T-2 y zearalenona. También se han desarrollado IACs que permiten la extracción y la purificación simultánea de varias micotoxinas,

como la OchraAflaprep para aflatoxinas y ocratoxina A. Sin embargo, estas columnas presentan el inconveniente de su elevado coste, lentitud y, en ocasiones, bajas recuperaciones, además de su limitado uso en determinaciones multirresiduo.

I.5.2. Extracción en Fase Sólida Dispersiva (dSPE) - QuEChERS

La SPE dispersiva (dSPE) es un método rápido y sencillo de limpieza de muestras, aplicado fundamentalmente en la determinación multirresiduo de plaguicidas en frutas y vegetales, pero que muestra interesantes perspectivas en otras matrices y compuestos.

Basándose en este tipo de extracción, Anastassiades y col. [190] desarrollaron el procedimiento comúnmente conocido como QuEChERS – *Quick* (rápido), *Easy* (fácil), *Cheap* (barato), *Effective* (eficaz), *Rugged* (robusto), *Safe* (seguro) – (Figura I.19) que implica un primer paso en el que se lleva cabo una extracción simple con acetonitrilo (AcN), seguida de un fenómeno de partición líquido-líquido por adición de $MgSO_4$ anhidro y NaCl.

Figura I.19. Esquema del método QuEChERS

El sulfato magnésico en concentraciones superiores a las de saturación, ayuda a mejorar las recuperaciones en el proceso de extracción, al favorecer el reparto de los

[190] M. Anastassiades, S. J. Lehotay, D. Stajbaher, F. J. Schenck, J. AOAC Int. 86 (2003) 412.

analitos en la capa orgánica. El NaCl ayuda a controlar la polaridad, aunque demasiada sal podría impedir el reparto. Además, el acetato sódico y otras sales actúan controlando el pH.

El segundo paso de esta metodología es el basado en la dSPE propiamente dicha e implica la eliminación de agua residual y limpieza (*clean-up*) mediante la adición de una mezcla basada en $MgSO_4$ (con objeto de eliminar el agua) y un sorbente, generalmente C_{18}, una amina primaria y secundaria (PSA) o carbono grafitizado, que proporciona una elevada capacidad de limpieza de los componentes de la matriz (azúcares, ácidos grasos, ácidos orgánicos, pigmentos, etc.). Después de este proceso de limpieza, se lleva a cabo una centrifugación y el extracto está listo para ser directamente analizado o sometido a evaporación y recomposición en el disolvente apropiado para su análisis.

El método QuEChERS se encuentra actualmente comercializado por diversas empresas [191,192], siendo posible encontrar diferentes kits de extracción y limpieza en función del tipo de muestra, y ha sido adoptado como método normalizado europeo para la determinación de plaguicidas [193].

Esta técnica ha sido empleada para la extracción de micotoxinas en diferentes matrices, como por ejemplo, en cereales [26,166,181,194-196], huevos [140], y durante la realización de esta Tesis además se ha aplicado en vinos [131], plantas medicinales [197], frutos secos [198], cereales y pseudocereales y melazas de cereal, obteniendo muy buenos resultados. Ésta metodología también ha sido utilizada en

[191] http://www.chem.agilent.com/en-US/Products/consumables/samplepreparation/

[192] http://www.sigmaaldrich.com/analytical-chromatography/sample-preparation/spe/dispersive-spe-tubes.html

[193] Norm EN 15662:2008. "Foods of plant origin - Determination of pesticide residues using GC-MS and/or LC-MS/MS following acetonitrile extraction/partitioning and clean-up by dispersive SPE - QuEChERS-method", 01-02-2009.

[194] S. C. Cunha, J. O. Fernandes, J. Sep. Sci. 33 (2010) 600.

[195] I. Sospedra, J. Blesa, J. M. Soriano, J. Mañes, J. Chromatogr. A 1217 (2010) 1437.

[196] L. Vaclavik, M. Zachariasova, V. Hrbek, J. Hajslova, Talanta 82 (2010) 1950.

[197] N. Arroyo-Manzanares, A. M. García-Campaña, L. Gámiz-Gracia, J. Chromatogr. A 1282 (2013) 11.

[198] N. Arroyo-Manzanares, J. F. Huertas-Pérez, L. Gámiz-Gracia, A. M. García-Campaña, Talanta 115 (2013) 61.

métodos multirresiduo para la determinación de distintas familias de contaminantes, entre las que se incluyen micotoxinas [145,199-201].

I.5.3. Microextracción Líquido-Líquido Dispersiva (DLLME)

Tal y como se ha comentado anteriormente y siguiendo la tendencia actual de disminuir el consumo de disolventes, recientemente se han propuesto diversas técnicas de extracción miniaturizadas como alternativas a los procedimientos de preparación de muestra más convencionales. Entre ellas se encuentra la microextracción líquido-líquido (LLME) [202], una técnica simple y económica en la que se requieren sólo unos microlitros de disolvente para concentrar a los analitos a partir de las muestras, en lugar de los elevados volúmenes requeridos en la extracción líquido-líquido convencional, y que es compatible con GC, HPLC y CE.

Una de las modalidades de este tipo de extracción es la microextracción líquido-líquido dispersiva (*dispersive liquid-liquid microextraction*, DLLME), introducida en 2006 por Assadi *et al*. [203]. Esta técnica está basada en la rápida inyección de una mezcla, en proporciones adecuadas, de unos pocos microlitros de disolvente extractante y un disolvente dispersivo en el seno de una muestra acuosa que contiene los analitos de interés. Esta rápida inyección produce una gran turbulencia, originándose la formación de finas gotas de extractante que se dispersan a través de la muestra acuosa. Tras centrifugación, las gotas se depositan en el fondo de un tubo cónico, siendo posible el análisis de esta fase para la determinación de los analitos de interés (Figura I.20).

Las ventajas de esta técnica radican en su simplicidad de operación, rapidez, bajo coste, altas recuperaciones, elevados factores de enriquecimiento y alta compatibilidad

[199] A. L. Capriotti, C. Cavaliere, S. Piovesana, R. Samperi, A. Laganà, J. Chromatogr. A 1268 (2012) 84.
[200] O. Lacina, M. Zachariasova, J. Urbanova, M. Vaclavikova, T. Cajka, J. Hajslova, J. Chromatogr. A 1262 (2012) 8.
[201] J. M. Zhang, Y. L. Wu, Y. B. Lu, J. Chromatogr. B 915-916 (2013) 3.
[202] A. Sarafraz-Yazdi, A. Amiri, TrAC-Trends Anal. Chem. 29 (2010) 1.
[203] M. Rezaee, Y. Assadi, M.R. Milani Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, J. Chromatogr. A 1116 (2006) 1.

con el medioambiente, debido al reducido volumen de disolventes orgánicos requerido. Sus aplicaciones han sido recogidas en diversos artículos de revisión [204-209].

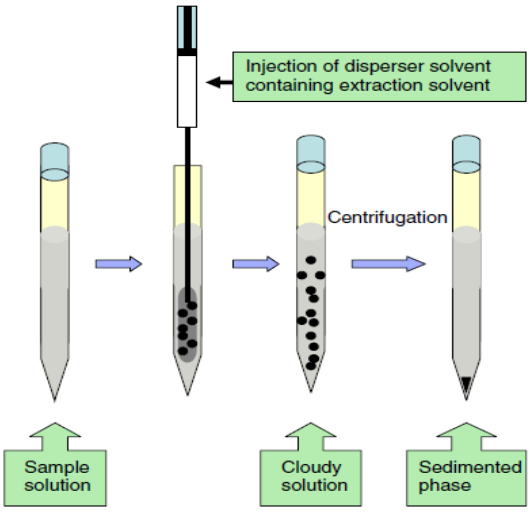

Figura I.20. Esquema del procedimiento de DLLME [207]

Hasta la fecha son pocas las aplicaciones de la DLLME para la determinación de micotoxinas en alimentos, concretamente se ha determinado OTA en vinos [210] y cereales [211], aflatoxinas en cereales [212], zearalenona en cereales [213], patulina en zumos [31], y las llevadas a cabo durante la realización de esta Tesis para la determinación de OTA en vinos [130], y varias micotoxinas en cardo mariano [197] y frutos secos [198].

[204] M. Rezaee, Y. Yamini, M. Faraji, J. Chromatogr. A 1217 (2010) 2342.
[205] A. V. Herrera-Herrera, M. Asensio-Ramosa, J. Hernández-Borges, M. A. Rodríguez-Delgado, TrAC-Trends Anal. Chem. 29 (2010) 728.
[206] X. H. Zang, Q. H. Wu, M. Y. Zhang, G. H. Xi, Z. Wang Z, Chinese J. Anal. Chem. 37 (2009)161.
[207] C. Bosch-Ojeda, F. Sánchez-Rojas, Chromatographia 69 (2009) 1149.
[208] A. Zgoła-Grzeskowiak, T. Grzeskowiak, TrAC-Trends Anal. Chem. 30 (2011) 1382.
[209] V. Andruch, I.S. Balogh, L. Kocú rová, J. Sandrejová, Appl. Spectrosc. Rev. 48 (2013)161.
[210] L. Anna, L. Piccinelli, L. Rastrelli, Anal. Bioanal. Chem. 399 (2010) 1279.
[211] L. Campone, A. L. Piccinelli, R. Celano, L. Rastrelli, Anal. Chim. Acta 754 (2012) 61.
[212] L. Campone, A. L. Piccinelli, R. Celano, L. Rastrelli, J. Chromatogr. A 1218 (2011) 7648.
[213] H. M. Antep, M. Merdivan, Anal. Meth. 4 (2012) 4129.

I.5.4. Microextracción Líquido-Líquido Dispersiva con Líquidos Iónicos (IL-DLLME)

Los líquidos iónicos (IL) son un grupo de compuestos orgánicos formados exclusivamente por un catión y un anión y que son líquidos a temperaturas moderadas, ya que su punto de fusión oscila entre -81 a 125 ºC. En la mayoría de los casos, los cationes tienen naturaleza aromática con átomos de nitrógeno en el anillo (imidazol, pirrolidina, piridina, etc.), mientras que los aniones suelen estar constituido por diferentes elementos químicos, normalmente conteniendo halógenos (Cl^-; Br^-, I^-, $[BF_4]^-$, $[AlCl_4]^-$, $[PF_6]^-$). Debido a la baja estabilidad y toxicidad de los cationes que contienen halógenos, se están desarrollando IL con cationes de bajo contenido en halógenos o incluso libres de éstos, como el bis(2-etilhexil)sulfosuccinato $[BEHSS]^-$, trifluroetanoato $[CF_3CO_2]^-$, acetato $[CH_3CO_2]^-$, etc. (Figura I.21).

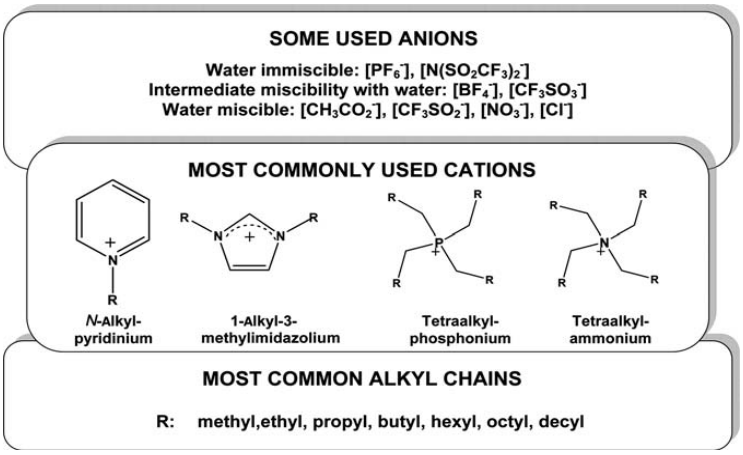

Figura I.21. Cationes y aniones comunes en IL [218]

Los IL presentan importantes características, como elevada estabilidad térmica y química, no inflamabilidad, amplio rango de viscosidad, conductividad electrolítica y capacidad de disolver compuestos orgánicos, inorgánicos e incluso materiales poliméricos, debido a su naturaleza iónica y a su composición orgánica. Cambiando la naturaleza del anión y del catión es posible modificar su miscibilidad en agua y en disolventes orgánicos y su viscosidad, siendo posible disponer de un "IL diseñado" para cada aplicación. Estas características hacen de los IL una excelente alternativa

como disolventes en los procesos de tratamientos de muestras y extracción [214,215,216] o en técnicas separativas [217,218], pudiendo también emplearse unidos a soportes de sílice en fibras para microextracción en fase sólida y fases estacionarias en HPLC, como aditivos a bajas concentraciones en CE, en la fase móvil en HPLC o como agentes complejantes para la detección de aniones por MS [219].

Los IL pueden remplazar los disolventes extractantes en DLLME (IL-DLLME), incorporándose directamente en el sistema cromatográfico con una simple dilución, obteniéndose así métodos rápidos y menos contaminantes que otros procesos de extracción convencionales. Un artículo de revisión reciente describe esta metodología [220].

Actualmente se encuentran escasas aplicaciones de la IL-DLLME en la determinación de contaminantes orgánicos en alimentos [221,222] y, por lo que sabemos, la única aplicación existente para micotoxinas ha sido la desarrollada en esta Tesis para la determinación de OTA en vinos [131].

I.6. Metabolómica en la investigación de micotoxinas

La metabolómica es la ciencia encargada del estudio sistemático del perfil metabolómico (metaboloma) de un proceso biológico. El término fue acuñado por primera vez a finales de los 90 [223,224] y desde entonces el número de publicaciones relacionadas con la metabolómica ha crecido exponencialmente [225].

[214] R. Liu, J. F. Liu, Y. G. Yin, X. L. Hu, G. B. Jiang, Anal Bioanal Chem 393 (2009) 871.
[215] L. Ruiz-Aceituno, M. L. Sanz, L. Ramos, TrAC - Trends Anal. Chem. 43 (2013) 121.
[216] J. Xu, J. Zheng, J. Tian, F. Zhu, F. Zeng, C. Su, G. Ouyang, TrAC-Trends Anal. Chem. 47 (2013) 68.
[217] A. Berthod, M. J. Ruiz-Ángel, S. Carda-Broch, J. Chromatogr. A 1184 (2008) 6.
[218] B. Buszewski, S. Studzinska, Chromatographia 68 (2008) 1.
[219] P. Sun, D. W. Armstrong, Anal. Chim. Acta 661 (2010) 1.
[220] D. Han, B. Tang, Y. Ri Lee, K. Ho Row, J. Sep. Sci. 35 (2012) 2949.
[221] L. M. Ravelo-Pérez, J. Hernández-Borges, M. Asensio-Ramos, M. A. Rodríguez-Delgado, J. Chromatogr. A 1216 (2009) 7336.
[222] L. M. Ravelo-Pérez, J. Hernández-Borges, A. V. Herrera-Herrera, M. A. Rodríguez-Delgado, Anal. Bioanal. Chem. 395 (2009) 2387.
[223] J. K. Nicholson, J. C. Lindon, E. Holmes, Xenobiotica 29 (1999) 1181.

El metaboloma es el conjunto dinámico constituido por el conjunto de metabolitos (moléculas de pequeño tamaño, <1500 Dalton), presentes en un biofluido, célula u organismo [226]. Los metabolitos son productos finales de los procesos regulatorios de las células, y sirven como indicadores directos de su actividad bioquímica [227], a diferencia de las proteínas o los genes, las cuales para ser activas necesitan modificaciones post-traduccionales o epigenéticas, respectivamente. Además, el número de metabolitos es inferior al de genes y proteínas, lo que reduce notablemente la complejidad del análisis.

La Figura I.22 muestra un diagrama esquemático detallando la complejidad de la función celular y las tecnologías -ómicas asociadas a cada proceso.

En el estudio del metaboloma se han utilizado dos estrategias fundamentales dependiendo de la información previa disponible acerca del problema biológico: aproximaciones dirigidas, cuando se conoce el número y tipo de metabolitos de interés, o aproximaciones no dirigidas, cuando no se tiene información previa de los metabolitos que puedan estar implicados en un proceso biológico/bioquímico en concreto.

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[224] O. Fiehn, J. Kopka, P. Dormann, T. Altmann, R. N. Trethewey, L. Willmitzer. Nat. Biotechnol. 18 (2000) 1157.

[225] A. Carrasco Pancorbo, M. Gómez Romero, Boletín GRASEQA 2 (2012) 22.

[226] J. C Lindon, J. K Nicholson, E Holmes. Handbook of Metabonomics and Metabolomics. Oxford. Elsevier, London, 2007.

[227] L. Botros, D. Sakkas, E. Seli. Mol. Hum. Reprod. 14 (2008) 679.

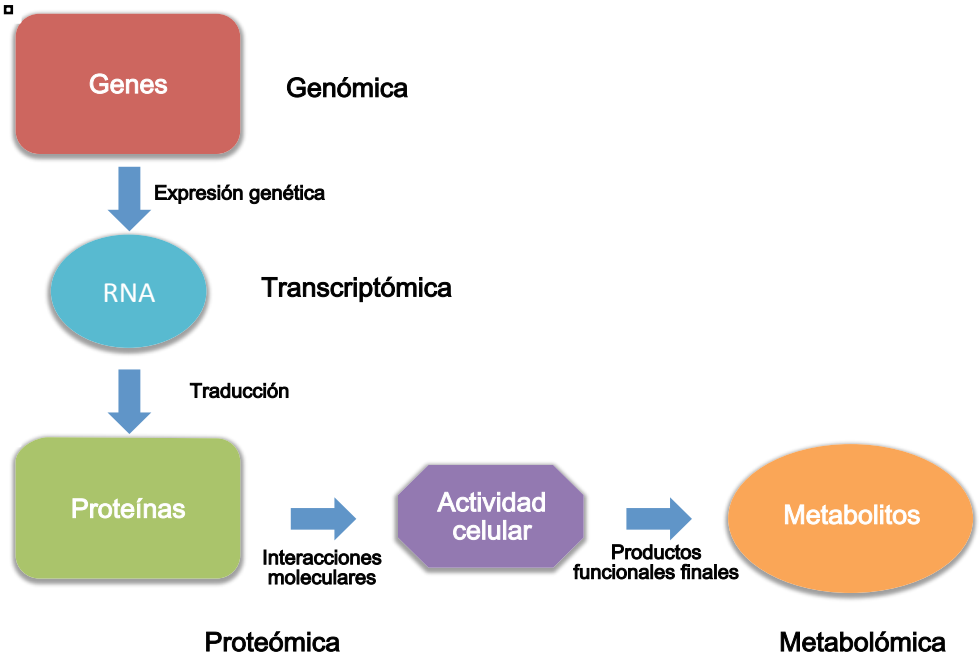

Figura I.22. Diagrama de la función celular, incluyendo algunas de las tecnologías -ómicas asociadas: genómica, transcriptómica, proteómica y metabolómica, que contribuyen al estudio y comprensión de los sistemas biológicos [228].

Para el análisis de metabolitos no existen metodologías globales. La identificación, detección y cuantificación de un número elevado de metabolitos presentes, a diferentes niveles de concentración, requieren la optimización exhaustiva de las condiciones de operación de los métodos analíticos. En las aproximaciones dirigidas será más fácil la elección del método analítico, ya que inicialmente se puede optimizar tanto el método de extracción como la técnica analítica a utilizar para su medida. Sin embargo, en las aproximaciones no dirigidas el objetivo es determinar simultáneamente el mayor número de metabolitos para obtener la máxima información posible; para ello es necesario el empleo de diferentes técnicas analíticas, aunque las más utilizadas para realizar estudios son LC-MS [229] y RMN [230]. En el caso de la LC-MS, serán necesarias herramientas informáticas y de tratamiento estadístico para

[228] M. G. Katz-Jaffe, S. McReynolds, D. K. Gardner, W. B. Schoolcraft. Mol. Hum. Reprod. 15 (2009) 271.
[229] M. Bedair, L.W. Sumner. TrAC-Trends Anal. Chem. 27 (2008) 238.
[230] D. S. Wishart. TrAC-Trends Anal. Chem. 27 (2008) 228.

manejar tal complejidad de datos, que permitan el alineamiento de los cromatogramas para detectar diferencias entre grupos, ya que es demasiado tediosa la inspección manual de todos los picos obtenidos. Para esta labor, en el desarrollo de esta Tesis se ha utilizado el programa SIEVE, sin embargo existen otros como MetAlign, MZMine o XCMS. Con la aplicación de esta técnica se han detectado muchos metabolitos no descritos anteriormente, lo que demuestra que el conocimiento del metabolismo celular es parcial y que aún quedan muchos metabolitos por identificar.

Para la identificación de metabolitos se procede de manera distinta en el caso de RMN o de LC-MS. En RMN, una vez obtenidos los puntos espectrales que difieren entre las distintas condiciones, se procede a asignar a qué metabolitos pertenecen dichas señales para su posterior identificación. En RMN se dispone de bases de datos comerciales así como una amplia bibliografía con numerosas reseñas de distintos metabolitos, por lo que el análisis resulta menos tedioso que mediante LC-MS. Para LC-MS será necesario conocer las masas y el tiempo de retención, así como el patrón de fragmentación. A continuación (y en el mejor de los casos), se compara dicho patrón de fragmentación con patrones disponibles en algunas bases de datos tales como HMDB, Metlin o MassBank entre otras. En el caso que no aparezca el patrón de fragmentación, se tiene que recurrir a métodos de elucidación estructural para identificar el metabolito [231].

Como se ha comentado anteriormente, son muchos los metabolitos que quedan por identificar, por lo que las aplicaciones metabolómicas serán muy importantes a la hora de identificar metabolitos secundarios producidos por hongos, y que en ciertos casos, dependiendo de su toxicidad, serán considerados micotoxinas. Basándonos en esto, en esta Tesis se ha desarrollado un método para la para la identificación de alcaloides ergóticos (alcaloides del cornezuelo de centeno) nuevos o poco explorados, usando espectrometría de masas de alta resolución y de múltiples etapas [232].

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[231] A. Beltran Carbó, O. Yanes Torrado, Boletín GRASEQA 2 (2012) 5.

[232] N. Arroyo-Manzanares, S. Malysheva, J. Vanden Bussche, L. Vanhaecke, J. D. Di Mavungu, S. De Saeger, Talanta (submitted for publication).

Otra de las grandes aplicaciones de la metabolómica en este campo es el estudio de la ruta biosintética de las micotoxinas. Para ello será necesario identificar y caracterizar inicialmente los genes implicados en la producción de micotoxinas.

La mayoría de los metabolitos secundarios producidos por hongos son sintetizados por pequeños complejos enzimáticos incluyendo terpeno sintasas (*ts*), policétido sintasas (*pks*), péptidos no ribosomales sintasas (*nrps*) e híbridos *pks-nrps* [233]. La mayoría de los productos de estas reacciones enzimáticas sufren modificaciones catalizadas por enzimas, como la oxigenación, ciclación e isomerización, para completar su respectiva ruta biosintética. Así, el primer intermedio en la biosíntesis de aflatoxinas, fumonisinas o zearalenona es un policétido, un terpeno en la biosíntesis de deoxinivalenol, y un híbrido polícetido-peptido en la biosíntesis de fusarina C [234].

Un gran número las rutas biosintéticas de los metabolitos secundarios producidos por hongos que han sido estudiados hasta la fecha, demuestran que tanto el gen que codifica la enzima de síntesis como la modificadora se encuentran en el mismo conjunto de genes (*cluster*).

En esta Tesis se ha estudiado el perfil metabolómico de uno de los *clusters* de *Aspergillus* flavus [235]. Utilizando técnicas bioinformáticas han sido identificados 55 clusters asociados con metabolitos secundarios, pero solamente 20 metabolitos han sido identificados, encontrándose que únicamente 6 de los 55 clusters están implicados en su producción, por lo que es necesario el estudio de los restantes.

Para el estudio del perfil metabolómico se han utilizado usualmente dos estrategias. En la primera aproximación se lleva a cabo la identificación de reacciones bioquímicas catalizadas por enzimas codificadas por genes de función desconocida. La segunda aproximación es el análisis funcional mediante co-respuesta, que usa los perfiles metabólicos de los genes de función conocida para elucidar las posibles funciones de

[233] N. P. Keller, G. Turner, J. W. Bennet, Nat. Rev. Microbiol. 3 (2005) 937.

[234] D. W. Brown, R. A. E. Butchko, R. H. Proctor en: S. De Saeger (ed.), Determining mycotoxins and mycotogenic fungi in food and feed, Woodhead Publishing, Limited, Cambrige, 2011, pp. 332.

[235] N. Arroyo-Manzanares, S. V. Malysheva, J. W. Cary, K. C. Ehrlich, J. Vanden Bussche, L. Vanhaecke, D. Bhatnagar, J. D. Di Mavungu, S. De Saeger (in the process of drafting).

genes de función desconocida. Esto se realiza por comparación de los perfiles metabólicos entre mutantes defectivos en el gen desconocido y mutante en un gen cuya función se conoce experimentalmente. Si el mutante en el gen desconocido presenta el mismo perfil metabólico que el mutante en el gen de función conocida, quiere decir que los productos de los genes actúan en la misma vía metabólica.

En el estudio del perfil metabolómico de un cierto *cluster* en un hongo, se pueden utilizar cepas mutantes, donde ese *cluster* es bloqueado, y comparar los metabolitos producidos con los obtenidos a partir de cepas silvestres del mismo hongo.

En concreto, en el trabajo realizado y descrito en esta Tesis, se han utilizado cepas de *Aspergillus flavus* mutantes (donde el *cluster* 27 fue bloqueado) y silvestres (Figura I.23), y se ha utilizado la espectrometría de masas para el estudio de los metabolitos secundarios producidos por ambas cepas, observando las diferencias y semejanzas entre los perfiles cromatográficos correspondientes a las dos cepas empleando UHPLC- HRMS (Figura I.24).

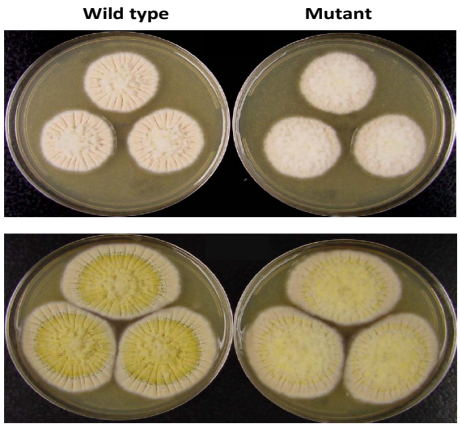

Figure 1.23. Diferencias fenotópicas entre cepa mutante y silvestre del hongo *Aspergillus flavus*

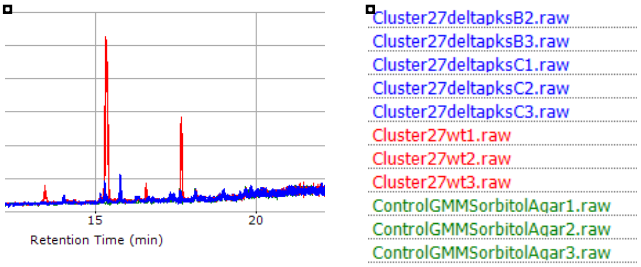

Figura I.24. Alineamiento del los cromatogramas usando el programa SIEVE

Para la identificación de los metabolitos, fue necesario el empleo de un espectrómetro de masas con analizador IT que permitió el estudio de la fragmentación MS^n de estos compuestos, junto con la masa exacta obtenida con HRMS.

CHAPTER 1

Preconcentración en línea para la determinación de aflatoxinas en arroz mediante cromatografía capilar electrocinética micelar con fluorescencia inducida por láser

On-line preconcentration for the determination of aflatoxins in rice samples by micellar electrokinetic capillary with laser induced fluorescence

Resumen

Se propone la cromatografía capilar electrocinética micelar con detección por fluorescencia inducida por láser para la determinación de cuatro aflatoxinas (aflatoxina B_1, aflatoxina B_2, aflatoxina G_1 y aflatoxina G_2) en muestras de arroz. La separación se llevó a cabo en un capilar de sílice fundida (70 cm × 75 µm I.D., 55 cm de longitud efectiva), usando un tampón borato 20 mM con 30 mM de dodecilsulfato sódico (pH 8.5) y 7% de acetonitrilo. Con el fin de aumentar la sensibilidad, se llevó a cabo una preconcentración en el propio capilar, basada en la metodología de barrido de analitos ("sweeping"), usando el mismo tampón de separación pero sin dodecilsulfato sódico como disolvente de la muestra. Gracias a la preconcentración en línea se obtuvieron límites de detección entre 0.04 y 0.52 µg L^{-1}. La precisión del método se evaluó en términos de repetitividad y precisión intermedia, obteniendo resultados aceptables, con desviaciones estándar relativas inferiores al 12%. El estudio de recuperación se llevó a cabo usando extractos de muestras de arroz dopadas con las cuatro aflatoxinas, obteniéndose recuperaciones entre 93.0-105.4%. Finalmente, el método se ha aplicado a la determinación de aflatoxinas en muestras de arroz empleando columnas de inmunoafinidad para la extracción, comparándose los resultados con los obtenidos tras aplicar un método normalizado para la determinación de aflatoxinas en cereales, basado en cromatografía líquida con detección fluorescente y derivatización previa, no observándose diferencias significativas en ninguna de las muestras analizadas.

Abstract

Micellar electrokinetic chromatography coupled with laser induced fluorescence detection has been used for the determination of four aflatoxins (aflatoxin B_1, aflatoxin B_2, aflatoxin G_1 and aflatoxin G_2) in rice samples. Separations were performed in an uncoated fused-silica capillary (70 cm×75 μm I.D., 55 cm effective length), using 20 mM borate buffer with 30 mM sodium dodecyl sulphate (pH 8.5) and 7% acetonitrile. In order to increase sensitivity, an on-line preconcentration procedure was applied, based on sweeping, using the same separation buffer without sodium dodecyl sulphate as solvent of the sample. With the on-line preconcentration, limits of detection were comprised between 0.04-0.52 μg L^{-1}. The precision of the method was evaluated in terms of repeatability and intermediate precision and the results were acceptable in all cases (relative standard deviations lower than 12%). Recovery studies were developed with extracts of rice samples spiked with aflatoxins, being in the range between 93.0-105.4%. The method has also been applied to the determination of aflatoxins in rice samples using immunoaffinity columns for the extraction, and the results were compared to those obtained by a standard method for the determination of aflatoxins in cereals, based on liquid chromatography with fluorescence detection previous derivatization, being in good agreement.

1.1. Introduction

Mycotoxins are toxic natural secondary metabolites produced by several species of fungi on agricultural commodities in the field or during storage [1]. Among them, aflatoxins are produced primarily by fungi *Aspergillus flavus* (which produces aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2)) and *Aspergillus parasiticus* (which produces aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), AFB_1 and AFB_2) [2,3]. Aflatoxins are considered as mutagenic agents; particularly, AFB_1 has been included in the Group 1 of carcinogenic to humans by the International Agency by Research on Cancer (IARC) [4]. Major food commodities susceptible to aflatoxin contamination include maize and other cereals such as wheat and rice, groundnuts and other nuts such as pistachios and Brazil nuts, cottonseed, copra and spices [5].

Rice (*Oryza sativa*) is one of the most consumed cereals in the world. Thus, the world rice trade in 2013 is forecast to reach 37.5 million tonnes [6]. Moreover, rice is one of the matrices of interest in mycotoxin determination [7]. Rice is grown in the wet season and frequent and heavy rainfall and floods, particularly near harvest, wet the crop and make panicles more prone to invasion by fungi and bacteria. During the wet season, sun drying practiced by most farmers may not adequately reduce the moisture content in grains. Thus, rice grains with moisture content higher than the desired level enter the storage system. As a result invasion by both, field and storage fungi takes place. Therefore, mycotoxin-producing moulds could contaminate the grain and produce important quantities of mycotoxins during storage [8].

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[2] C. M. Maragos, J. I. Greer, J. Agric. Food Chem. 45 (1997) 4337.

[3] G. S. Shephard, Anal. Bioanal. Chem. 395 (2009) 1215.

[4] IARC, Monographs on the evaluation of carcinogenic risks to humans, Volume 56, Lyon 1993.

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[6] Food and Agriculture Organization of the United Nations (2013). Available on: http://www.fao.org/economic/est/publications/rice-publications/rice-market-monitor-rmm/en/

[7] K. Tanaka, Y. Sago, Y. Zheng, H. Nakagawa, M. Kushiro, (2007). Int. J. Food Microbiol. 119 (2007) 59.

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European Union (EU) food safety legislation on mycotoxin levels is becoming increasingly strict and has established maximum level for these compounds by means of the Regulation (CE) No. 1881/2006 [9] and subsequent amends. For instance, these maximum permitted levels are 2 μg kg^{-1} for AFB_1 and 4 μg kg^{-1} for the sum of AFB_1, AFB_2, AFG_2 and AFG_1 for most of the cereals, including rice. This fact has motivated the necessity of very sensitive analytical methods for aflatoxin determination, as only trace amounts of aflatoxins are present in the samples, making their determination very difficult. The earliest analytical methods were based on solvent extraction, clean-up on open-ended packed-silica columns and separation of the analytes by thin layer chromatography (TLC). Although these methods continue in use, many others have been developed, most of them based on high performance liquid chromatography (HPLC) with UV, fluorescent (FL) or mass spectrometry (MS) detection. Different reviews compiled extensively these methodologies and applications [3,5,10,11], some of them devoted to environmental [12] or food [13] samples.

Capillary electrophoresis (CE) with UV/Vis detection has also been proposed for the determination of some mycotoxins, such as ochratoxin A (OTA) in wine [14,15], moniliformin in maize [16], or patulin in apple cider [17] or juice [18,19]. Regarding detection techniques, FL has been used in a flow system to establish a screening method for total determination of aflatoxins in feed samples, coupled with micellar electrokinetic chromatography (MEKC) as separation technique [20]. As a more sensitive detection, laser induced fluorescence (LIF) has also been coupled to CE in

[9] Commission Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs, Off. J. Eur. Commun. L364 (2006) 5.

[10] M. W. Trucksess, A. E. Pohland, Molecular Biotechnology 22 (2002) 287.

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the analysis of OTA in different food samples [21], AFB_1 in corn [22], and T-2 toxin in maize [23].

Concerning sample preparation, different approaches have been proposed, being the most common methodologies solid phase extraction (SPE) and immunoaffinity columns (IACs) containing specific antibodies to the analyte of interest, which allow faster clean-up and a higher degree of sample purification [13]. An overview of the different methodologies proposed for sample preparation in the analysis of aflatoxins in food has been compiled in a revision [24].

In the present chapter, MEKC with LIF detection has been proposed for the determination of AFB_1, AFB_2, AFG_1 and AFG_2 in rice samples. To improve the sensitivity, an on-line sample concentration method based on sweeping has been developed [25]. This approach is designed to focus the analytes into a narrow band within the capillary, thereby increasing the sample volume that can be injected without any loss of efficiency. It is based on the different interactions between an additive (i.e., a pseudostationary phase or micellar media) in the separation buffer and the sample in a matrix that is free of the used additive. It involves the accumulation of charged and neutral analytes by the pseudostationary phase that penetrates the sample zone and "sweeps" the analytes, producing a focusing effect [26]. To the best of our knowledge this is the first time that an on-line preconcentration technique has been proposed for the determination of aflatoxins by CE. The method has been applied to the analysis of the four main aflatoxins in rice samples and it has been validated by comparison of the results to those obtained by an standard method based on HPLC-FL with post-column

[21] S. Corneli, C. M. Maragós, J. Agric. Food Chem. 46 (1998) 3162.
[22] C. M. Maragós, J. I. Greer, J. Agric. Food Chem. 45 (1997) 4337.
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[25] J. P. Quirino, J. B. Kim, S. Terabe, J. Chromatogr. A, 956 (2002) 357.
[26] A. T. Aranas, A. M. Jr. Guidote, J. P. Quirino, Anal. Bioanal Chem. 394 (2009) 175.

derivatisation of the compounds with iodine [27]. A previous purification of the sample based on the use of IACs was used.

1.2. Experimental

1.2.1. Chemicals and solvents

All reagents were of analytical reagent grade and solvents were HPLC grade. Methanol (MeOH), acetonitrile (MeCN), toluene, sodium dodecyl sulphate (SDS), potassium chloride, potassium dihydrogen phosphate, disodium phosphate, sodium chloride, 35% nitric acid, and sodium hydroxide were purchased from Panreac-Química (Madrid, Spain). Sodium tetraborate, imidazol and cyclodextrins (CDs) were obtained from Sigma-Aldrich (St. Louis, MO, USA), tris(hydroxymethyl)aminomethane (Tris) and hydrochloric acid were obtained from Merck (Darmstadt, Germany), and iodine from Fluka (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the work.

Individual stock standard solutions of 200 µg mL^{-1} of aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 (analytical standard grade, Sigma-Aldrich) were prepared following the AOAC recommendation [28], by dissolving each aflatoxin in MeOH. The MeOH solvent was evaporated to dry film, and the residue was re-dissolved in toluene:MeCN (98:2) and stored in the dark at -18 ºC. Intermediate solutions (50 µg mL^{-1}, 1 µg mL^{-1}) were prepared by dissolving the appropriate amount of each stock standard solution in MeCN and stored refrigerated. Working solutions were prepared by diluting the intermediate solutions in buffer (20 mM sodium borate pH 8.5, 7% MeCN (v/v)) to the desired concentration prior to use.

The phosphate buffer used for purification of the samples in the IACs (Aflaprep immunoaffinity columns, R-Biopharm, Darmstadt, Germany) was prepared by dissolving 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g Na_2HPO_4 and 8 g NaCl in 1 L of water, and adjusting the pH at 7.4. The iodine solution for post-column derivatisation in the

[27] ISO 16050:2003 “Foodstuffs. Determination of aflatoxin B_1, and the total content of aflatoxins B_1, B_2, G_1 and G_2 in cereals, nuts and derived products. High performance liquid chromatographic method”

[28] K. Helrich, in: 15th ed, Official Method of Analysis, Vol. 49, AOAC, 1990, pp. 1184.

standard HPLC-FL method [27] was prepared by dissolving 200 mg of iodine in 4 mL of MeOH. Subsequently, 400 mL of water were added and the solution was shaken for 30 min and filtered. This solution was light protected.

1.2.2. Instrumentation and software

All CE experiments were carried out on an HP3D CE instrument (Agilent Technologies, Waldbronn, Germany), coupled to a Zetalif fluorescence detector (Picometrics S.A., Toulouse, France), shown in Figure 1.1. The excitation was performed with a He-Cd Laser at 325 nm. Separations were performed in an uncoated fused-silica capillary (70 cm×75 µm I.D., 55 cm effective length, Polymicro Technologies, Phoenix, AZ, USA).

HPLC experiments were carried out on an 1100 HPLC instrument equipped with a fluorescence detector (Agilent Technologies). An auxiliary pump (Waters reagent manager model RMA, Waters, Milford, MA, USA) was used for post-column derivatisation. A Spherisorb ODS C_{18} column (250×4 mm, 3µm, Waters) was used for separation.

Data were collected using the software provided with the HP ChemStation version A.09.01. Additional programs, as Excel (Microsoft) and Statgraphics Plus 5.1 (Statistical Graphics, Rockville, MD, USA), were used for data processing.

The pH of the different buffers was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit. A Visiprep SPE vacuum manifold (Supelco, Schnelldorf, Germany), a centrifuge (Hettich Universal 320, Leipzig, Germany) and a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), were used for sample treatment and preparation of standard solutions.

1.2.3. Electrophoretic procedure

Before the first use, the new capillary was rinsed with 1 M NaOH for 10 min, then with deionised water for 10 min, and finally with the background electrolyte (BGE) solution for 15 min. At the beginning of the day, the capillary was cleaned with 0.1 M NaOH for

7 min, then with deionised water for 1 min and finally with the running buffer for 20 min. Before each run, the capillary was pre-washed with 0.1 M NaOH for 2 min, deionised water for 1 min and finally with the BGE for 10 min. In all instances, a N_2 pressure of 1 bar was applied.

Aflatoxins were analysed using a BGE consisting of an aqueous solution of 20 mM borate and 30 mM SDS adjusted to pH 8.5 with 1 M HCl and with 7% MeCN (v/v). A voltage of 20 kV (normal mode) was applied for the electrophoretic separation using a ramp of 0-20 kV in 0.5 min. The temperature of the capillary was kept constant at 25 ºC. Samples and standard solutions were prepared in the BGE buffer but without SDS, and were introduced by hydrodynamic injection at a pressure of 35 mbar for 38 s.

1.2.4. HPLC-FL standard method

Chromatographic separation of the aflatoxins was based on the recommended standard method for the determination of aflatoxins in cereals, using HPLC-FL with post-column derivatisation with iodine [27]. The separation was performed on a Spherisorb ODS C_{18} column (250×4 mm, 3 µm) using an isocratic mobile phase of water:MeOH (49:51) at a flow rate of 0.5 mL min^{-1}. The column temperature was kept at 40 ºC, and the injection volume was 100 µL. The derivatisation was carried out by mixing the eluent of the column with an iodine solution at a flow rate of 0.3 mL min^{-1}. The mixed solution passed through a reaction coil (5 m) placed in a bath at 80 ºC and finally was detected by fluorescence (λ_{ex}= 360 nm; λ_{em}= 420 nm).

1.2.5. Sample treatment

The treatment used for rice samples was based on the recommended method for determination of aflatoxins in cereals [27], consisted of an extraction using IAC in order to preconcentrate the analytes and remove interfering substances.

Rice samples were grinded to a particle size less than 0.5 mm and homogenized. Approximately a portion of 5 g were accurately weighted and placed in a falcon tube (50 mL) and 25 mL of MeOH:water (70:30) were added. The mixture was vortexed for 30 s twice. An additional volume of 25 mL of water was added, the sample was gentle

mixed and then centrifuged at 4500 rpm for 10 min. Then, 10 mL of the extract was submitted to clean-up through an IAC, at a flow rate of 1 drop per second, approximately. After that, the column was washed twice with phosphate buffer (prepared as described in Section 1.2.1.) and subsequently dried. Finally, the aflatoxins were eluted with 1 mL of MeOH, with three backflushing, followed by 1 mL of water. The eluate was collected in a vial and directly analysed by the standard HPLC-FL method for validation purposes. For the analysis by the proposed MEKC-LIF method, 1.3 mL of the final eluate was evaporated to near dryness using a gentle stream of N_2, and then reconstituted with 0.5 mL of buffer (20 mM sodium borate pH 8.5, 7% MeCN (v/v)). The extract was vortexed, mixed and analysed. The sample treatment is summarized in Figure 1.1.

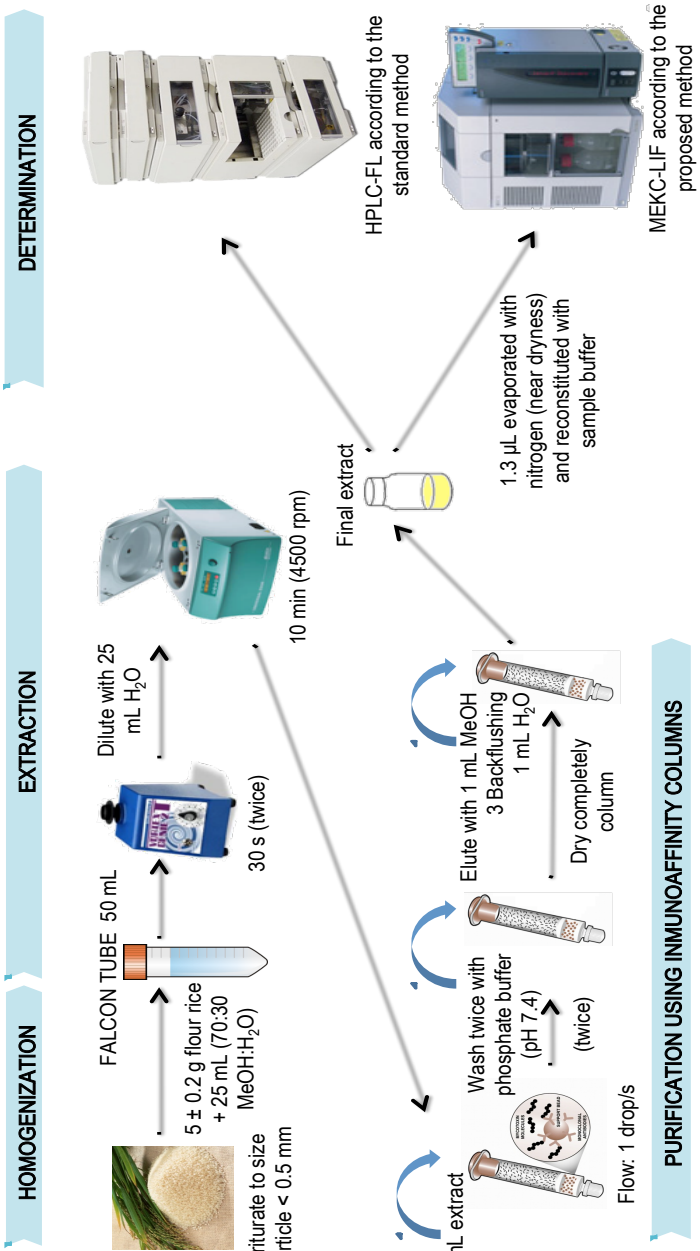

Figure 1.1. Diagram of the sample treatment

1.3. Results and discussion

1.3.1. Optimization of the sweeping-MEKC experimental conditions

Using capillary-zone electrophoresis (CZE), aflatoxins are not separated since they are neutral substances. On the contrary, when SDS is added to the BGE, different migration behaviours can be achieved, due to the different interactions between the mycotoxins and the micelles. Thus, MEKC was the separation mode selected for this work. The optimisation of the main variables affecting the separation and simultaneous quantification of the four aflatoxins by LIF detection was carried out considering the resolution, the sensitivity (peak area) and analysis time as response variables, and keeping the generated current at optimum values (below 100 μA).

First of all, the effect of pH of the BGE was investigated over the range 6.5-10.0 using different buffer solutions: 10 mM Na_2HPO_4 with 50 mM SDS was used for pH values between 6.5-8, and 10 mM $Na_2B_4O_7$ with 50 mM SDS was used for pH values between 8.5-10. The pH was adjusted using HCl 1 M.

The study showed that at neutral pHs, the intensity of peaks of AFB_2 and AFG_2 was higher. However, the signals for AFB_1 and AFG_1 decrease to hardly be detectable (see Figure 1.2). According to these results, a pH 8.5 was finally selected.

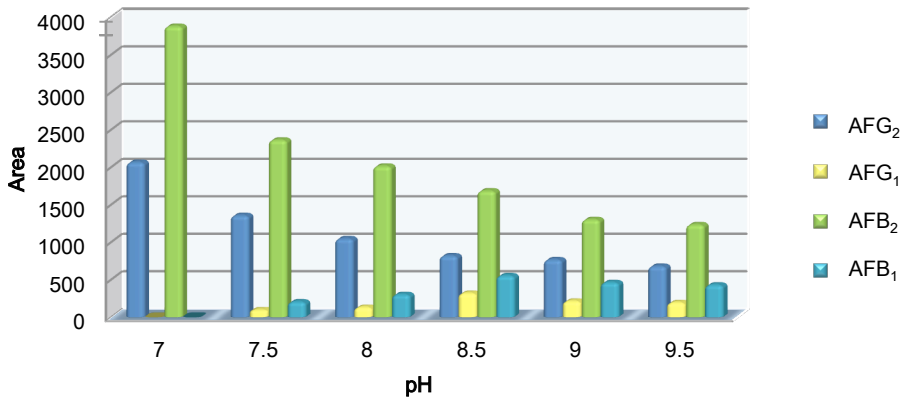

Figure 1.2. Influence of the pH of the BGE on the peak area

Then, the nature of the buffer at the optimum pH was studied. Different buffer solutions ($Na_2B_4O_7$, Na_2HPO_4, imidazol and tris), were tested, all of them at a concentration of 25 mM and 50 mM SDS. As shown in Figure 1.3., the best results were obtained using $Na_2B_4O_7$. The results obtained using imidazol buffer are not showed because no peaks were obtained.

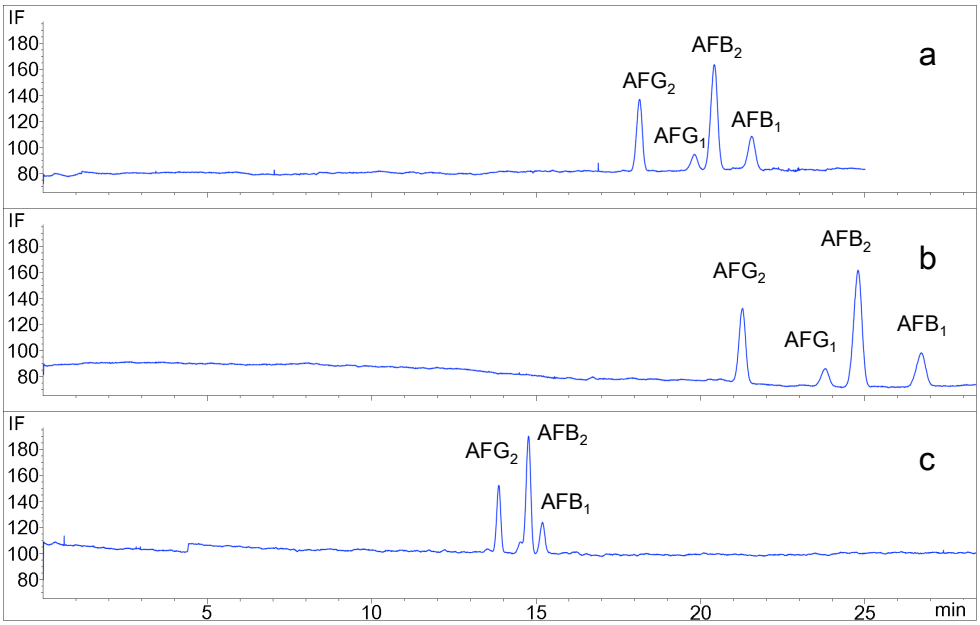

Figure 1.3. Electropherograms obtained with different buffer solutions (50 mM SDS, pH 8.5): 25 mM, sodium tetraborate (a), 25 mM, sodium phosphate (b), 25 mM, tris (c)

Subsequently, sodium tetraborate concentration was modified between 10 and 35 mM, keeping the concentration of SDS at 50 mM and the pH at 8.5. From this experience, a concentration of 20 mM was selected, as it provided the highest signals, keeping also an adequate electric current, around 70 μA (Figure 1.4).

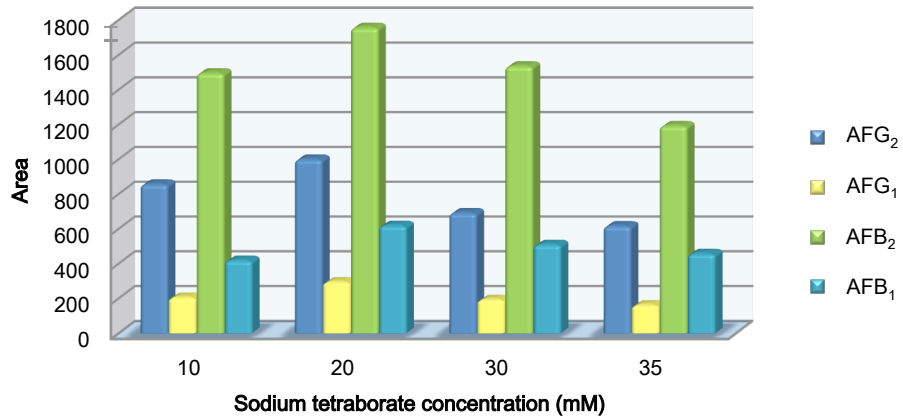

Figure 1.4. Influence of sodium tetraborate concentration on peak area (50 mM SDS, pH 8.5)

The SDS concentration was modified between 20 and 60 mM; the best signals for most of the aflatoxins (including AFB_1, the most critical one), as well as an optimum resolution between AFG_1 and AFB_2, were obtained with a concentration of 30 mM (Figure 1.5).

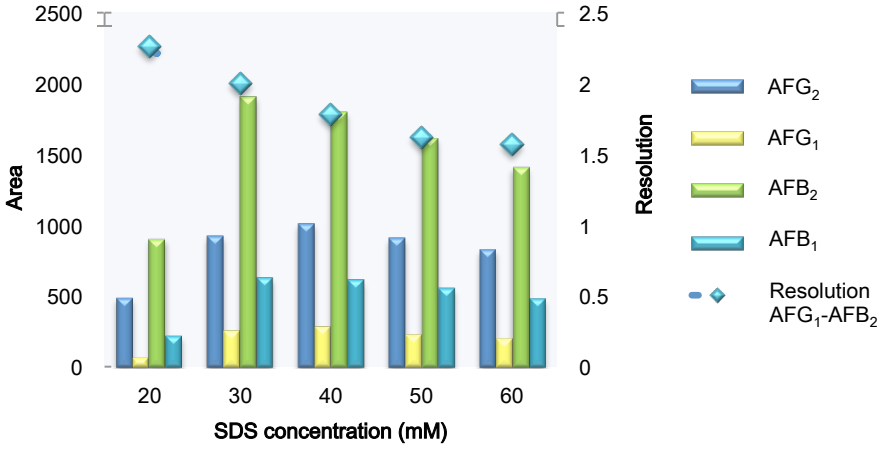

Figure 1.5. Effect of SDS concentration on peak area and resolution between AFG_1 and AFB_2

Also, the addition of different modifiers to the BGE, such as CDs (7 mM of α-cyclodextrin (α-CD), γ-cyclodextrin (γ-CD), β-cyclodextrin (β-CD), 2-hydroxi-propil-β-

cyclodextrin (2-hpβ-CD) and heptakis(2,6-di-O-methyl)-β-cyclodextrin) were tested in order to improve sensitivity. Figure 1.6 shows the results, except for heptakis(2,6-di-O-methyl)-β-cyclodextrin) because an effective separation of the analytes was not obtained. As can be seen, the addition of CDs reduced the analysis time but caused the decrease of signal, so its use was discarded

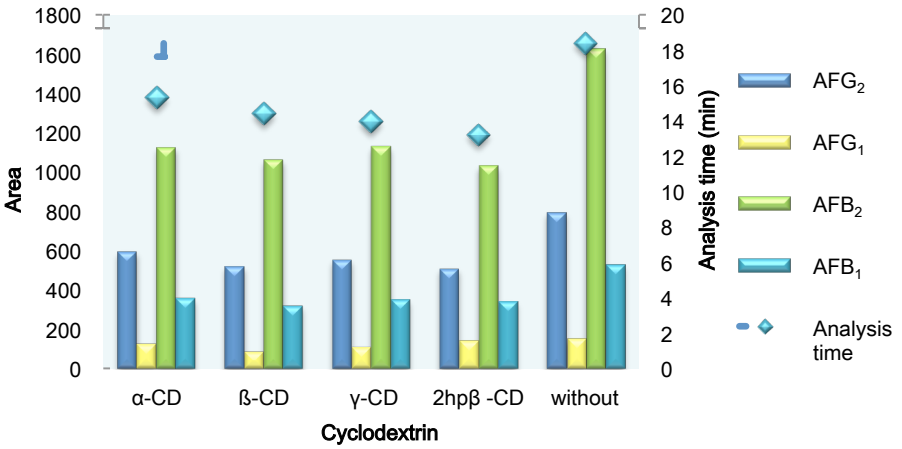

Figure 1.6. Effect of addition of 7 mM CDs on the BGE

The addition of organic modifiers, as MeCN and MeOH, was also considered. A significant improvement in resolution and shorter analysis times were obtained in the presence of MeCN (Figure 1.7). Concentrations of 2, 5, 7, 10 and 15% were tested (Figure 1.8). Although there were no significant differences in the peak areas, higher peaks were obtained with 7% MeCN.

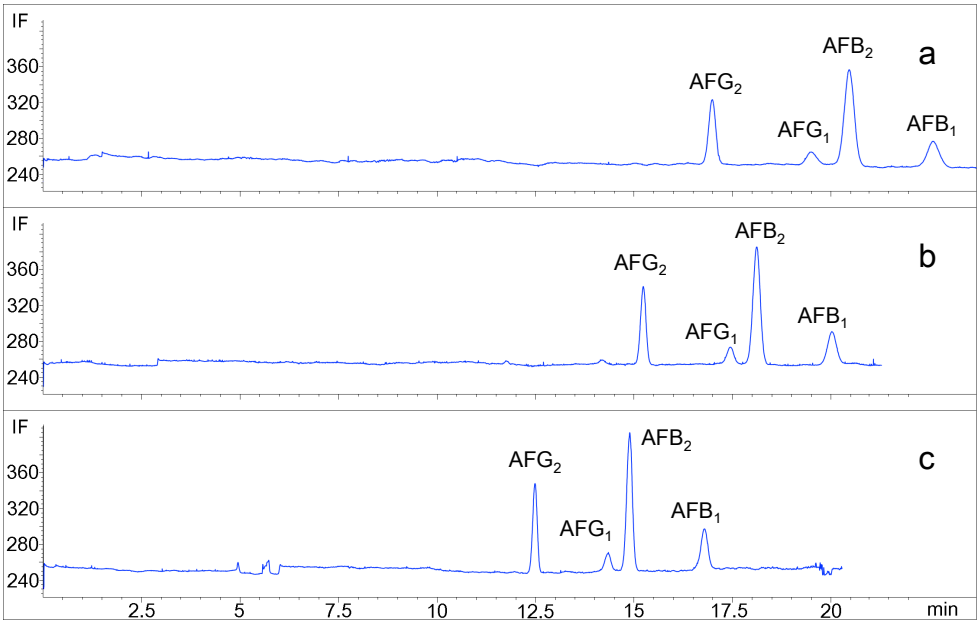

Figure 1.7. Electropherograms obtained with different organic modifiers: without organic modifier (a), 5% MeOH (b), 5% MeCN (c)

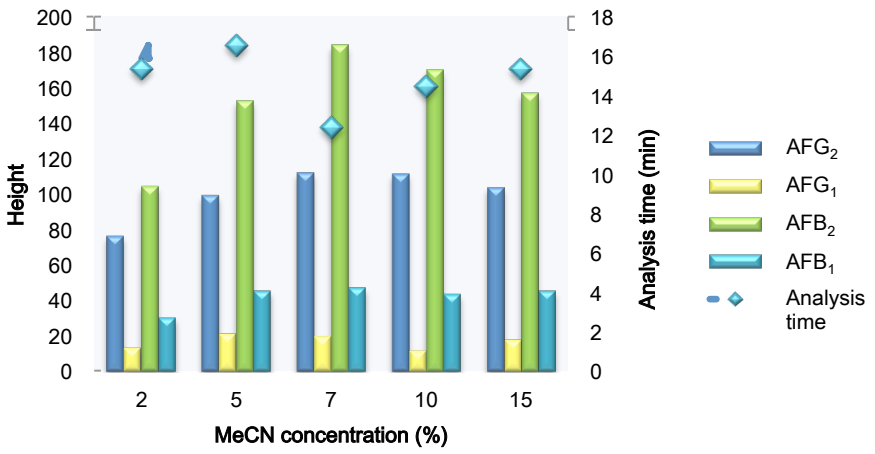

Figure 1.8. Effect of MeCN concentration on peak height and analysis time

Once the BGE had been optimised, the separation voltage was modified between 18 and 28 kV. A voltage of 20 kV was applied as optimum to achieve a short running time with a low electric current (Figure 1.9).

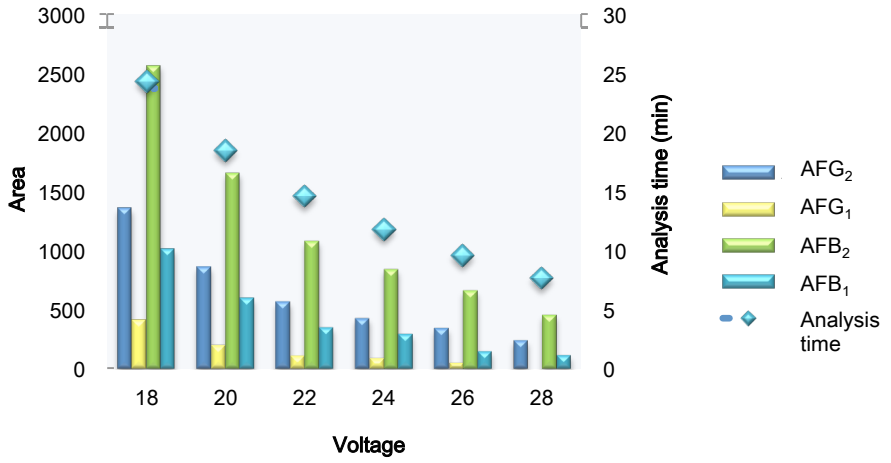

Figure 1.9. Effect of voltage on peak area and analysis time

Finally, the effect of the temperature on the separation was investigated in the range of 15-31 ºC, observing that the capillary temperature did not influence the separation significantly (Figure 1.10), so 25ºC was selected.

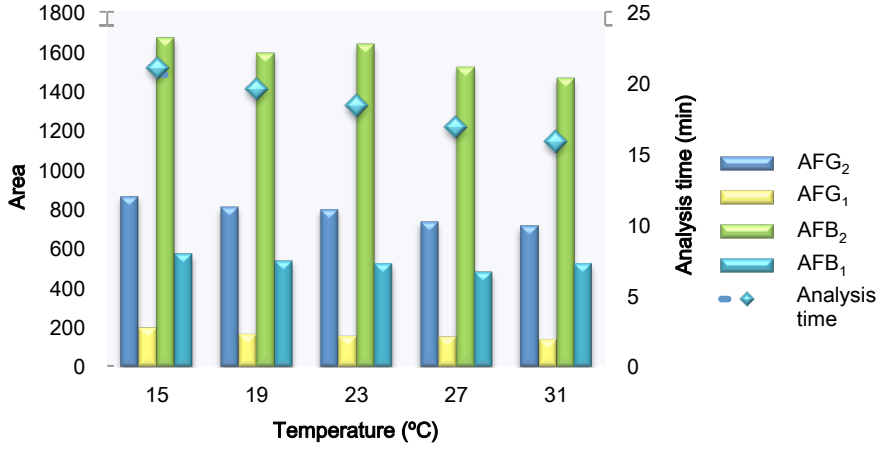

Figure 1.10. Effect of the temperature on peak area and analysis time

Regarding the sweeping-MEKC preconcentration step, and in order to obtaining the maximum sensitivity, the sample solvent and the injection time, which are related with the sample injection volume, were optimised. The adequate sample solvent for an

optimum collection and accumulation of analyte molecules by the pseudostationary phase that penetrates in this sample zone was the BGE but in absence of additive (SDS) [25]. The injection time was increased from 10 to 200 s. Finally, 38 s at 35 mbar was used as optimum, as it was not possible to fill the capillary over 147 nL, which was equivalent to approximately 4.8% of the total volume of the capillary (Table 1.1). At these conditions, a preconcentration factor of 4 was achieved, compared to a normal injection of 5 s, without sweeping (Figure 1.11).

Table 1.1. Sample volume and percentage of capillary filled at different injection times

Injection time (s)	Injected sample volume (nL)	Capillary filled (%)
5	19.4	0.63
10	38.8	1.26
20	77.7	2.51
35	135.9	4.39
38	147.5	4.77
40	155.3	5.02
797	3094.7	100

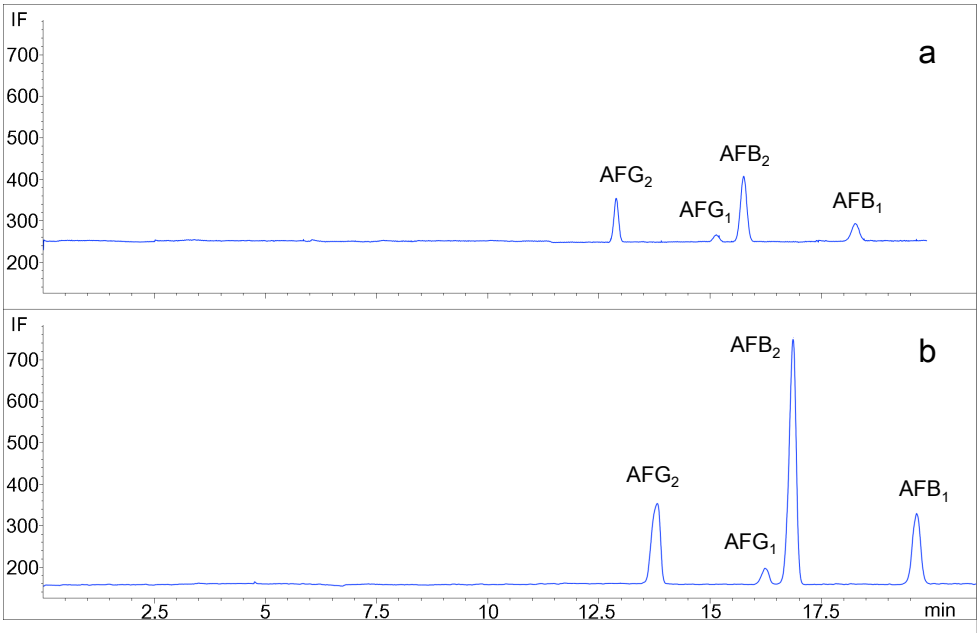

Figure 1.11. Electropherograms obtained for comparing: normal injection (5 s) (a), sweeping mode injection (38 s) (b)

Moreover, in order to illustrate the effect of sweeping in the electrophoretic separation, two injections of 38 s, one of them made using the sweeping mode and the other one with the sample dissolved in the BGE, were performed. The results are shown in Figure 1.12, highlighting the obtained preconcentration effect.

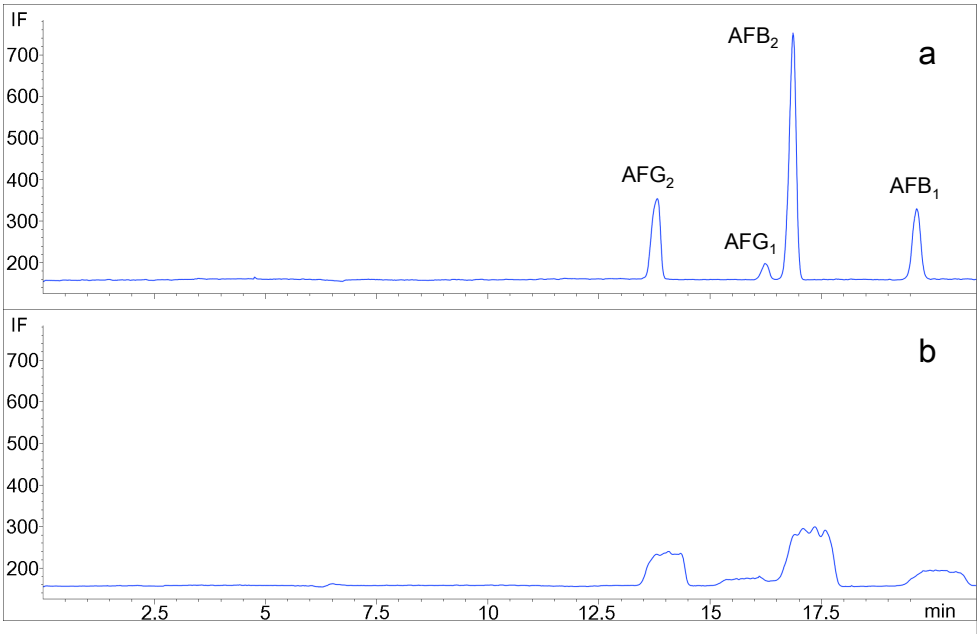

Figure 1.12. Electropherograms obtained with and without on-line preconcentration: sweeping (38 s) (a), without sweeping (38 s) (b)

1.3.3. Performance characteristics of the sweeping-MEKC-LIF method

The characterization of the proposed sweeping-MEKC-LIF method was carried out by the establishment of the calibration curves, estimation of the limits of detection (LOD) and quantification (LOQ) and precision study (including repeatability and reproducibility). To assess the trueness of the method for the analysis of rice samples, two approaches were carried out: (a) recovery studies with spiked samples free of analytes; and (b) analysis of rice samples naturally contaminated by aflatoxins and comparison of the obtained results with those obtained with the standard method.

1.3.3.1. Calibration curves, limits of detection and quantification

The calibration curves were established from standard solutions (ranging from 0.25-50.0 μg L^{-1} for AFG_2; 2.5 - 150 μg L^{-1} for AFG_1; and 0.25 - 25 μg L^{-1} for AFB_2 and AFB_1) injected in triplicate, considering the peak areas as analytical signal.

The statistics parameters, calculated by least-square regression, LODs and LOQs are shown in Table 1.2. The satisfactory determination coefficients confirmed that the analytical responses were linear over the studied ranges. LODs and LOQs were calculated using 3×signal-to-noise ratio (S/N) and 10×S/N, respectively. As can be seen, very low LOQs were obtained for all the studied aflatoxins.

Table 1.2. Statistics and performance characteristics for the sweeping-MEKC-LIF method

Compound	Linear range (μg L^{-1})	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})
AFG_2	0.37 – 50	0.995	0.11	0.37
AFG_1	1.74 – 150	0.997	0.52	1.74
AFB_2	0.13 – 25	0.998	0.04	0.13
AFB_1	0.32 – 25	0.995	0.10	0.32

1.3.3.2. Precision study

The instrumental precision of the method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by means of repetitive application of the sweeping-MEKC-LIF method to three standard solutions (experimental replicates) injected in triplicate (instrumental replicates), at two concentration levels for each aflatoxin, and analysed the same day. Intermediate precision was evaluated with a similar procedure, but the standard solutions were analysed in five consecutive days. The results, expressed as relative standard deviation (% RSD) of the peak areas are shown in Table 1.3. Acceptable

precision (fulfilling current demands about analytical methods for determination of mycotoxins [29]) were obtained in all cases.

Table 1.3. Instrumental precision study (%RSD of peak areas)

Concentration level	AFG_2	AFG_1	AFB_2	AFB_1
Repeatability		RSD (%) (n=9)		
2 μg L^{-1}	7.1	8.7	4.8	3.9
10 μg L^{-1}	9.2	11.2	4.3	5.7
Intermediate precision		RSD (%) (n=15)		
2 μg L^{-1}	8.5	9.0	9.8	8.4
10 μg L^{-1}	7.1	9.7	6.7	9.5

1.3.3.3. Recovery studies

In order to check the applicability of the proposed method, different extracts of rice samples free of aflatoxins, previously obtained by using IACs and analysed by the HPLC-FL standard method [27] were spiked at two concentration levels (level 1: AFB_1 and AFG_1 5 μg L^{-1}, AFB_2 and AFG_2 1.5 μg L^{-1}; level 2: AFB_1 and AFG_1 25 μg L^{-1}, AFB_2 and AFG_2 7.5 μg L^{-1}) and analysed by the proposed sweeping-MEKC-LIF method. The results are shown in Table 1.4, and can be considered well enough for analytical purposes [29]. A typical electropherogram corresponding to the separation of the analytes in spiked extracts of rice samples under developed conditions is shown in Figure 1.13.

[29] Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Commun., L70/12 (9.3.2006).

Table 1.4. Recovery study in extracts of rice samples free of aflatoxins (n=3)

	Level 1[a]		Level 2[b]	
	R (%)	RSD (%)	R (%)	RSD (%)
AFG_2	105.4	6.3	104.5	1.7
AFG_1	98.8	8.8	95.6	4.0
AFB_2	101.1	5.0	93.0	2.9
AFB_1	104.3	1.8	98.4	6.0

[a] Level 1: AFB_1 and AFG_1 5 μg L^{-1}; AFB_2 and AFG_2 1.5 μg L^{-1}

[b] Level 2: AFB_1 and AFG_1 25 μg L^{-1}; AFB_2 and AFG_2 7.5 μg L^{-1}

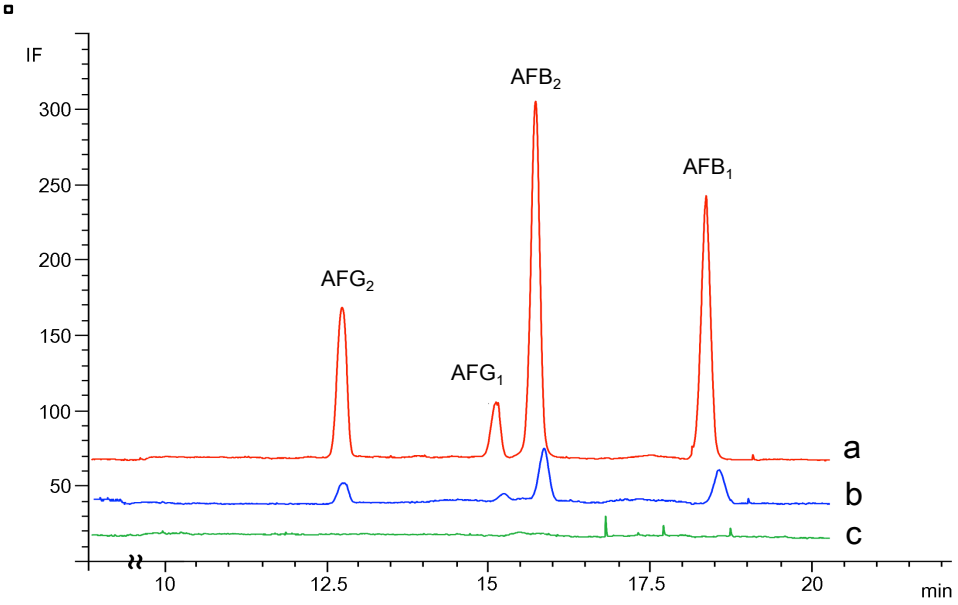

Figure 1.13. Electropherogram of an extract of rice sample analysed by the proposed method: extract spiked at 25 μg L^{-1} of AFB_1 and AFG_1 and 7.5 μg L^{-1} of AFB_2 and AFG_2 (a); extract spiked at 5 μg L^{-1} of AFB_1 and AFG_1 and 1.5 μg L^{-1} of AFB_2 and AFG_2 (b); blank sample (c)

1.3.3.4. Analysis of rice samples

Three different naturally contaminated rice samples (supplied by Biosearch S.A. and stored frozen in the dark until analysis) where submitted to IAC extraction and analysed by the proposed sweeping-MEKC-LIF method and the standard HPLC-FL method (analysis performed at the laboratories of Biosearch S.A.). The results were statistically

compared by means of the Student t-test, being in good agreement (see Table 1.5), showing the trueness of the method for rice samples. None of the samples gave a positive result for AFG_1 and AFG_2, but they were positive for AFB_2 and AFB_1, with concentrations for the latter above the maximum contents established by EU regulation in rice (2 μg L^{-1}) [9], making these samples unfit for human consumption.

Table 1.5. Determination of aflatoxins in three different rice samples. Comparison with the HPLC-FL standard method

		MEKC-LIF (n = 3)		HPLC-FL (n=1)		
		μg kg^{-1}	RSD (%)	μg kg^{-1}	Relative error (%)	P-value[a]
Sample 1	AFB_2	0.93	2.99	0.97	-4.52	0.11
	AFB_1	2.91	4.69	2.86	1.65	0.61
Sample 2	AFB_2	0.55	1.10	0.56	-0.22	0.80
	AFB_1	7.47	3.82	7.42	0.67	0.79
Sample 3	AFB_2	0.51	6.21	0.51	1.32	0.78
	AFB_1	4.81	3.43	4.63	4.05	0.19

[a] P-Value (5%) for Student t-test

1.4. Conclusions

CE has been proposed as an alternative to HPLC for the simultaneous determination of four aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) in rice samples. They can be separated and detected in less than 20 min using MEKC with LIF detection and on-line sample preconcentration based on sweeping to improve sensitivity. Satisfactory precision (intraday and interday-precision) was obtained. A great improvement in the LODs of 77-3000 times for the four aflatoxins compared to a previous CE-FL method [20] and up to 5 times for AFB_1, compared to other CE-LIF method [22] has been achieved. The method has been validated for the analysis of rice samples by means of recovery assays in samples free of analytes and by comparison of the results obtained from the proposed method with those obtained from a standard method based on HPLC-FL detection. In both cases, a previous standard protocol for sample treatment using IAC was used. The values of the recovery percentages as well as the good agreement between the results obtained by both methods confirmed the trueness of the methodology established in this work. This method is accurate and sensitive and

avoids the inconvenient of the derivatisation required by the HPLC-FL method, saving solvents and being more environmentally friendly.

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CHAPTER 2

Comparación de diferentes tratamientos de muestra para el análisis de ocratoxina A en vino mediante HPLC capilar con detección por fluorescencia inducida por láser

Comparison of different sample treatments for the analysis of ochratoxin A in wine by capillary HPLC laser-induced fluorescence detection

Resumen

La ocratoxina A es una micotoxina presente de forma natural en varios alimentos, incluyendo el vino. Está considerada como un posible carcinógeno humano, habiéndose establecido una concentración máxima permitida en vino para este compuesto de 2 μg kg^{-1} (Directiva (CE) Nº 1881/2006). Las columnas de inmunoafinidad han sido utilizadas habitualmente para su extracción. Sin embargo, son necesarios sistemas de extracción más simples, más eficientes y menos contaminantes. En este capítulo se han evaluado y comparado tres tratamientos de muestra para la determinación de ocratoxina A en vino: microextracción líquido-líquido dispersiva, microextracción líquido-líquido dispersiva usando líquidos iónicos como disolvente de extracción y el procedimiento QuEChERS. Para su determinación se ha propuesto la cromatografía de líquidos capilar acoplada a un detector de fluorescencia inducida por láser (He-Cd con excitación a 325 nm), añadiendo a la fase móvil un medio micelar aniónico para aumentar la intensidad de la fluorescencia y mejorar la eficacia. Se establecieron las curvas de calibrado en presencia de matriz para los tres métodos propuestos, obteniendo límites de detección de 5.5, 5.2 y 85.7 ng L^{-1} para microextracción líquido-líquido dispersiva, microextracción líquido-líquido dispersiva usando líquidos iónicos como disolvente de extracción y el procedimiento QuEChERS, respectivamente. La precisión fue evaluada en términos de repetitividad y precisión intermedia, siendo satisfactoria en todos los casos. El método se aplicó a muestras de vino blanco, tinto y rosado, obteniéndose buenas recuperaciones en todos los casos. Las metodologías propuestas podrían incluirse dentro de la química analítica verde, combinando los bajos volúmenes de disolventes orgánicos necesarios en estos tratamientos con el reducido consumo de fase móvil requerido por la cromatografía líquida capilar.

Abstract

Ochratoxin A is a mycotoxin naturally found in various foods, including wine. As it is considered as a possible human carcinogen, the maximum concentration for this compound has been established at 2 μg kg^{-1} in wine by the European Union (Directive (CE) Nº 1881/2006). Typically, immunoaffinity columns have been used for its extraction. However, simpler, more efficient and less contaminant extraction systems are demanding. In this chapter, dispersive liquid–liquid microextraction, dispersive liquid–liquid microextraction using ionic liquid as extraction solvent and the QuEChERS procedure, have been evaluated and compared for extraction of ochratoxin A in wine samples. Laser–induced fluorescence (He–Cd Laser, excitation at 325 nm) coupled with capillary liquid chromatography has been proposed as analytical technique. An anionic micellar medium was added to the mobile phase for increasing the fluorescence intensity and peak efficiency. Matrix-matched calibration curves were established for three methods, obtaining limits of detection (3 x S/N) of 5.5, 5.2 and 85.7 ng L^{-1} for dispersive liquid–liquid microextraction, dispersive liquid–liquid microextraction using ionic liquid as extraction solvent and the QuEChERS procedure, respectively. Clean extracts were obtained for white, rose and red wines with the three methods, with good recoveries in all cases. The precision was evaluated in terms of repeatability and intermediate precision, being in agreement with current legislation. The proposed methodologies could be classified as green analytical chemistry alternatives, combining the low organic solvent volumes required in these sample treatments with the reduced consumption of mobile phase in capillary liquid chromatography.

2.1. Introduction

OTA is a mycotoxin produced by several *Aspergillus (A. ochraceus*, *A. niger*, *A. carbonarius*, *A. terreus)* and *Penicillium* species (*P. verrucosum*, *P. nordicum*). OTA has immunosuppressive, hepatotoxic and teratogenic activity. Moreover, there are sufficient evidences in experimental animals, but inadequate evidences in humans, for its carcinogenicity. Consequently, it has been classified by IARC as a possible human carcinogen (Group 2B) [1]. The occurrence of OTA in food and feed has been reported world-wide. Among them, wine is considered one of the major food commodities susceptible to OTA contamination [2], after cereals and derived [3]. Thus, the European Commission has approved the Directive (EC) 1881/2006 [4], the Directive (EC) 105/2010 [5] and the Directive (UE) 594/2012 [6] which establish OTA limits for different food matrices: cereal, coffee, wine, beer, spices and liquorice and their derivatives. The maximum permitted level for wine is 2.0 μg kg^{-1} [4]. As a consequence, very sensitive analytical methods for OTA detection, with performance characteristics fulfilling the established legislation, are needed [7].

The most common analytical technique for OTA determination in foodstuff is high performance liquid chromatography (HPLC) coupled to FL [8-12], including the

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reference method described by the European Standard EN 14133 for the determination of OTA in wine [13]. Alternative detection methods, such as diode array detection (DAD) [14,15], MS and tandem mass spectrometry (MS/MS) [16] have also been proposed. Others applied techniques are thin layer chromatography (TLC) [17,18], gas chromatography (GC) with MS [19], enzyme-linked immunosorbent assay (ELISA) [20,21] and FL immunoassay [22,23]. Also, CE with UV/Vis detection has also been proposed for the determination of OTA in wine [24,25]. Moreover, the use of LIF as a more sensitive detection system has been proposed for the determination of OTA in different food samples (roasted coffee, corn, and sorghum) using CE [26]. Some reviews devoted to the analysis of OTA [2,27-30] and mycotoxins in general [31-36], compile most of these analytical methodologies.

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Regarding sample treatment and clean-up, IACs have been widely used for selective mycotoxin extraction [37,38,39]. Other methods have been reported for the extraction of OTA, such as liquid-liquid extraction (LLE) in wine, beer, must and milk [8,10,40], SPE in milk, wine, beer, must and cereals [8-10,40-42], molecular imprinted polymers as sorbent in SPE (MISPE) in wine, beer, orange juice, and wheat [43-47], solid-phase microextraction (SPME) in coffee, grape juice and urine [48], pressurized liquid extraction (PLE) in cereals [49,50] and bread [51] or immune-ultrafiltration in cereal samples [52]. However, some of these treatments present some disadvantages, for example, IACs are expensive, not recyclable and have a limited storage time, LLE requires long extraction times and a high volume of organic solvents and MISPE shows high cost and time to produce them.

In this sense, simpler, more efficient and environmentally friendly extraction systems are demanding. For instance, coacervative extraction using reverse micelles has been proposed as a valuable strategy to replace organic solvent in the determination of OTA in wine [53]. Also, several novel microextraction techniques have been developed in

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order to reduce the analysis step, increasing the sample throughput and improving the quality and sensitivity of the analytical methods [54,55]. Dispersive liquid–liquid microextraction (DLLME) is one of this emerging techniques introduced for treatment of liquid samples [56-61] and has been used in the determination of OTA in wine by HPLC-MS [62] and in cereals by HPLC-FL [12]. DLLME is based on the use of a ternary component solvent system, where an appropriate mixture of a few microliters of an organic extraction solvent (usually with a density higher than water), and a small volume of a disperser solvent (miscible with the extraction solvent and with water), is injected rapidly into an aqueous sample, resulting in the formation of a stable emulsion. The organic analytes present in the aqueous sample are rapidly extracted into the extraction solvent as a result of the large contact surface between the organic and the aqueous phases. Phase separation is performed by centrifugation and an organic phase with the analytes of interest is settled in the bottom of a conical tube and subsequently analysed by an appropriate technique.

However, the search for new extraction solvents is a key trend in DLLME. In the last decade, the use of ionic liquids (ILs) as extraction solvent has been found to be especially important in analytical chemistry [63,64]. ILs are ionic media resulting from the combination of organic cations and various anions [65], and show interesting properties, such as: negligible vapor pressure, good solubility for organic and inorganic compounds, non-flammability, high thermal stability, wide temperature range as a liquid phase, etc. Due to these characteristics, ILs can replace the volatile organic solvents

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used during sample preparation procedures, including DLLME, providing a fast and easy to operate procedure that avoids using highly toxic solvents [66,67]. However, to the best of our knowledge, IL-DLLME has not been explored yet for the extraction of mycotoxins.

Another popular treatment is a fast and inexpensive method, so called QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe), which has been widely used in the last years, mainly for the extraction of pesticides, but also for other compounds [68]. QuEChERS methodology presents some advantages, such as its simplicity, minimum steps, and effectiveness for cleaning-up complex samples [69]. It involves two steps: the first one is an extraction step based on partitioning via salting-out extraction involving the equilibrium between an aqueous and an organic layer, and the second one is a dispersive SPE (dSPE) that involves further clean-up using combinations of $MgSO_4$ and different sorbents, such as C_{18} or primary and secondary amine (PSA), to remove interfering substances. QuEChERS based methods have been recently reported for the extraction of different mycotoxins in cereal products [70-74], bread [75] and eggs [76], and in the multiresidue extraction of different contaminants, including mycotoxins, in organic food products [77].

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[76] A. Garrido-Frenich, R. Romero-González, M. L. Gómez-Pérez, J. L. Martínez-Vidal, J. Chromatogr. A, 1218 (2011) 4349.
[77] R. Romero-González, A. Garrido-Frenich, J. L. Martínez-Vidal, O. D. Prestes, S. L. Grio, J. Chromatogr. A 1218 (2011) 1477.

In this chapter, a comparison between DLLME, IL-DLLME and QuEChERS for the extraction of OTA in wine has been carried out, in terms of efficiency, recovery, sensitivity and precision. Moreover, the coupling of capillary HPLC with LIF detection (offering better resolution, lower detection limits and lower solvent consumption) using a SDS micellar solution in the mobile phase to increase the native fluorescence of OTA, is also proposed as an alternative to the conventional HPLC-FL methods. The combination of these sample treatments (DLLME, IL-DLLME and QuEChERS) with a miniaturized technique such as capillary HPLC is an environmentally friendly alternative to the determination of OTA, as the consumption of organic solvent is reduced in both steps of the method (sample treatment and determination), being in agreement with the new trends of green analytical chemistry [78,79].

2.1. Experimental

2.2.1. Chemicals and solvents

All the reagents were analytical reagent grade, solvents were HPLC grade and OTA was analytical standard grade. MeOH, ethanol (EtOH), MeCN, sodium chloride, phosphoric acid (85%), sodium hydroxide, sodium dihydrogen phosphate monohydrate and SDS were supplied by Panreac (Madrid, Spain); acetic acid, chloroform and tetrachloroethane were purchased from VWR BDH Prolabo (West Chester, Pensilvania, USA); carbon disulfide was obtained from Carlo Erba (Rodano, MI, Italy); tetrahydrofuran (THF), formic acid and acetone (ACO) was supplied by Merck (Darmstadt, Germany); chlorobenzene was obtained from Alfa Aesar (Karlsruhe, Germany) and β-cyclodextrin, 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4MIM][PF_6]) and 1-hexyl-3-methylimidazoliumhexafluorophosphate ([C_6MIM][PF_6]) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the work.

[78] M. Tobiszewski, A. Mechlinska, B. Zygmunt, J. Namiesnik, TrAC-Trends Anal. Chem. 28 (2008) 943.

[79] C. J. Welch, N. Wu, M. Biba, R. Hartman, T. Brkovic, X. Gong, R. Helmy, W. Schafer, J. Cuff, Z. Pirzada, L. Zhou, TrAC-Trends Anal. Chem. 28 (2010) 667.

Kits SampliQ QuEChERS (Agilent Technologies Inc., Wilmington, DE, USA) consisted of 50 mL buffered QuEChERS extraction tubes (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) and 15 mL dispersive tubes containing different mixtures: A (150 mg C_{18} and 900 mg $MgSO_4$); B (400 mg C_{18}, 400 mg PSA and 1200 mg $MgSO_4$); or C (400 mg C_{18}, 400 mg PSA, 400 mg graphitized carbon (GCB) and 1200 mg $MgSO_4$).

Acrodisc 13 mm syringe filters with 0.2 µm nylon membrane (Pall Corp., MI, USA) were used for filtration of samples prior to the injection in the chromatographic system. A 0.2 µm nylon membrane filter (Supelco, Bellefonte, PA, USA) was used for filtration of mobile phase.

A stock standard solution of 10 µg mL^{-1} of OTA in MeCN was obtained from Supelco. Working solutions were prepared by evaporation of the stock standard solution to near dryness using a gentle stream of N_2, and diluting with the appropriate amount in MeOH and H_2O (50:50, v/v). These solutions were stored at -20ºC.

2.2.2. Instrumentation and software

All experiments were carried out using an Agilent HP-1200 series capillary HPLC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump (20 µL min^{-1} maximum flow-rate), online degasser, autosampler (8 µL loop), and a column thermostat. This system was coupled with a LIF detector (Zetalif Evolution model LIF UV-01, Picometrics S.A., Ramonville, France) equipped with a 325 nm He-Cd laser (see Figure 2.1). A fused-silica capillary (75 µm I.D.) from Polymicro Technologies (Phoenix, AZ, USA) was used to couple the HPLC and LIF detector. ChemStation software (A.10.20 [1757] version) was used for data acquisition and processing. A Luna C_{18} (150×0.5 mm, 5 µm) chromatographic column from Phenomenex (Torrance, CA, USA) was used for separation.

The pH of the different buffers was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of ±0.01 pH unit. A centrifuge Model Universal 320R (Hettich, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA), a mechanical shaker (model 384 from Vibromatic, Noblesville, USA) and a

Evaporator EVA EC-S/EVA LS-S (VLM GmbH, Bielefeld, Germany) were also used for sample treatment.

The statistical software STATGRAPHICS Centurion XV.II was used for data treatment.

2.2.3. Chromatographic conditions

The analyses were performed on a Luna C_{18} column (150×0.5 mm, 5 µm, 100Å). An isocratic mobile phase of water (2% acetic acid, 0.2 M SDS):MeOH (30:70, v/v) at a flow rate of 14 µL min^{-1} was used; the column temperature was kept at 40 ºC and the injection volume was 1.20 µL. Under optimum conditions, the analysis took around 4 min. SDS micellar solution was used in the mobile phase to increase the fluorescence intensity and the efficiency.

2.2.3. Sample treatment

In this study, we have tested three different methodologies for sample treatment in the determination of OTA in wine: DLLME, IL-DLLME and QuEChERS. With this purpose, different wine samples (white, rose, and red) were purchased from local markets. The samples were stored refrigerated in their original bottles or containers at 4-5°C until analysis.

2.2.3.1. DLLME procedure

An aliquot of 5.0 mL of sample was placed into a 10 mL screw cap test tube with conical bottom and 0.25 g of NaCl (5%; w/v) was added. The mixture of the disperser solvent (940 µL of MeCN) and the extraction solvent (660 µL of chloroform) was rapidly injected into the test tube with a 2.0 mL syringe. The solution was shaken and a cloudy solution was formed in the tube. In this step, the OTA was extracted into the fine droplets of chloroform. Later, the mixture was centrifuged at 5000 rpm for 1 min and the fine particles were sedimented at the bottom of the tube. The sedimented phase (approximately 700 µL) was removed using a 1 mL syringe, evaporated to near dryness using a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The

solution was filtered and injected into the capillary HPLC–LIF system for analysis. After sample treatment, a preconcentration factor of 5 was achieved. Figure 2.1 shows the DLLME procedure.

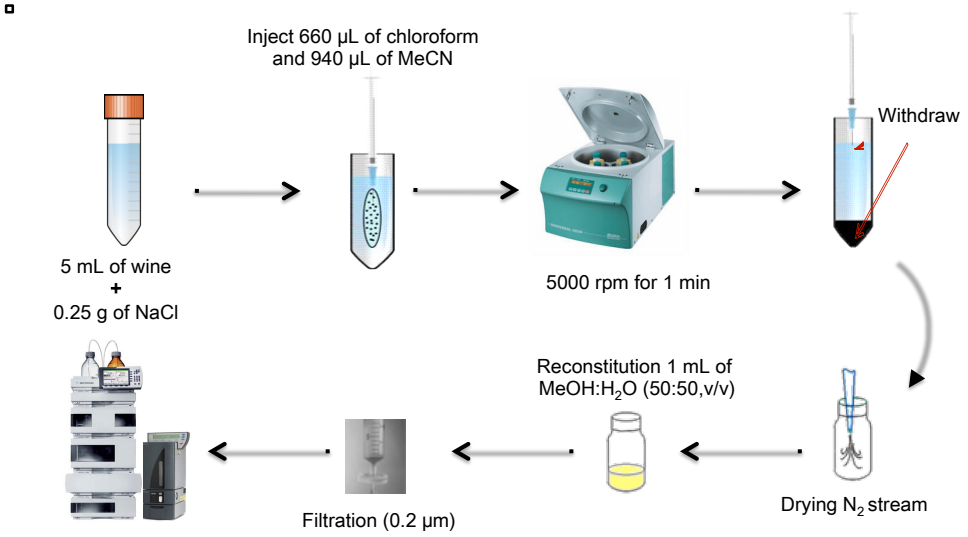

Figure 2.1. Diagram of DLLME for the determination OTA in wine samples

2.2.3.2. IL-DLLME procedure

An aliquot of 5.0 mL of sample at pH 2.8 (adjusted with phosphoric acid) was placed into a 10 mL screw cap test tube with conical bottom. A mixture of 100 mg of [C_6MIM][PF_6] (extraction solvent) and 700 µL of MeOH (disperser solvent) was immediately injected into the sample solution with a 2.0 mL syringe, in order to induce the formation of a cloudy solution, formed by fine droplets of IL dispersed in the aqueous sample. The mixture was shaken by hand a few seconds and subsequently centrifuged for 5 min at 5000 rpm. Dispersive particles of IL were sedimented at the bottom of the centrifuge tube and the upper aqueous phase was removed with a syringe and discarded. After this process, the IL phase was dissolved in 1 mL of MeOH:H_2O (50:50, v/v). The solution was filtered with a 0.2 µm filter and injected into the capillary HPLC-LIF system for analysis. Thus, after sample treatment, a

preconcentration factor of 5 was achieved. The IL-DLLME procedure is summarized in Figure 2.2.

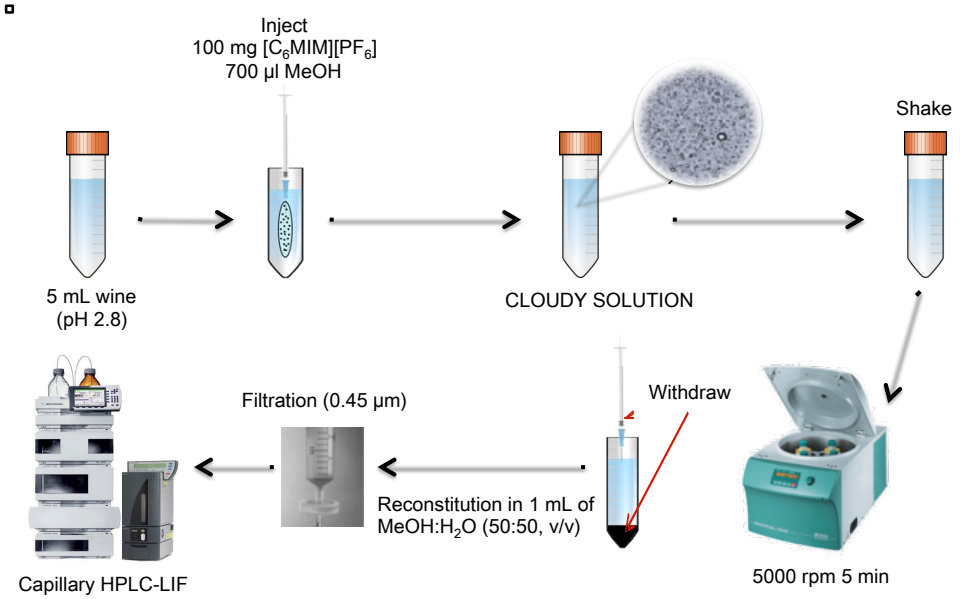

Figure 2.2. Diagram of IL-DLLME for the determination OTA in wine samples

2.2.3.3. QuEChERS procedure

An aliquot of 3 mL of wine and 8 mL of 30 mM NaH_2PO_4 buffer pH 7.0 was placed into a 50 mL screw cap test tube with conical bottom, shaking by vortex for 10 s. Subsequently, 10 mL of 5% formic acid in MeCN was added to the tube, shaking by vortex for 10 s. Agilent SampliQ EN QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) was added and the tube was shaken vigorously for 1 min. After that, the sample was centrifuged at 9000 rpm for 4 min; 4 mL of the upper MeCN layer was transferred to another tube containing the dSPE sorbent (400 mg C_{18}, 400 mg PSA and 1200 mg $MgSO_4$), shaking in vortex for 1 min. The tube was centrifuged at 9000 rpm for 2 min. Then, 1 mL of the supernatant was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). Finally, the samples were filtered

with a 0.2 µm filter before injection. After QuEChERS treatment, a dilution factor of 0.30 is obtained. A scheme of the procedure is shown in Figure 2.3.

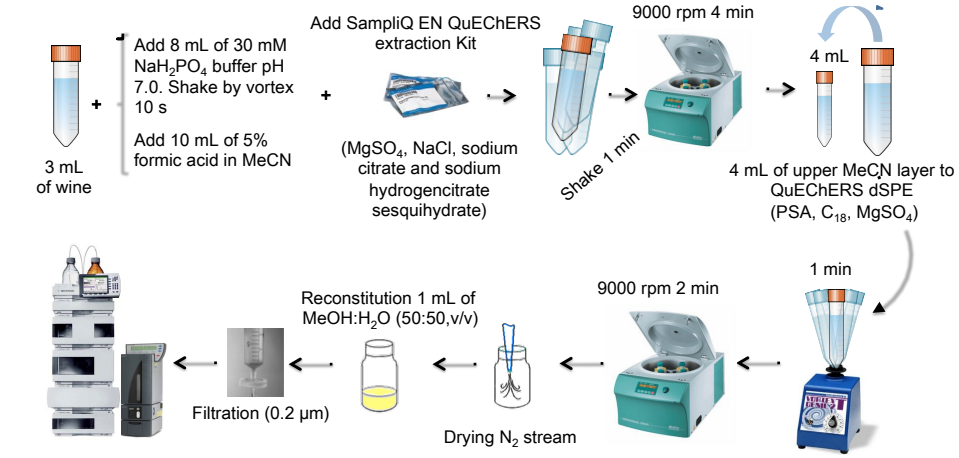

Figure 2.3. Diagram of QuEChERS treatment for the determination OTA in wine samples

2.3. Results and discussion

2.3.1. Optimization of capillary HPLC–LIF method

Peak area, efficiency and analysis time were taken into account to select the adequate determination procedure. Different natures of mobile phase (water with 2% acetic acid as solvent A, and MeCN, MeOH or a mixture of both of them as solvent B) were tested. The best results were obtained with MeOH as solvent B.

The use of CDs has been previously reported as modifiers of native fluorescence of mycotoxins [80]. Thus, the addition of 6 mM β-CD to solvent A was considered. However, the improvement in the sensitivity was not significant and a higher noise in the baseline was observed. As an alternative, the addition of a micellar anionic media (SDS) to solvent A was proposed. The presence of this anionic surfactant at a concentration above its critical micellar concentration (CMC) not only provided an

[80] C. M. Maragos, M. Appell, V. Lippolis, Z. Visconti, L. Catucci, M. Pascale, Food Addit. Contam. 25 (2008) 164.

increase on the fluorescence quantum yield of the analyte (as could be expected as effect of this organised medium on a fluorophore solution [81]) but also improved the chromatographic efficiency, giving a sharper peak. So, SDS was used for the rest of the work.

Subsequently, the percentages of solvent A and B were studied in order to get the best peak shape and sensitivity in the shortest time; the results are shown in Figure 2.4 and the following conditions were selected: water (2% formic acid, 0.1 M SDS): MeOH (30:70).

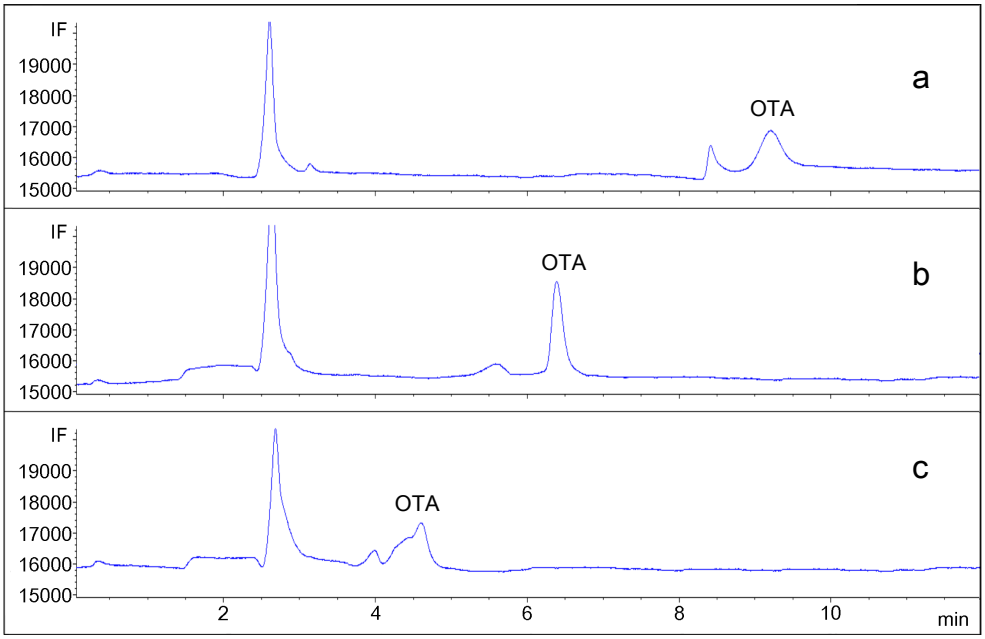

Figure 2.4. Optimization of percentage of solvent A (water, 2% acetic acid, 0.1 M SDS) and B (MeOH): A:B (40:60) (a); A:B (30:70) (b); A:B (20:80) (c). (10 μg L^{-1} OTA)

Then, the percentage of acetic acid in solvent A was optimized between 0 and 5%. The best results were obtained with 2% acetic acid (Figure 2.5).

[81] G. L. McIntire, Crit. Rev. Anal. Chem. 21 (1990) 257.

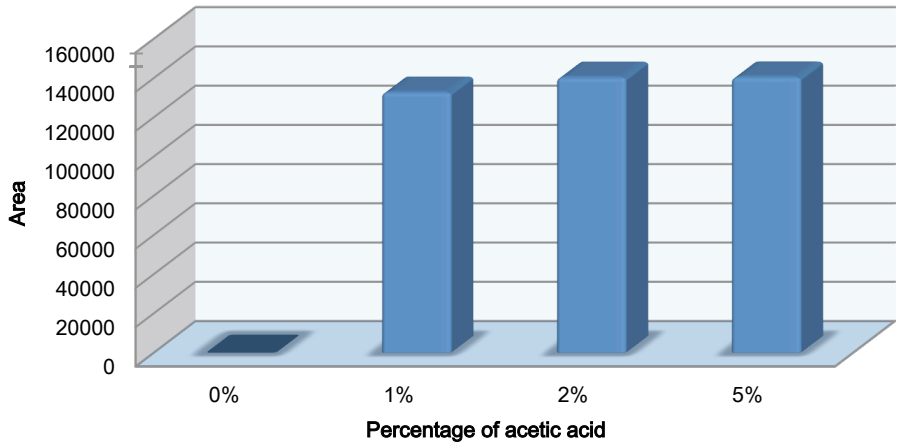

Figure 2.5. Influence of percentage of acetic acid (solvent A) on peak area (20 µg L^{-1} OTA)

Finally, concentrations of 0.1, 0.2 and 0.3 M SDS in solvent A were tested, obtaining the best efficiency and the highest signal and the shortest time with 0.2 M SDS. Higher concentrations did not improve the analysis; so 0.2 M was selected for the rest of the experimental work (see Figure 2.6).

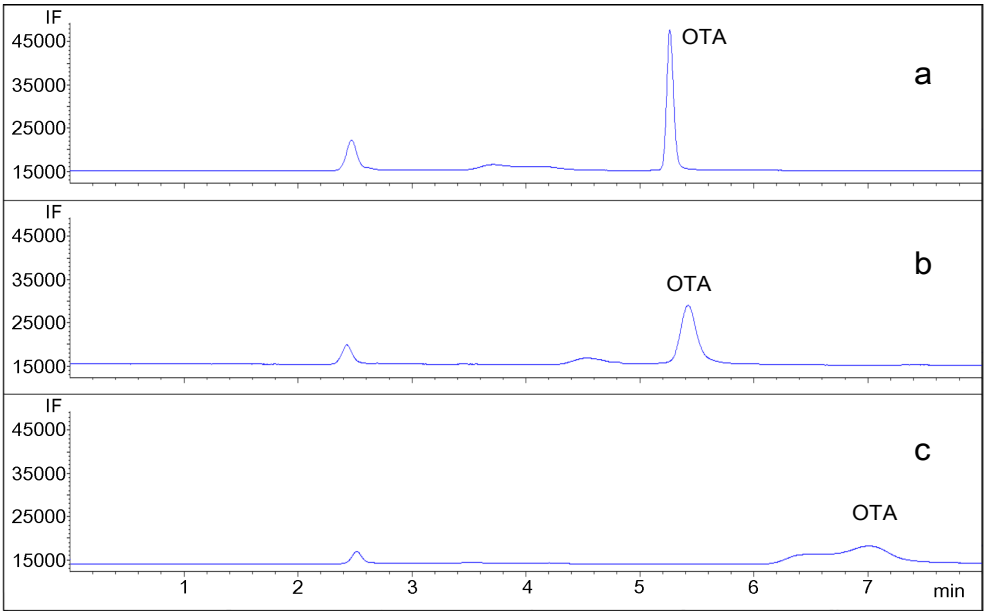

Figure 2.6. Optimization of SDS concentration (20 µg L^{-1} OTA): 0.2 M SDS (a); 0.1 M SDS (b); without SDS (c)

The effect of column temperature was examined in the range of 30–50 °C; an optimum value of 40 °C was chosen, as a compromise between analysis time and column life (Figure 2.7).

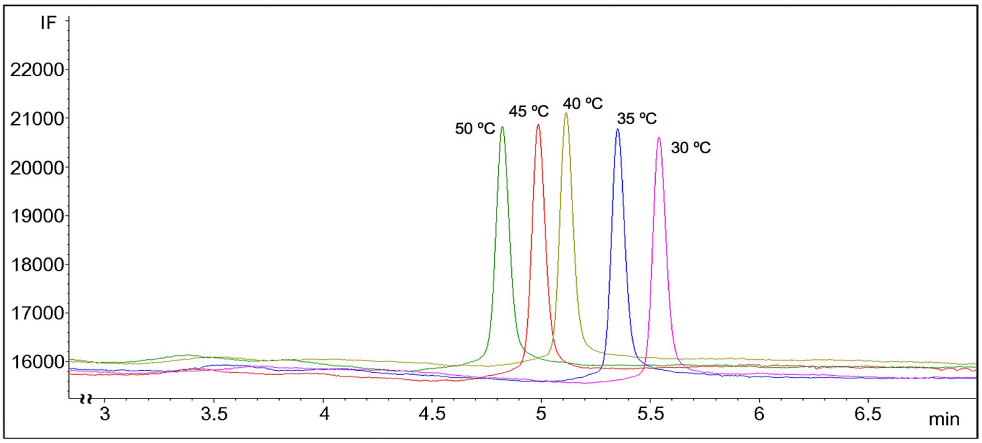

Figure 2.7. Optimization of column temperature (2 µg L^{-1} OTA)

The flow rate was also tested between 10–15 µL min^{-1}, selecting a final optimum value of 14 µL min^{-1}, as higher flow rates involved higher pressure without a significant improvement in the analysis (Figure 2.8).

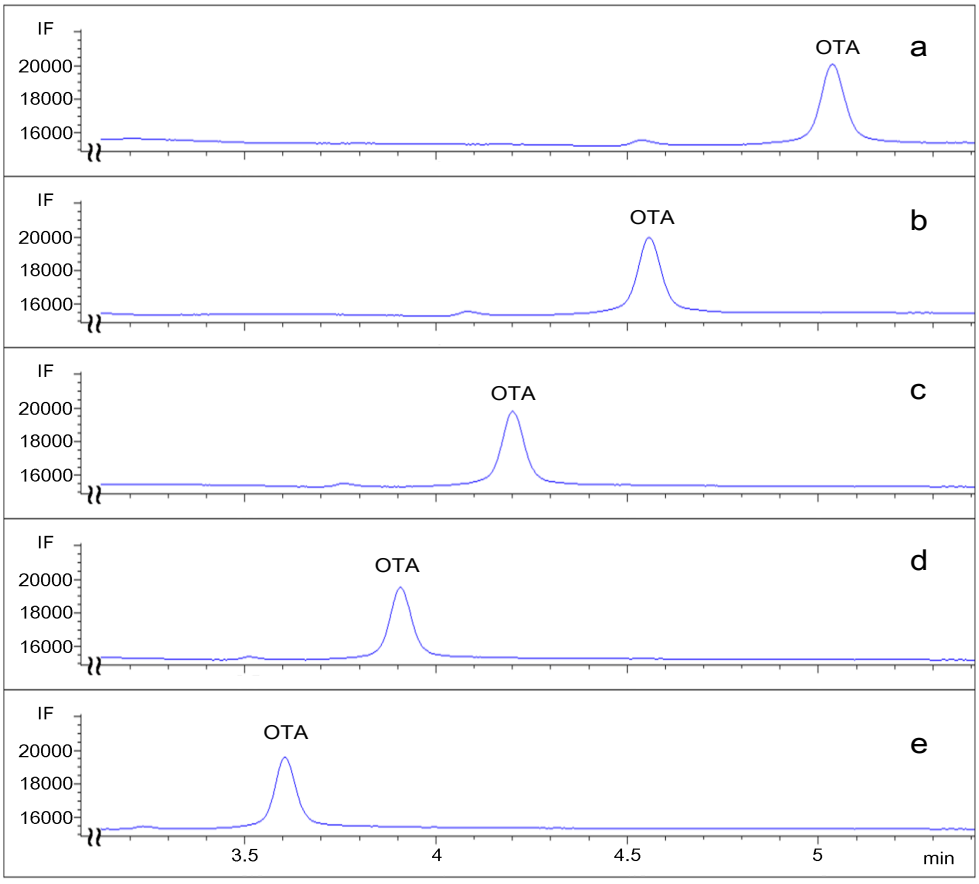

Figure 2.8. Optimization of flow rate (2 μg L^{-1} OTA): 10 μL min^{-1} (a); 11 μL min^{-1} (b); 12 μL min^{-1} (c); 13 μL min^{-1} (d); 14 μL min^{-1} (e)

Finally, the injection volume was increased from 0.6 to 1.4 μL (Figure 2.9), selecting 1.2 μL as optimum, as higher volumes involved peak splitting.

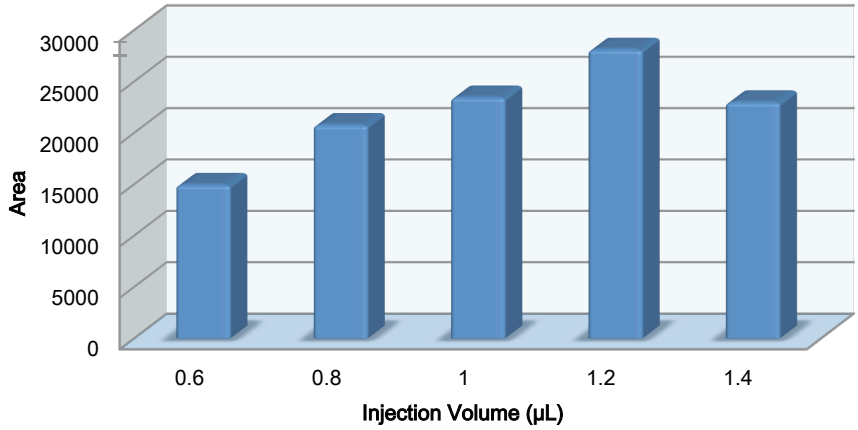

Figure 2.9. Influence of injection volume on peak area (2 µg L^{-1} OTA)

Table 2.1 summarizes the final optimum values for capillary HPLC-LIF detection. At these final conditions, the analysis of OTA took place in less than 3.5 min.

Table 2.1. Summary of the optimum values for the chromatographic procedure

Column	Luna C_{18} (150×0.5 mm, 5 µm)
Mobile phase	40% Water (2% acetic acid, 0.2 M SDS) 60% MeOH
Flow rate	14 µL min^{-1}
Injection volume	1.2 µL
Temperature	40 ºC

2.3.2. Optimization of sample treatments

The sample treatments were optimised separately taking into account the significant variables involved in each case, with the purpose to obtain the highest absolute recovery of OTA.

2.3.2.1. Optimization of the DLLME procedure

The optimization of DLLME involved the study of the following parameters: nature and volume of extraction solvent, nature and volume of disperser solvent, percentage of NaCl, extraction time and shaking mode. All the experiments were performed using samples of 5 mL of white wine spiked with 400 ng L^{-1} of OTA. First, it was necessary to select the extraction and disperser solvents. Aliquots of 600 µL of chloroform, carbon disulphide, chlorobenzene and tetrachloroethylene were tested as extraction solvents, using 1000 µL MeOH as disperser solvent; as can be observed in Figure 2.10. The best results were obtained with chloroform and it was used for the rest of the work.

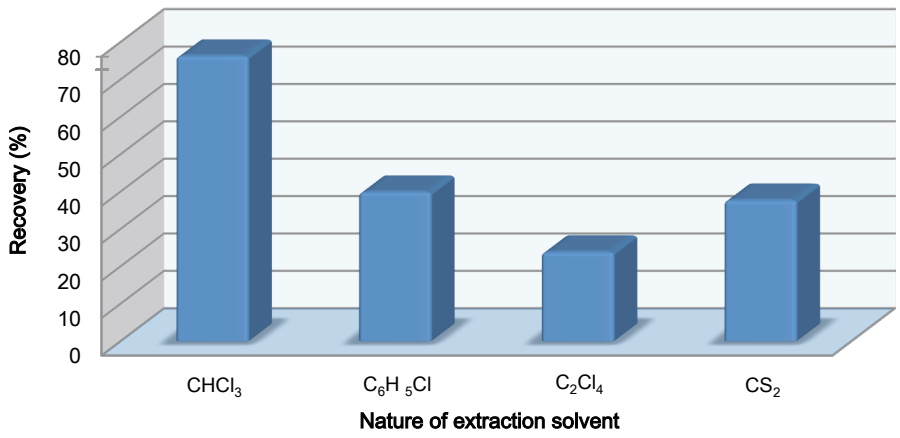

Figure 2.10. Influence of the nature of extraction solvents on the extraction efficiency

Then, aliquots of 1000 µL of MeOH, EtOH, ACO, MeCN and THF were tested as disperser solvents using 600 µL of chloroform as extraction solvent, and MeCN was selected because it offered higher recoveries (Figure 2.11).

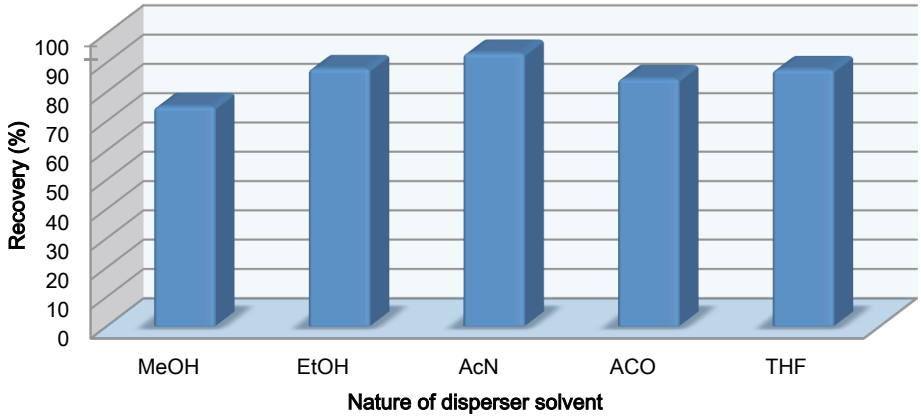

Figure 2.11. Influence of the nature of the disperser solvents on the extraction efficiency

Once the extraction and disperser solvents have been selected, their volumes and the percentage of NaCl were optimized by a multivariate approach using an experimental design, which takes into account possible interactions between the variables. A central composite design (2^3 + star, face centred), with three spaced central points, involving 17 runs, was used an as approach to generate the response surface, using the recovery percentage as analytical response. Different factors were studied in the following ranges: MeCN volume (800–1000 µL), chloroform volume (500–700 µL) and percentage of NaCl (0-5%). A Pareto chart (Figure 2.12) was obtained from the screening experimental design, showing the variables and/or interactions between them having significant effects when its value is changed in the selected experimental domain.

As can be observed, the extraction solvent volume and the quadratic terms of extraction solvent volume, disperser solvent volume and percentage of salt (AA, BB and CC, respectively) had a significant effect on the recovery of OTA. The obtained response surface (Figure 2.13) gave the optimum conditions for the studied parameters: extraction solvent = 660 µL, disperser solvent = 940 µL and percentage of salt = 5%. The P-value for the lack of fit test for the model was 38.0% and the determination coefficient (R^2) was 93.9%, showing the suitability of this design.

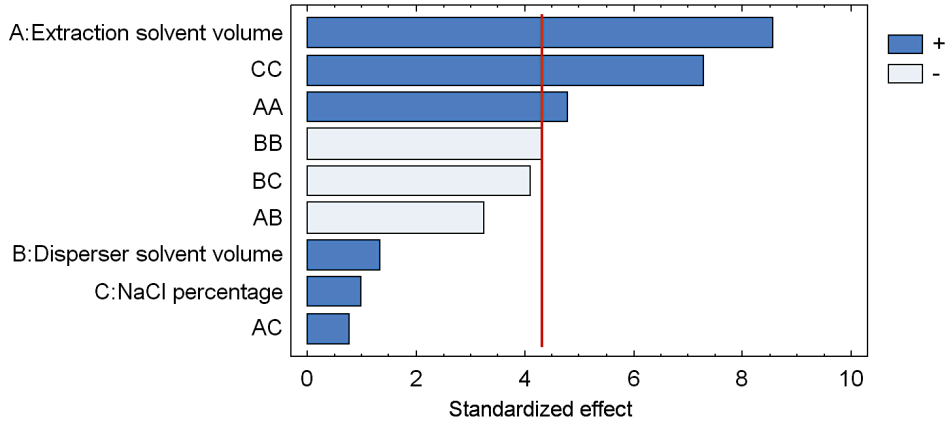

Figure 2.12. DLLME optimization: Pareto chart showing the effects of the studied variables on the recovery percentage: (+) Positive effects on the response; (-) negative effects on the response. Red line shows the limit of decision to consider the significance of the factors (based on the effect = estimated effect/standard error, P-value = 0.05 at 95% of confidence)

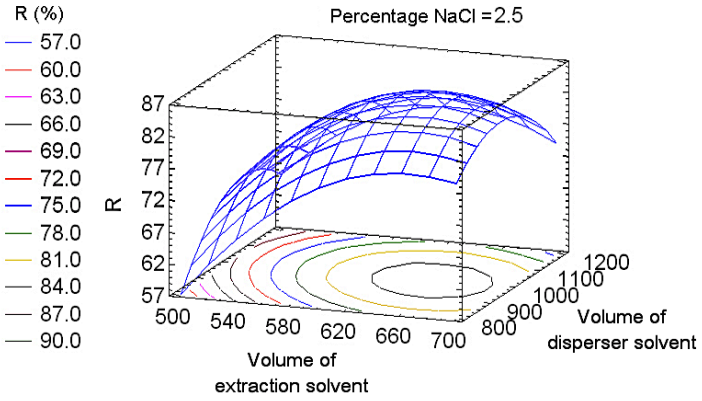

Figure 2.13. Estimated response surface obtained in the DLLME optimization procedure using a central composite design (2^3 + star, face centred), with three spaced central points

Under the previous optimum conditions, the effects of extraction time (defined as the interval between the injection of disperser and extraction solvents and centrifugation) and shaking mode (manual, vortex and mechanical shake) were tested in the range of 0–10 min. It was concluded that these variables had no influence in the extraction efficiency (Figure 2.14). The reason is that the extraction solvent can be evenly

dispersed after the formation of the cloudy solution, the transition of the analyte from the sample to the extraction phase can be very fast, and the equilibrium state can be subsequently achieved very quickly, resulting in a very short extraction time [206]. Thus, the extraction was carried out by shaking the sample manually for a few seconds. Finally, under the optimum conditions, an enrichment factor of 6.6 (calculated as the ratio of analyte concentration in the sediment, and the initial concentration of analyte in the aqueous sample) and a final preconcentration factor of 5 was achieved.

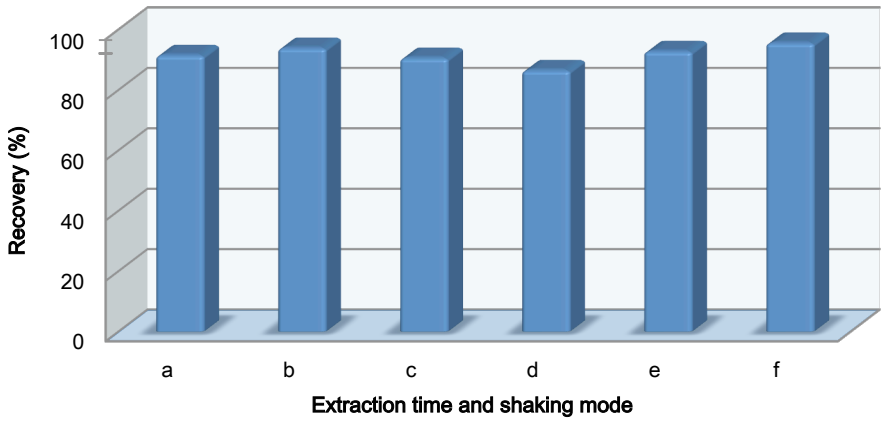

Figure 2.14. Effects of extraction time and shaking mode on the extraction efficiency. (a) without shaking, (b) manual shaking for few second; (c) shaking by vortex for 1 min; (d) mechanical shaking for 1 min; (e) mechanical shaking for 5 min; (f) mechanical shaking for 10 min

After optimization of all the variables, the whole DLLME was performed following the scheme previously presented in Figure 2.1. Chromatograms of a blank and a spiked white wine sample, treated following the proposed DLLME procedure, are shown in Figure 2.15. As can be seen, no interference signals were observed. Similar chromatograms were obtained for rose and red wine samples.

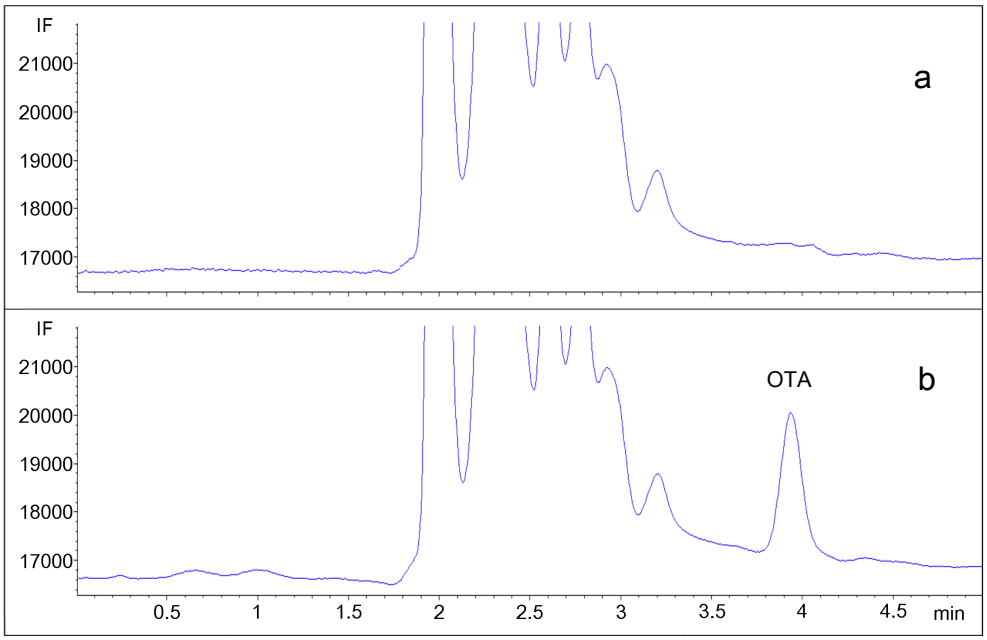

Figure 2.15. Chromatogram of a white wine sample: blank sample (a); sample spiked at 1 μg L^{-1} of OTA (b)

2.3.2.2. Optimization of the IL-DLLME procedure

In IL-DLLME, the extraction solvent of DLLME is substituted by an IL. The extraction solvents should have a higher density than water, extraction capability of compounds of interest and good chromatographic behaviour. Previous reported works about the use of IL-DLLME showed that [C_4MIM][PF_6] and [C_6MIM][PF_6] meet most of these requirements [66,82]. Thus, they were chosen and tested in the present study. All the experiments were performed using samples of 5 mL of white wine spiked with 2 ng mL^{-1} of OTA. Firstly, analyses were carried out using 80 mg of the selected ILs as extraction solvent and 700 μL of MeOH as disperser solvent. When using [C_4MIM][PF_6], the solution was always transparent and no sedimented phase appeared at the bottom of the tube after centrifugation. On the contrary, when [C_6MIM][PF_6] was tested, a

[82] J. G. Huddleston, A. E. Visser, W. M. Reichert, H. D. Willauer, G. A. Broker, R. D. Rogers, Green Chem. 3 (2001) 156.

sedimented phase was clearly obtained. Therefore, [C_6MIM][PF_6] was selected as extraction solvent.

Subsequently, 700 µl of MeOH, EtOH, ACO, MeCN, or THF were tested as disperser solvents, with 80 mg of [C_6MIM][PF_6]. When ACO and THF were used, no sedimented phase appeared after centrifugation. Figure 2.16 shows the results for the other solvents and, as can be seen, the best results in terms of recoveries were obtained with MeOH.

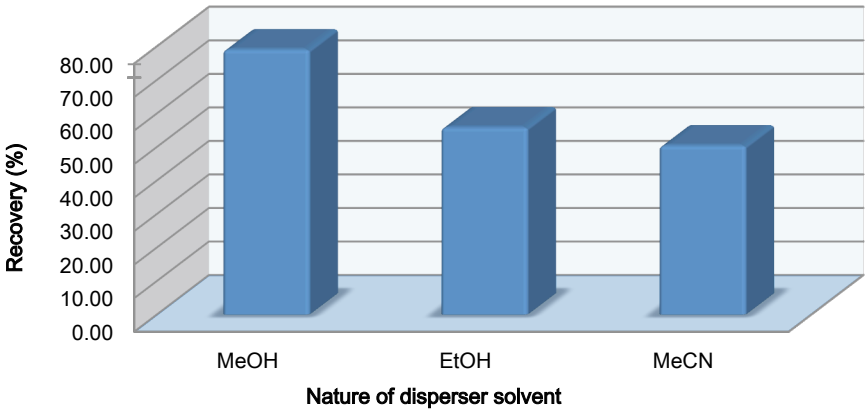

Figure 2.16. Influence of nature of disperser solvents on the extraction efficiency

Further experiments were carried out to evaluate the influence of sample pH, amount of IL, volume of disperser solvent, percentage of NaCl (which is frequently used to provide a salting-out effect), extraction time and shaking mode on the extraction efficiency (that is, on recovery percentages). The pKa of OTA is 3.29; above this pH, the molecule is charged and cannot be extracted with IL. Thus, the effect of pH sample was examined in the range of 2.3–3.3. An optimum value of 2.8 was chosen, adjusting the pH by additioning phosphoric acid (see Figure 2.17).

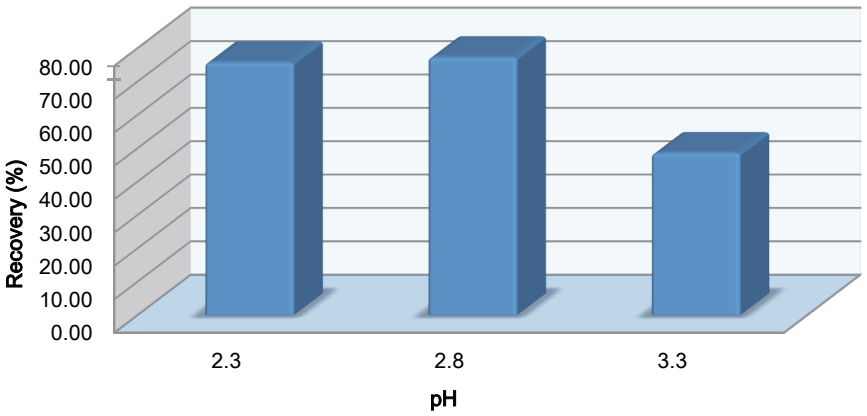

Figure 2.17. Influence of sample pH on the extraction efficiency

Subsequently, the amount of IL was tested between 60 and 120 mg, selecting 100 mg as optimum (Figure 2.18).

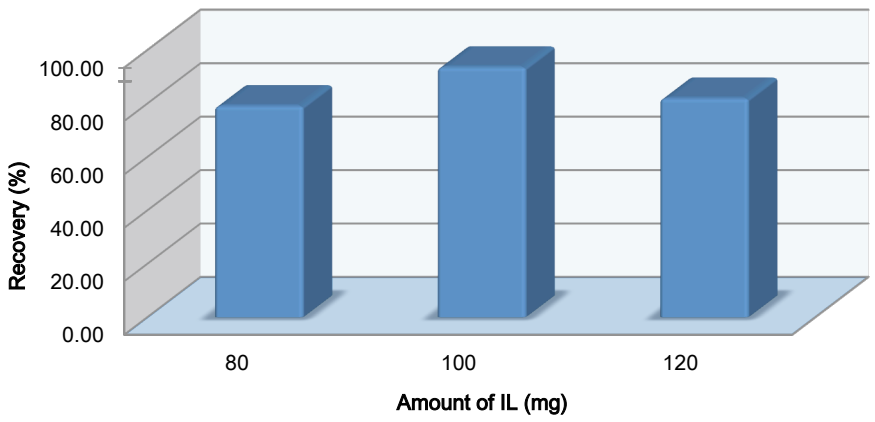

Figure 2.18. Influence of amount of IL on the extraction efficiency

The volume of MeOH was studied between 500 and 900 µL and a final optimum value of 700 µL was chosen (Figure 2.19).

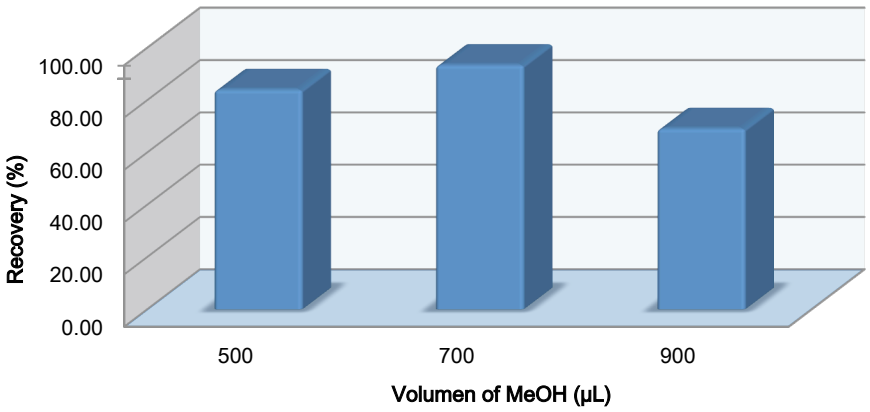

Figure 2.19. Influence of volume of MeOH on the extraction efficiency

Then, the addition of NaCl was tested in the range of 0–5%, but no improvement in the recovery percentage was observed. Thus, the subsequent experiments were carried out without NaCl.

Finally, the effects of extraction time and shaking mode (mechanical, vortex and manual shaking) were tested in the range of 0–10 min. As in the case of DLLME, it was concluded that the time and shaking mode did not affect significantly the extraction efficiency (Figure 2.20). Thus, the extraction was carried out by manually shaking the mixture for a few seconds.

The whole IL-DLLME procedure is described in Figure 2.2.

Figure 2.21 shows the chromatograms of a blank and a spiked white wine sample, treated following the proposed IL-DLLME procedure. As can be seen, a chromatogram free of interference signals was obtained. Similar chromatograms were obtained for rose and red wine samples.

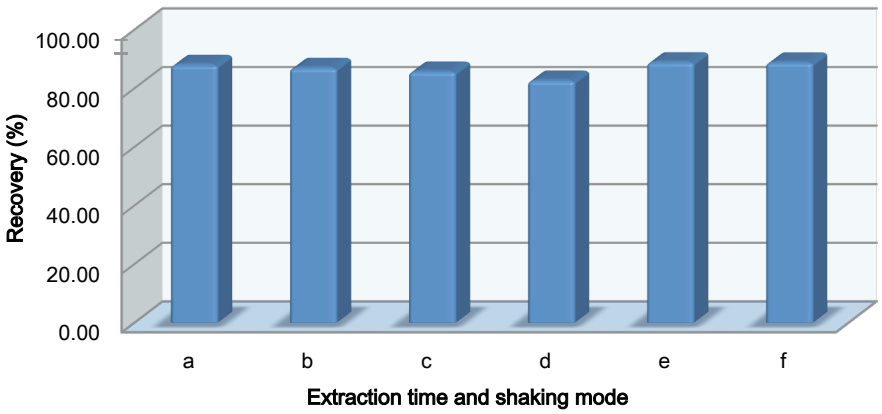

Figure 2.20. Effects of extraction time and shaking mode on the extraction efficiency. (a) without shaking; (b) manual shaking for a few second; (c) shaking by vortex for 1 min; (d) mechanical shaking for 1 min; (e) mechanical shaking for 5 min; (f) mechanical shaking for 10 min

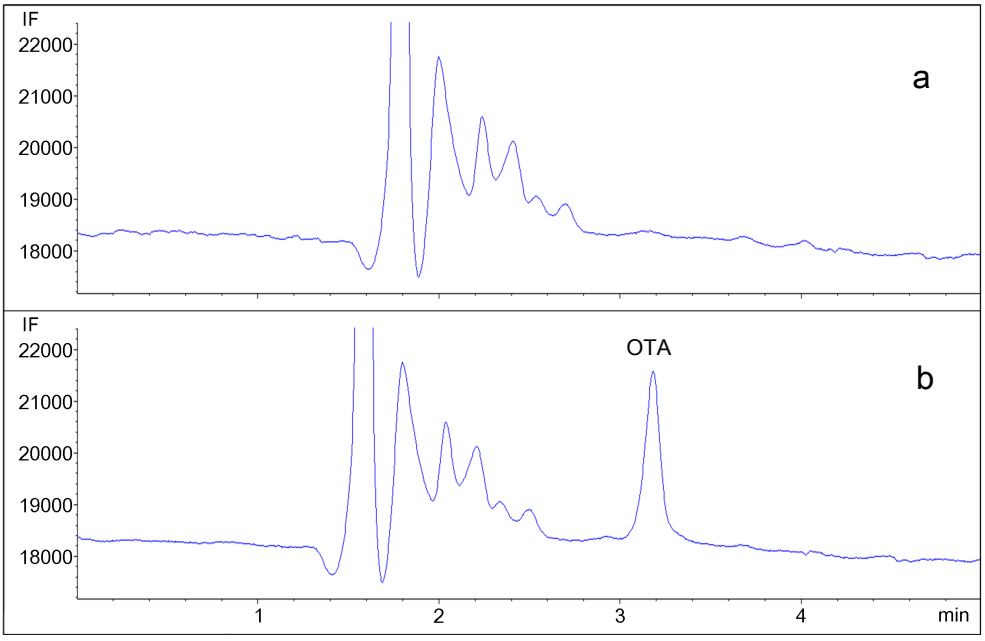

Figure 2.21. Chromatogram of a white wine sample: blank sample (a); sample spiked at 1 μg L^{-1} of OTA (b)

2.3.2.3. Optimization of the QuEChERS procedure

The QuEChERS procedure was adapted from a technical note proposed by Agilent Technologies for the determination of quinolones in bovine liver [83] and used in our laboratory for the determination of quinolones in milk [84]. The first step of the extraction/partition process was applied following the commercial proposed protocol: an aliquot of 3 mL of wine and 8 mL of 30 mM NaH_2PO_4 buffer pH 7.0 was placed into a 50 mL screw cap test tube with conical bottom, shaking by vortex. Subsequently, 10 mL of 5% formic acid in MeCN was added to the tube, shaking again. Agilent SampliQ EN QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium citrate sesquihydrate) was added and the tube was shaken vigorously for 1 min. After that, the sample was centrifuged at 9000 rpm for 4 min. For the second step, 4 mL of the extract was submitted to a subsequent clean-up by dSPE; three kits were tested containing salt and different sorbents, such as: a) C_{18} and $MgSO_4$; b) PSA, C_{18} and $MgSO_4$; and c) GCB, PSA, C_{18} and $MgSO_4$. OTA was lost when GCB was included in the clean-up step; on the other hand, the final extract was cleaner including PSA and the recovery was not affected. So, the best results were obtained with the kit containing PSA, C_{18} and $MgSO_4$.

After shaking, centrifugation and evaporation to dryness in N_2 stream of 1 mL of the supernatant, the final reconstitution step consisted of 1 mL of MeOH:H_2O (50:50, v/v). The procedure is described in detail in Figure 2.3.

Figure 2.22 shows the chromatograms of a blank and a spiked white wine sample analysed after the QuEChERS procedure. As can be seen, no interference peaks were found comigrating with the analyte. Similar chromatograms were obtained for rose and red wine samples.

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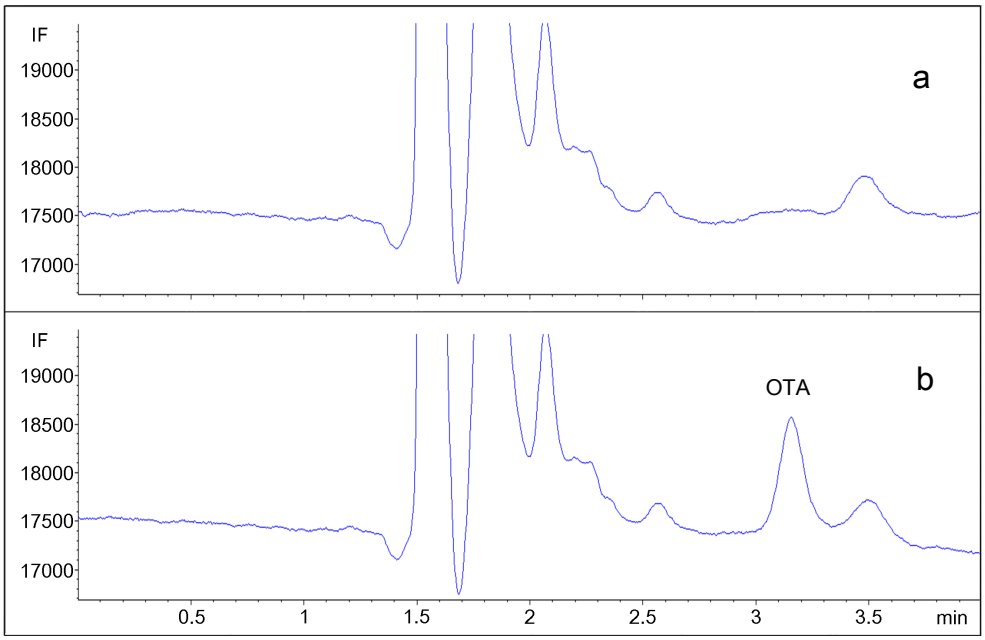

Figure 2.22. Chromatogram of a white wine sample: blank sample (a); sample spiked at 5 μg L^{-1} of OTA (b)

2.3.3. Performance characteristics and comparison of the three extraction methods

In order to check the suitability of the three proposed methods for the determination of OTA in wine samples, they were characterised in terms of linear dynamic range, LOD and LOQ, precision and trueness (by means of recovery assays). Subsequently, a comparison of the results was carried out.

2.3.3.1 Calibration curves, LOD and LOQ

Matrix-matched calibration curves were obtained using white wine samples spiked with the following concentrations of OTA, depending on the sensitivity provided by the sample treatment: DLLME: 0.02, 0.1, 0.2, 1, 2 and 4 μg L^{-1} (considering the preconcentration factor, those correspond to concentrations of OTA in the final extracts of 0.1, 0.5, 1, 5, 10, and 20 μg L^{-1}, respectively); IL-DLLME: 0.02, 0.2, 1, 2 and 4 μg L^{-1} (which correspond to concentrations of OTA in the final extracts of 0.1, 1, 5, 10, and 20

μg L^{-1}, respectively); and QuEChERS: 0.5, 5, 10, 20, and 30 μg L^{-1} (giving concentrations of OTA in the final extracts of 0.15, 1.5, 3, 6, and 9 μg L^{-1}, respectively). Each concentration level was prepared in duplicate, submitted to the subsequent extraction procedure and injected in triplicate.

The statistical parameters were calculated by least-square regression, and LODs and LOQs were considered as 3×S/N ratio and 10×S/N ratio, respectively. Table 2.2 summarizes the results.

Table 2.2. Statistics and performance characteristics of the capillary HPLC-LIF methods

Sample treatment	Linear range (ng L^{-1})	R^2	LOD (ng L^{-1})	LOQ (ng L^{-1})
DLLME	18.4 - 4000	0.998	5.5	18.4
IL-DLLME	17.5 - 4000	0.997	5.2	17.5
QuEChERS	28.6 - 30000	0.997	85.7	286

As can be seen, lower LODs and LOQs were obtained when DLLME or IL-DLLME procedures were used. This difference is due to the preconcentration obtained with both procedures, instead of the dilution produced by the QuEChERS methodology. Anyway, in all cases the high sensitivity provided by capillary HPLC with LIF detection allows the quantification of OTA at concentrations much lower than the maximum content established for wine [4].

2.3.3.2. Precision study

The precision of the whole methods was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by application of the whole procedures to three white wine samples (experimental replicates) spiked at three concentration levels of OTA: 0.2, 1 and 2 μg L^{-1} for DLLME and IL-DLLME and 1, 2 and 10 μg L^{-1} for QuEChERS. Each sample was injected in triplicate (instrumental replicates) and the whole procedure was developed on the same day. Intermediate precision was evaluated with a similar procedure, but the samples were treated and analysed in five different days.

The results, expressed as RSD of peak areas, are shown in Table 2.3. Although slightly higher RSD were obtained for IL-DLLME, in all cases good precision was achieved (RSD than 8.5%), being in agreement with current legislation [7].

Table 2.3. Precision study in white wine samples (%RSD of peak areas)

		Repeatability (n=9)	Intermediate Precision (n=15)
DLLME	Level 1 (0.2 μg L^{-1})	1.9	4.9
	Level 2 (1 μg L^{-1})	2.6	2.9
	Level 3 (2 μg L^{-1})	1.3	5.2
IL-DLLME	Level 1 (0.2 μg L^{-1})	6.7	8.0
	Level 2 (1 μg L^{-1})	6.5	7.9
	Level 3 (2 μg L^{-1})	8.1	8.5
QuEChERS	Level 1 (1 μg L^{-1})	4.2	5.4
	Level 2 (2 μg L^{-1})	4.0	5.2
	Level 3 (10 μg L^{-1})	3.8	3.7

2.3.3.3. Recovery studies

In order to check the trueness of the proposed methodology, recovery experiments were carried out. Thus, white, rose and red wine samples were spiked at three concentration levels, similar to those used in the precision study. Three samples were prepared at each concentration level, submitted to the DLLME, IL-DLLME and QuEChERS procedures and injected in triplicate in the capillary HPLC-LIF system. Blank samples were also analysed, and none of them gave a positive result for OTA.

The results are shown in Table 2.4, and, as can be seen, higher recoveries were obtained with the DLLME and IL-DLLME procedures, although the recoveries obtained with the three methods can be considered in agreement with current legislation, which establishes that the recoveries must be between 70 and 110% [7].

Table 2.4. Recovery study in wine samples with DLLME, IL-DLLME and QuEChERS treatments (n=9)

			White wine	Rose wine	Red wine
DLLME	Level 1 (0.2 μg L^{-1})	R (%)	94.9	94.4	98.3
		RSD (%)	1.9	3.9	4.1
	Level 2 (1 μg L^{-1})	R (%)	94.8	92.7	96.3
		RSD (%)	2.9	2.2	1.7
	Level 3 (2 μg L^{-1})	R (%)	91.7	95.8	98.1
		RSD (%)	1.4	1.7	1.8
IL-DLLME	Level 1 (0.2 μg L^{-1})	R (%)	93.5	94.2	93.1
		RSD (%)	6.7	7.3	4.0
	Level 2 (1 μg L^{-1})	R (%)	91.2	90.6	94.1
		RSD (%)	6.5	5.7	3.4
	Level 3 (2 μg L^{-1})	R (%)	81.3	85.4	83.7
		RSD (%)	4.2	5.1	4.8
QuEChERS	Level 1 (1 μg L^{-1})	R (%)	81.3	85.4	83.7
		RSD (%)	4.2	5.1	4.8
	Level 2 (2 μg L^{-1})	R (%)	82.6	83.8	84.5
		RSD (%)	4.0	4.9	4.3
	Level 3 (10 μg L^{-1})	R (%)	83.6	86.2	84.0
		RSD (%)	3.8	4.7	4.1

2.3.3.4. Other features of the proposed extraction methods

After comparison of the performance characteristics of the proposed extraction methods, other features were considered.

Regarding throughput, in general the QuEChERS procedure seems to be more time-consuming than DLLME and IL-DLLME, as it requires two centrifugations and the final evaporation step; however, the sample throughput could be higher than for the DLLME and IL-DLLME, in which the extraction of the upper aqueous phase must be carried out individually for each sample, while in the case of the QuEChERS it is possible to proceed with several samples simultaneously.

On the other hand, no extra clean-up procedure is required in DLLME and IL-DLLME, obtaining sample extracts slightly cleaner than using QuEChERS, and being a more environmentally friendly alternative because no contaminant organic solvents are

needed. In relation to the cost, both methodologies are similar and could be easily implemented in routine analytical laboratories.

2.4. Conclusions

A method based on a high sensitive and miniaturized technique, capillary HPLC with LIF detection, has been developed for the determination of OTA in different kinds of wine (white, rose and red). The addition of a micellar medium to the mobile phase has been used as an efficient way to improve sensitivity and efficiency. In addition, DLLME, IL-DLLME and QuEChERS methodologies have been proposed and compared as alternatives to the most frequent extraction methods for mycotoxins, based on SPE or IACs. General advantages of these methods with respect to IAC procedures are the shorter time and the relatively low-cost. DLLME has the advantages of simplicity of operation, rapidity, low-cost, and high-recovery, being also environmentally friendly. Also, the use of ILs in DLLME provides a fast and easy procedure that avoids using highly toxic solvents. On the other hand, QuEChERS is a very simple methodology that involves minimum steps, being very effective for the clean-up of complex samples. The LODs obtained with DLLME and IL-DLLME were lower than those obtained with QuEChERS procedure. This difference in the LODs is due to the preconcentration obtained with IL-DLLME, instead of the dilution produced by QuEChERS. Better recoveries were obtained with DLLME and IL-DLLME; however, the precision, evaluated in terms of repeatability and intermediate precision, was slightly better with QuEChERS and DLLME treatments. The three extraction methods have shown to be a good alternative for the extraction of OTA from wines, reducing extraction time, providing good recoveries and precisions and being environmentally friendly. It can be concluded that the methods fulfil the requirements established by the legislation for the determination of mycotoxins in foodstuff.

This work was published as:

"Determination of Ochratoxin A by capillary HPLC with laser induced fluorescence detection". N. Arroyo-Manzanares, L. Gámiz-Gracia, A. M. García-Campaña, Luminescence 25 (2010) 271.

“Comparison of different sample treatments for the analysis of ochratoxin A in wine by capillary HPLC with laser-induced fluorescence detection”. N. Arroyo-Manzanares, A. M. García-Campaña, L. Gámiz-Gracia, Anal. Bioanal. Chem. 401 (2011) 2987.

“Determination of ochratoxin A in wines by capillary liquid chromatography with laser induced fluorescence detection using dispersive liquid-liquid microextraction”. N. Arroyo-Manzanares, L. Gámiz-Gracia, A. M.

CHAPTER 3

Determinación de micotoxinas en cardo mariano mediante UHPLC-MS/MS usando un procedimiento basado en QuEChERS y microextracción líquida-líquida dispersiva

Multiclass mycotoxin determination in milk thistle by UHPLC-MS/MS using a procedure based on QuEChERS and dispersive liquid-liquid microextraction

Resumen

En este capítulo se propone un método de cromatografía líquida de ultra resolución acoplado a espectrometría de masas en tándem para la determinación de 15 micotoxinas (incluyendo aflatoxinas, fumonisinas, tricotecenos, ocratoxina A, citrinina, esterigmatocistina y zearalenona) en cardo mariano (*Silybum marianum*). Las micotoxinas fueron detectadas mediante ionización por electrospray en modo positivo y monitorización de reacción múltiple, consiguiéndose la separación de los analitos en menos de 4 min. El tratamiento de la muestra consistió en un primer paso basado en el procedimiento QuEChERS (que permitía la determinación de fumonisina B_1, fumonisina B_2, nivalenol, deoxinivalenol y fusarenona-X) y una posterior limpieza basada en la microextracción líquido-líquido dispersiva para la determinación del resto de las micotoxinas. El método se ha caracterizado en extracto y semillas de cardo mariano, obteniéndose límites de detección y cuantificación por debajo de los permitidos en legislación para un gran número de matrices alimentarias. La precisión fue inferior al 10% y las recuperaciones oscilaron entre el 62.3% y el 98.9 %, excepto para la zearalenona en semillas y para la citrinina en extracto de cardo mariano. Finalmente se analizaron diversas muestras comerciales (una muestra de extracto de cardo mariano y seis muestras de semillas), algunas de las cuales dieron positivo para las toxinas HT-2 y T-2 y zearalenona. Esta es la primera vez que este tipo de tratamiento, que permite el análisis de las micotoxinas más importantes, se ha propuesto para el análisis de estas complejas matrices alimentarias.

Abstract

An UHPLC-MS/MS method has been developed for the determination of 15 mycotoxins in milk thistle (*Silybum marianum*), including aflatoxins, fumonisins, trichothecenes, ochratoxin A, citrinin, sterigmatocystin and zearalenone. The mycotoxins were detected by electrospray ionization in positive ion mode and multiple reaction monitoring, achieving the separation in about 4 min. Sample treatment consisted of a modified method based on a first step using a QuEChERS-based procedure which allowed the determination of fumonisin B_1, fumonisin B_2, nivalenol, deoxynivalenol and fusarenon-X, and a subsequent clean-up based on dispersive liquid-liquid microextraction for the determination of the rest of mycotoxins. The method has been validated in extract and seeds of milk thistle, obtaining limits of quantification lower than those usually permitted by legislation in food matrices, with precisions lower than 10%. Recoveries were between 62.3% and 98.9%, except for zearalenone in seed samples and citrinin in extract. The method was also applied for studying the occurrence of these mycotoxins in market samples (six samples of seeds and one natural extract of milk thistle), and HT-2 and T-2 toxins and zearalenone were found in some of the samples. To the best of our knowledge, this is the first time that this type of treatment has been used for these complex food matrices, allowing the analyses of the most important mycotoxins.

3.1. Introduction

During the last decade, both offer and consumption of products with specific nutritional and/or functional characteristics, declared to have positive effects on our health have significantly increased. They can be found in pharmacies, supermarkets, food shops, and even advertised on the radio/TV or by internet, through websites located inside but also outside Europe. Among these products, food supplements are intended to provide a concentrated source of nutrients and other substances and contain, in many cases, herbal products and/or their derivatives as ingredients (i.e. ginseng, echinacea, gingko, green tea, etc.). Previous studies have demonstrated that these materials can suffer from fungi and mycotoxin contamination [1-4], which could lead to diverse human health problems. The principal fungi genera and species that can produce toxins and the mycotoxicosis symptoms of some of the most common mycotoxins are reported by Capriotti *et al.* [5].

In the EU, herbals are registered either as food ingredients or as drugs, depending on the Member State. This fact hampers the standardization of the legislation to regulate the production and commercialization of these products within the common market. Although the EFSA has published a series of documents to facilitate the safe evaluation of plants intended to be used in food supplements [6], regarding contaminant content, including mycotoxins, EFSA only specifies that herbal products must comply with the current food legislation of the EU. In this sense, EU has set maximum levels for aflatoxins, ochratoxin A, patulin, deoxynivalenol, zearalenone and fumonisins for several foodstuffs derived from fruits and cereals by the Commission

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Regulation (CE) Nº 1881/2006 [7] and subsequent amended. However, it must be pointed out that none of these Regulations set maximum levels for mycotoxins in food supplements. Moreover, recently the EFSA gave a "call for continuous collection of chemical contaminants occurrence data in food and feed", including mycotoxins and plant toxins among the target substances to be controlled [8].

Regarding the contamination of herbal products used as drug ingredients, the European Pharmacopeia only sets a maximum level for AFB_1 (2 μg kg^{-1}) and for the total content of AFB_1, AFB_2, AFG_1 and AFG_2 (4 μg kg^{-1}) [9]. For other mycotoxins, the European Pharmacopeia neither recommends nor establishes any allowed maximum content. Therefore, there seems to be evidences of a legal loophole in this field. This all suggests that developing efficient, high sensitive, fast and multianalyte methods to control these residues in food supplements based on herbal products, fulfilling the established legislation [10] is indispensable.

Different analytical methods have been proposed for mycotoxin determination in foods, such as TLC [11,12], ELISA [13,14], GC-MS [15] or CE [16]. However, the most popular techniques are HPLC with UV/Vis or FL detection [17-21] or MS detection [22-

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25]. Ultra-high performance liquid chromatography (UHPLC) coupled with MS/MS has become very popular in the last years for the multiclass analysis of mycotoxins [3,26,27,28] and for their determination with other contaminants [29-32].

For extraction and clean-up, different approaches have been proposed, the most common methodologies being solid-liquid extraction followed by SPE and IACs containing specific antibodies to the analyte of interest [33]. Several reviews present an overview of the different methodologies proposed for the determination of mycotoxins in foods, including the most frequent sample treatments [34-41].

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The scarce methods proposed for the determination of mycotoxins in herbal products are based mainly on LC-FL for aflatoxins (involving post-column derivatization) and OTA [42-46] or LC-MS for multiresidue analysis [3,47]. Most of these methods use solid-liquid extraction and IAC for clean-up, including the method recommended by the Pharmacopoeia for the determination of AFB_1, which has been validated only for devil's claw roots, ginger and senna pods [9]. However, IAC is an expensive and complex purification system which suffers from low recoveries for some mycotoxins, due to the complexity of these matrices. Moreover, the multiclass analysis is quite limited with this selective extraction. As a consequence, simpler, more efficient, multiclass and environmentally friendly extraction systems are demanding. Increasingly popular treatments are the so called QuEChERS and the DLLME, described in Chapter 2 where they were applied to the determination of OTA in wine with excellent results [48,49].

Considering the above described situation about the determination of mycotoxins in herbal products, we propose a multiclass method based on UHPLC-MS/MS for the simultaneous determination of 15 mycotoxins in *Silybum Marianum*, commonly known as milk thistle. This botanical is one of the most consumed food supplements due to its protective effects on the liver and to greatly improve its function; moreover, although analytical methods for studying the occurrence of mycotoxins in other herbal products (such as ginseng, ginger and others) has been previously reported, this is not the case of milk thistle. The studied mycotoxins are included in regulation (EC) 1881/2006 or

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considered by the IARC [50]: AFB_1, AFB_2, AFG_1, AFG_2, fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), nivalenol (NIV), deoxynivalenol (DON), fusarenon-X (F-X), HT-2 toxin (HT-2), T-2 toxin (T-2), OTA, citrinin (CIT), sterigmatocystin (STE) and zearalenone (ZEN). In addition, an extraction method based on QuEChERS procedure for determination of FB_1, FB_2, NIV, DON and F-X with an additional clean-up DLLME step for determination of AFB_1, AFB_2, AFG_1, AFG_2, OTA, T-2, HT-2, STE, CIT and ZEN, has been proposed. To the best of our knowledge, this is the first time that this type of treatment has been used for these food matrices.

3.2. Experimental

3.2.1. Chemicals and solvents

Solvents were LC–MS grade and mycotoxins were analytical standard grade. Formic acid eluent additive for LC–MS, MeCN, MeOH, tetrachloroethylene, dibromomethane, carbon tetrachloride, ammonium formate and individual standards of each mycotoxin were obtained from Sigma Aldrich (St. Louis, MO, USA). Formic acid (analysis grade), THF, EtOH and ACO were supplied by Merck (Darmstadt, Germany). Sodium chloride, sodium hydroxide, sodium dihydrogen phosphate monohydrate and dichloromethane were supplied by Panreac (Madrid, Spain); chloroform and acetic acid were purchased from VWR BDH Prolabo (West Chester, Pensilvania, USA).

Ultrapure water (18.2 MΩ cm^{-1}, Milli-Q Plus system, Millipore Bedford, MA, USA) was used throughout the work.

Kits SampliQ QuEChERS (supplied by Agilent Technologies Inc., Wilmington, DE, USA) consisted of QuEChERS extraction packed (buffered: 4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate; non-buffered: 4 g $MgSO_4$, 1 g NaCl) and 15 mL dispersive tubes A (400 mg C_{18}, 400 mg PSA and 1200 mg $MgSO_4$) or B (150 mg C_{18} and 900 mg $MgSO_4$).

[50] International Agency for Research on Cancer (IARC) (2012). Available on: http://www.iarc.fr

Acrodisc 13 mm syringe filters with 0.2 µm nylon membrane (Pall Corp., MI, USA) were used for filtration of samples prior to the injection into the chromatographic system.

A 30 mM phosphate buffer solution pH 7.1 was prepared by dissolving 2.07 g of sodium dihydrogen phosphate monohydrate in 500 mL of water, adjusting the pH with NaOH solution.

3.2.2. Instrumentation and software

Separations were performed on an Extreme Pressure Liquid Chromatography (XLC) system (two pumps, oven, auto sampler, mixer and degasser unit) from Jasco (Easton, MD, USA). The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (AB SCIEX, Toronto, ON, Canada) with electrospray ionization (ESI). A Zorbax Eclipse Plus RRHD (50 mm × 2.1 mm, 1.8 µm) was used as chromatographic column.

A pH-meter with a resolution of ±0.01 pH unit (Crison model pH 2000, Barcelona, Spain), a Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and an evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used.

The instrumental data were collected using the Analyst® Software version 1.5 with Schedule multiple reaction monitoring (MRM) TM Algorithm (AB Sciex). The statistical software Statgraphics Centurion XV.II was used for data treatment.

3.2.3. Sample treatment

Milk thistle samples (seeds and extract) were purchased in local markets from Granada (Spain) and stored at room temperature. The QuEChERS procedure was adapted from that used in our laboratory for the determination of OTA in wine [49], described in Chapter 2.

Milk thistle seeds were crushed and homogenized with an automatic coffee grinder. A portion of 2 g and 8 mL of 30 mM NaH_2PO_4 buffer pH 7.1 were placed into a 50 mL

screw cap test tube with conical bottom, shaking by vortex for 10 s. Subsequently, 5 mL of 5% formic acid in MeCN was added to the tube, shaking by vortex for 2 min. Agilent SampliQ EN QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the tube was shaken vigorously for 1 min. After that, the sample was centrifuged at 4500 rpm for 5 min.

Then, 1 mL of the upper MeCN layer was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2 µm filter before injection and NIV, DON, F-X, FB_1 and FB_2 were determined by UHPLC–MS/MS.

For the analysis of the rest of mycotoxins, 2 mL of the upper MeCN layer (obtained after extraction/partitioning step) was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). This solution was placed into a 15 mL screw cap test tube with conical bottom and 4 mL of water and 0.21 g of NaCl (4.2%; w/v) were added. The mixture of the disperser solvent (950 µL of MeCN) and the extraction solvent (620 µL of chloroform) was rapidly injected into the test tube with a 2.0 mL syringe. The solution was shaken and a cloudy solution was formed in the tube. In this step, the mycotoxins were extracted into the fine droplets of chloroform. The mixture was centrifuged at 6000 rpm for 3 min and the fine particles were sedimented at the bottom of the tube. The sedimented phase (approximately 400 µL) was removed using a 1 mL syringe, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The solution was filtered and injected into the UHPLC–MS/MS system for the analysis of AFB_1, AFB_2, AFG_1, AFG_2, OTA, T-2, HT-2, STE, CIT and ZEN. Figure 3.1 shows a diagram of the whole sample treatment.

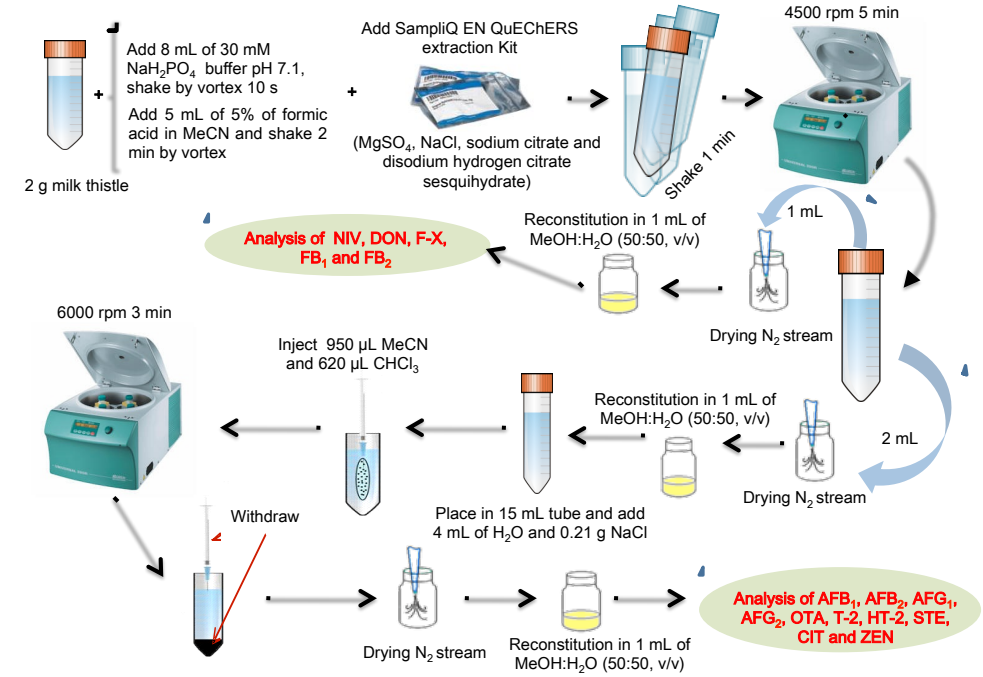


Figure 3.1. Sample treatment for the determination of mycotoxins in milk thistle

3.2.4. UHPLC-MS/MS analysis

UHPLC separations were performed in a C_{18} column (Zorbax Eclipse Plus RRHD 50 mm × 2.1 mm, 1.8 µm) using a mobile phase consisting of 0.3% aqueous formic acid solution with 5 mM ammonium formate (solvent A), and MeOH with 0.3% formic acid and 5 mM ammonium formate (solvent B) at a flow rate of 0.4 mL min^{-1}.

The eluent gradient profile was as follows: 0 min: 5% B; 1 min: 50% B; 2 min: 72% B; 4 min: 80% B and 6 min: 90% B. Finally it was back to 5% B in 0.2 min and maintained for 1.8 min for column equilibration. The temperature of the column was 35 °C and the injection volume was 5 µL (full loop).

The MS was working with ESI in positive mode (ESI (+)) under the MRM conditions shown in Table 3.1.

Table 3.1. Monitored ions of the target analytes and MS/MS parameters

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CEn[a]	CXP[a]
NIV	1.36	313.1	$[M+H]^+$	41.0	5.0	14.0	174.9 (Q)	17.0	4.0
							128.0 (I)	75.0	6.0
DON	1.70	297.1	$[M+H]^+$	36.0	5.5	16.0	249.2 (Q)	17.0	4.0
							161.0 (I)	29.0	4.0
F-X	2.02	355.1	$[M+H]^+$	26.0	12.0	18.0	174.7 (Q)	23.0	4.0
							137.1 (I)	31.0	4.0
AFG_2	2.52	331.1	$[M+H]^+$	61.0	6.0	42.0	245.1 (Q)	39.0	4.0
							313.1 (I)	27.0	6.0
AFG_1	2.61	329.0	$[M+H]^+$	76.0	9.5	16.0	243.1 (Q)	39.0	6.0
							311.1 (I)	29.0	6.0
AFB_2	2.72	315.1	$[M+H]^+$	81.0	4.0	34.0	286.9 (Q)	33.0	6.0
							259.0 (I)	39.0	8.0
AFB_1	2.79	313.1	$[M+H]^+$	46.0	12.0	26.0	241.0 (Q)	41.0	4.0
							284.9 (I)	39.0	4.0
CIT	2.90	251.2	$[M+H]^+$	26.0	11.0	18.0	233.0 (Q)	23.0	23.0
							204.8 (I)	73.0	10.0
HT-2	3.18	442.0	$[M+NH_4]^+$	21.0	5.5	21.0	262.8 (Q)	22.0	8.0
							215.4 (I)	19.0	4.0
FB_1	3.24	722.2	$[M+H]^+$	71.0	10.0	30.0	334.2 (Q)	51.0	6.0
							352.2 (I)	47.0	6.0
T-2	3.44	484.0	$[M+NH_4]^+$	21.0	10.0	22.0	215.0 (Q)	22.0	4.0
							185.0 (I)	29.0	4.0
ZEN	3.71	319.0	$[M+H]^+$	26.0	8.0	20.0	282.9 (Q)	19.0	4.0
							301.0 (I)	15.0	10.0
OTA	3.76	404.0	$[M+H]^+$	41.0	7.5	16.0	238.9 (Q)	31.0	6.0
							102.1 (I)	91.0	6.0
FB_2	3.86	706.2	$[M+H]^+$	71.0	10.5	20.0	336.3 (Q)	43.0	14.0
							318.3 (I)	45.0	12.0
STE	3.88	325.1	$[M+H]^+$	66.0	3.5	26.0	281.0 (Q)	43.0	4.0
							310.0 (I)	37.0	4.0

[a] Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CEn). All expressed in voltage.

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

The ionization source parameters were: source temperature 500 °C; curtain gas (nitrogen) 30 psi; ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi. In all cases a precursor ion and two product ions (the most

abundant for quantification and the other one for confirmation) were studied, obtaining four identification points, fulfilling the requirements established by EU for confirmation of contaminants in foodstuff [51].

3.3. Results and discussion

3.3.1. Optimization of chromatographic conditions and MS/MS detection

In order to get the highest sensitivity, MS/MS detection was optimized for each analyte. With this purpose, standard solutions of 1 mg L^{-1} in 0.1% aqueous formic acid:MeCN (50:50, v/v) of each analyte were individually infused into the mass spectrometer. All the compounds were tested using ESI positive/negative mode. ESI (+) showed the best results in terms of sensitivity for most of the mycotoxins, including aflatoxins, so it was selected for the rest of the work. Precursor ions were protonated or ammonium adducts.

During the infusion, the parameters Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CEn) were optimized for each compound in order to obtain the maximum sensitivity (see results in Table 3.1). Each compound was characterized by its retention time and by two product ions. The target scan time established for each transition was 0.1 s. The most intense product ion was used for quantification, while the second one was used to complete the identification.

In order to optimize the chromatographic parameters, mobile phases consisted of water with 0.1% formic acid (solvent A) and MeCN or MeOH with 0.1% formic acid (solvent B) were tested. MeOH in solvent B provided better results, so it was selected for subsequent studies.

The gradient was studied in order to get the best separation, peak shape and sensitivity in the shortest time. The following gradient was selected: 0 min: 5% B; 1 min: 50% B; 2

[51] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC), Off. J. Eur. Commun. L221 (2002) 8.

min: 72% B; 4 min: 80% B and 6 min: 90% B. Finally it was back to 5% B in 0.2 min. Although the run time for each injection was 8 min (including the cleaning and conditioning of the column), under optimum conditions all analytes were eluted in about 4 min.

The use of acid in the mobile phase is required to improve the ionisation step in ESI (+) and different percentages of formic acid were tested (0% to 0.5%). Without any acid, ionization was not achieved and the irreproducibility increased. Figure 3.2 shows the results of the influence of the percentage of formic acid on peak area, and, as can be observed, a concentration of 0.3% formic acid in both, solvents A and B, gave the highest signals and good peak shape.

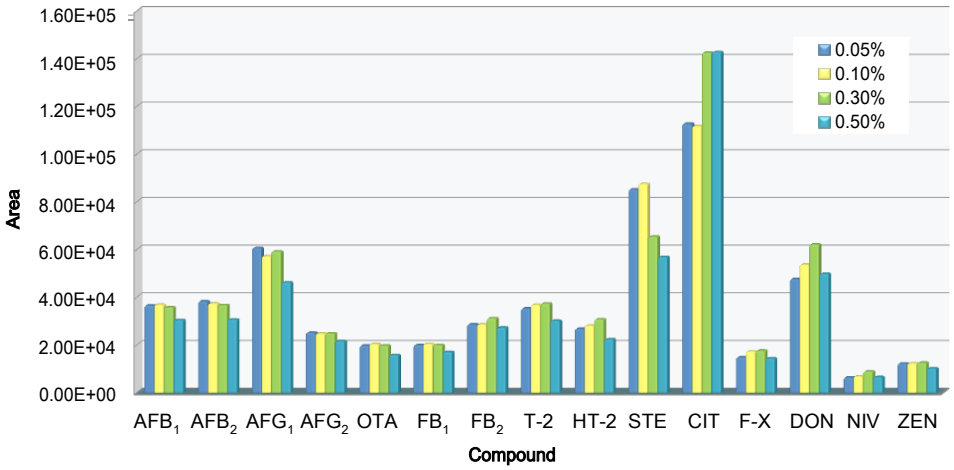


Figure 3.2. Influence of the percentage of formic acid in the mobile phase on peak area

To ensure proper formation of ammonium adducts (for T-2 and HT-2 determination) ammonium formate was added to the mobile phase. The concentration was studied between 0 and 10 mM. For some mycotoxins a maximum was found at 5 or 10 mM (see Figure 3.3); however, these increase in the concentration had a negative effect on fumonisins and ZEN (mycotoxins with the lowest signals). So, a concentration of 5 mM in solvent A and B was selected as a compromise.

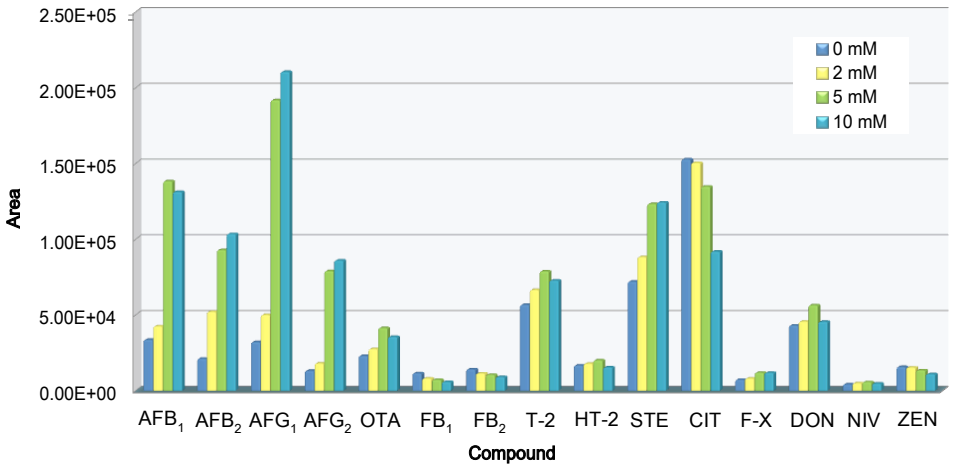


Figure 3.3. Influence of the ammonium formiate concentration in the mobile phase on peak area

Subsequently, the flow rate was studied from 0.3 mL min^{-1} to 0.5 mL min^{-1} and finally 0.4 mL min^{-1} was selected as a compromise between signal, peak shape and run time. The temperature of the column was studied between 25°C and 45°C. The temperature did not affect the signal, and although a temperature rise involved a reduction of analysis time, this decrease was not significant (a difference of 0.35 min between 25 ºC and 45 ºC) so 35°C was selected as optimum because higher temperatures worsened the resolution. The injection volume was 5 µL (full loop) for all the experiments.

Finally, under the chromatographic conditions described previously, the ionization source parameters were optimized following the recommendations of the manufacturer. Source temperature was tested between 300 and 600 °C. As can be seen in Figure 3.4, the signal increased with the temperature for the most of analytes. However, the signal for T-2, HT-2, F-X and NIV decreased at temperatures above 500 °C, so 500 °C was selected as optimum.

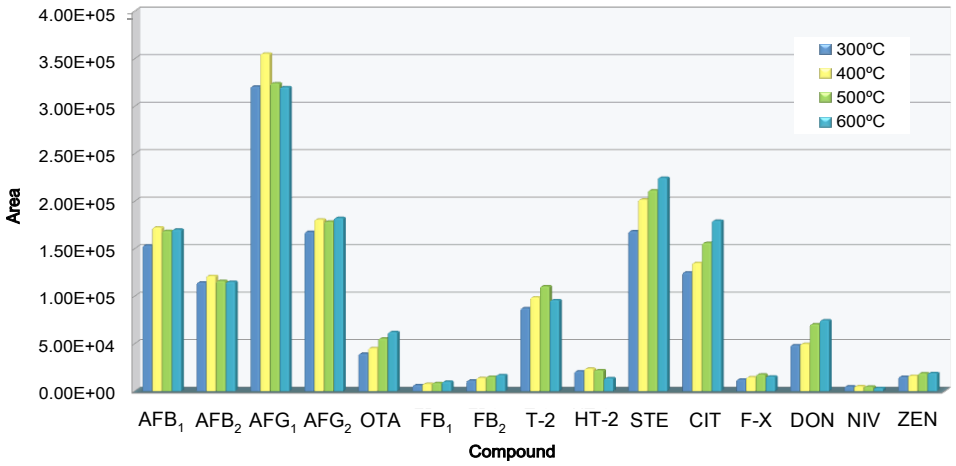


Figure 3.4. Influence of the source temperature on peak area

Curtain gas (nitrogen) was studied between 25 and 45 psi and the best results for most of the compounds were obtained with 30 psi, or have no significant influence (Figure 3.5).

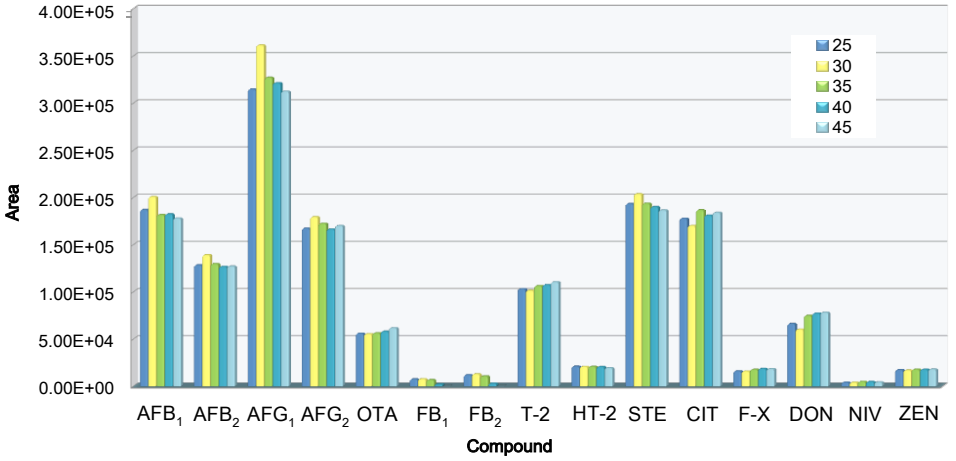


Figure 3.5. Influence of the curtain gas on peak area

Finally, ion spray voltage was evaluated from 4500 to 5500 V and 5000 V was selected as optimum (Figure 3.6). GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi.

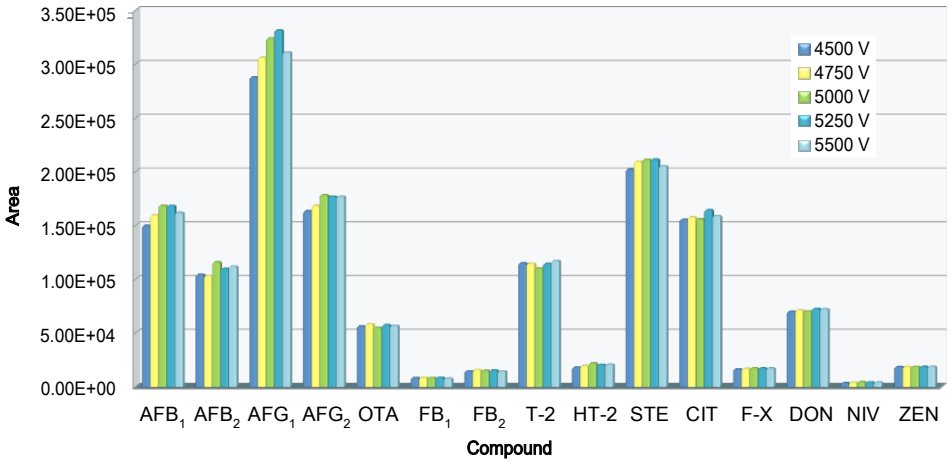


Figure 3.7. Influence of the spray voltage on peak area

3.3.2. Optimization of sample preparation

Initially, the first step of the extraction/partitioning process of the QuEChERS procedure was evaluated in standard solutions of mycotoxins. Two different extraction solvents were tested: (a) 8 mL of 30 mM NaH_2PO_4 buffer pH 7.1 and 5% formic acid in MeCN, used for the determination of OTA in wine (see in Chapter 2) [20]; and (b) 10 mL of MeOH:H_2O (80:20) with 1% acetic acid, reported for the determination of mycotoxins in eggs [140]. For most of the compounds, the best results in terms of extraction efficiency were obtained with the former (Figure 3.7).

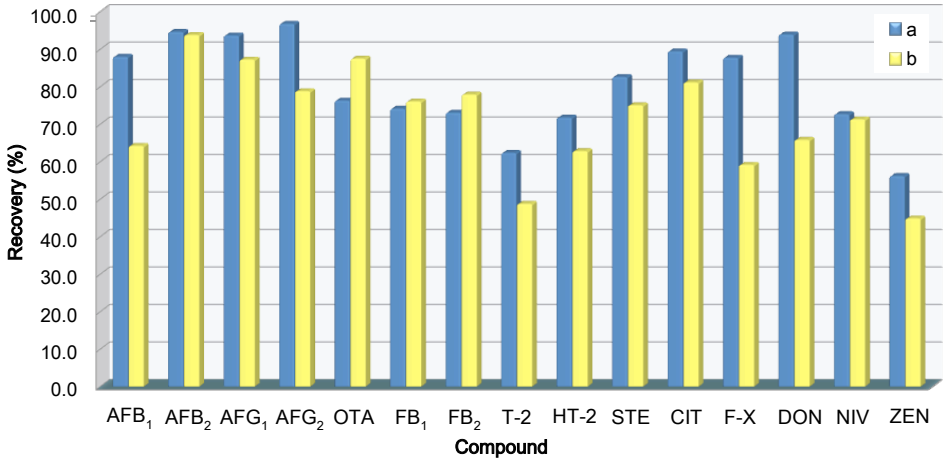


Figure 3.7. Study of the extraction solvent in standard solutions of mycotoxins: 8 mL of 30 mM NaH_2PO_4 buffer pH 7.1 and 5% formic acid in MeCN (a); 10 mL of MeOH:H_2O (80:20) with 1% acetic acid (b)

Then, the percentage of formic acid in the extraction solvent was studied (0 – 5%) and 5% was selected as optimum (see Figure 3.8).

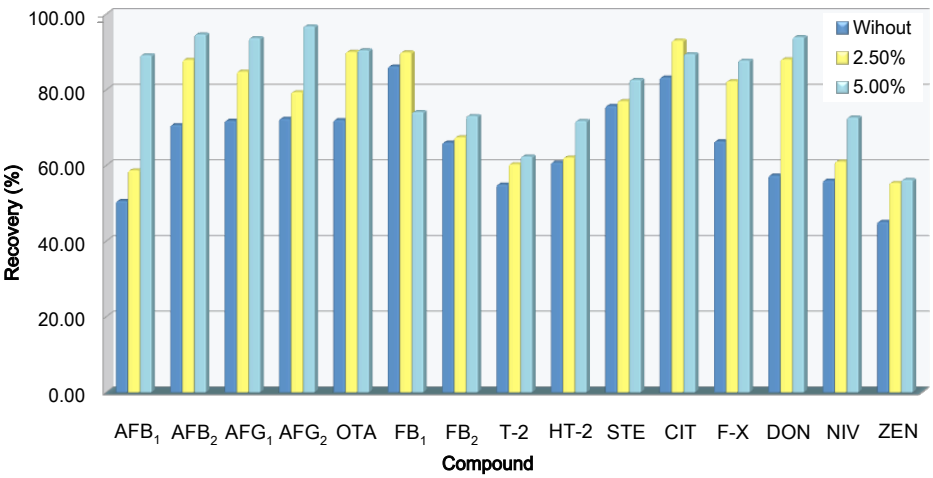


Figure 3.8. Study of the percentage of formic acid in the extraction solvent in standard solutions of mycotoxins

The second step of the QuEChERS procedure, involving sample clean-up by dSPE, was also evaluated, testing two commercial kits containing different sorbents (C_{18}, $MgSO_4$ and PSA). However, as can be seen in Figure 3.9, this clean-up step caused a decrease on the recovery of almost all the studied mycotoxins, being higher if PSA was included (in this case fumonisins disappeared completely) so this dSPE step was discarded.

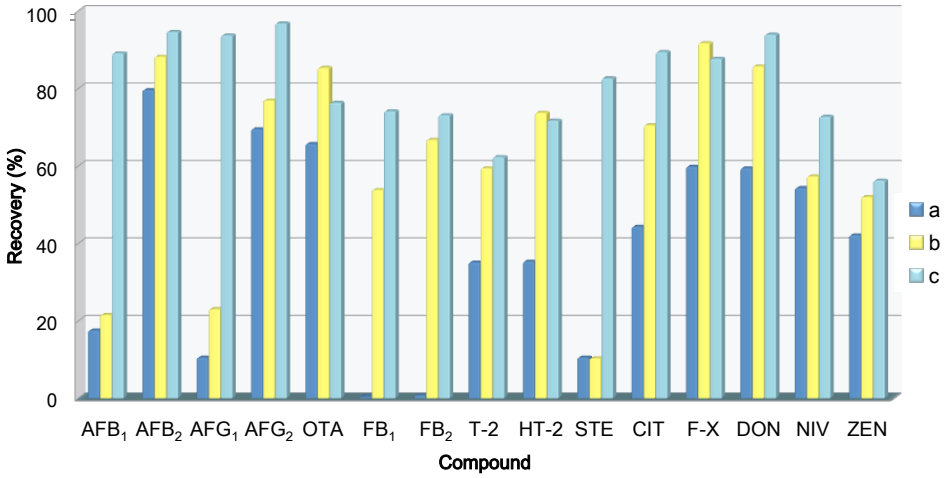


Figure 3.9. Study of sample dSPE clean-up in standard solutions of mycotoxins: 400 mg PSA (a), 400 mg C_{18} and 1200 mg $MgSO_4$; 150 mg C_{18} and 900 mg $MgSO_4$ (b); without any clean-up step (c)

Once this preliminary studied had been carried out, and in order to consider the matrix effect, from this moment, all the experiments were performed using 2 g of milk thistle seed samples spiked with the studied mycotoxins. In all cases, preliminary analyses were performed in order to check that the samples were free of analytes.

Thus, the extraction/partitioning process of the QuEChERS procedure was optimized for milk thistle seeds testing different solvents and different Agilent SampliQ EN QuEChERS extraction kits: (a) 10 mL of MeOH with 5% formic acid; (b) 10 mL of MeCN with 5% formic acid; (c) 8 mL of H_2O + 10 mL of MeCN with 5% formic acid + QuEChERS extraction tube (4 g $MgSO_4$, 1 g NaCl); (d) 8 mL of H_2O + 10 mL of MeCN with 5% formic acid + buffered QuEChERS extraction tube (4 g $MgSO_4$, 1 g

NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate); (e) 8 mL of 30 mM NaH_2PO_4 buffer pH 7.1 + 10 mL of MeCN with 5% formic acid + buffered QuEChERS extraction tube. As it was observed in Figure 3.10, the best results were obtained with option (e) so, those conditions were selected. However, the volume of extraction solvent (MeCN with 5% formic acid) was studied between 5 and 10 mL, selecting 5 mL as final optimum value as no significant differences were observed on recoveries (see Figure 3.11), thus improving LODs and LOQs.

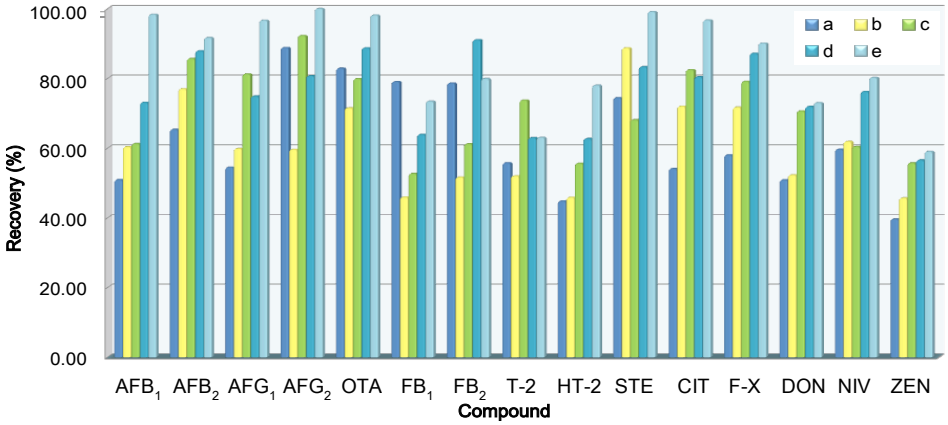


Figure 3.10. Study of different solvents and different Agilent SampliQ EN QuEChERS extraction kits in milk thistle: (a), (b), (c), (d) and (e) extraction conditions as indicated in the text

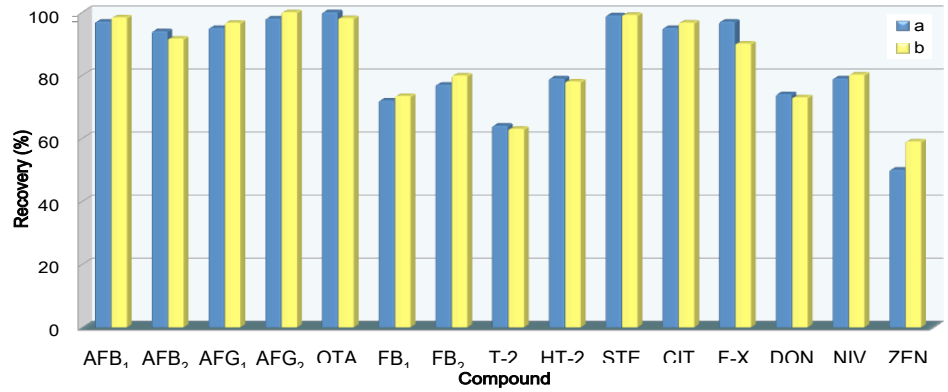


Figure 3.11. Study of the volume of extraction solvent in milk thistle: 5 mL of MeCN with 5% formic acid (a); 10 mL of MeCN with 5% formic acid (b)

After this extraction/partitioning step, NIV, DON, F-X, FB_1 and FB_2 were determined by UHPLC–MS/MS. However, some mycotoxins, as aflatoxins, could not be determined at low concentrations, probably due to the high oil content of the seeds (about 30%) [58] that could be the reason of a strong matrix effect and interfering peaks; thus, a subsequent clean-up step was required for their determination. As the second step of the QuEChERS procedure (based on dSPE) has been discarded in the previous studies with standard solutions and considering the excellent results described in Chapter 2 for the determination of OTA in wine samples [21], DLLME was checked for cleaning-up the extract.

The optimization of DLLME involved the study of the following parameters: nature and volume of extraction solvent, nature and volume of disperser solvent and percentage of NaCl. The aim was to obtain the highest extraction efficiency for all the studied mycotoxins, but with special interest on aflatoxins (as they are the only group of mycotoxins legislated in herbal products by Pharmacopeia) and OTA because due to the matrix effect, the usual established limits set by EU legislation in different foodstuff were not reached only with the first step of QuEChERS procedure.

Thus, the seeds spiked with mycotoxins were treated with the previously described extraction/partitioning step and 2.0 mL aliquots of the final extract were dried, recomposed in 1 mL of MeOH:H_2O (50:50), diluted in 4 mL of H_2O and used in the optimization of DLLME.

Firstly, it was necessary to select the extraction and disperser solvents. Taking into the account the previous considerations, only aflatoxins and OTA were selected as target analytes for this study. Volumes of 660 µL of chloroform, tetrachloroethylene, dibromomethane, carbon tetrachloride and dichloromethane were tested as extraction solvents, using 940 µL of MeOH as disperser solvent. As can be seen in Figure 3.12, the best results were obtained with chloroform. Then, 940 µL of MeOH, EtOH, ACO, MeCN, THF and MeOH:MeCN (50:50, v/v) were tested as disperser solvents, selecting MeCN as optimum (see Figure 3.13).

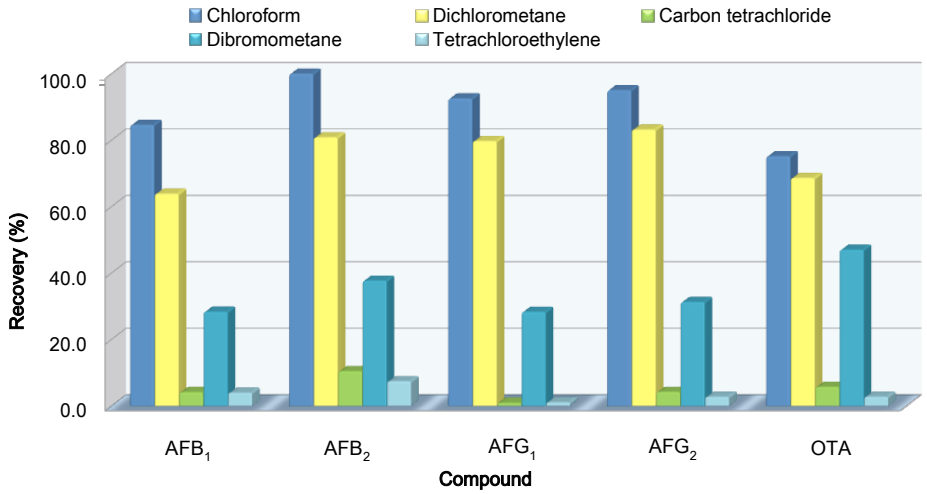


Figure 3.12. Influence of different extraction solvents on extraction efficiency

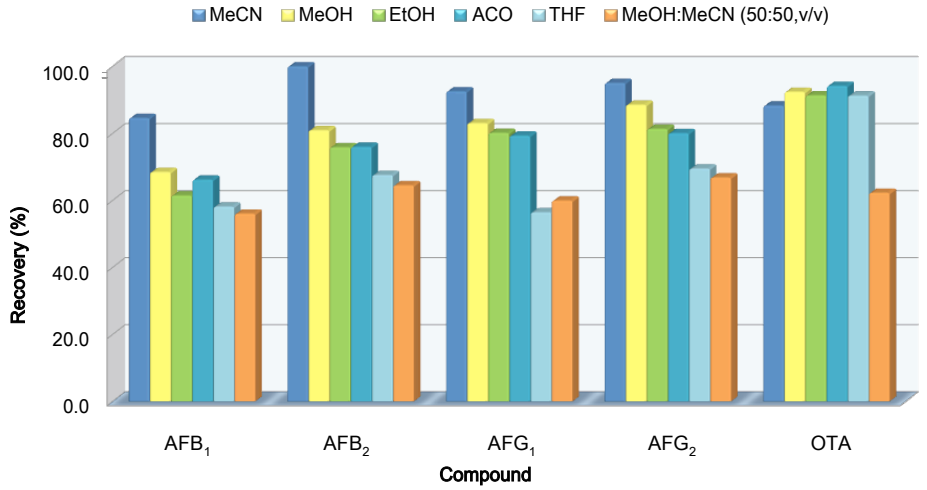


Figure 3.13. Influence of different disperser solvents on the extraction efficiency

Subsequently, the volumes of extraction and disperser solvents, and the percentage of NaCl were optimized by a multivariate approach, taking into account possible interactions between the variables. A central composite design (2^3 + star, face centred), with three spaced central points, involving 17 runs, was used as an approach to generate the response surface, using the recovery percentage of all the mycotoxins as analytical response. The different factors were studied in the following ranges: MeCN

volume (750–1250 µL); chloroform volume (200–800 µL); and percentage of NaCl (0–5%). The extraction solvent volume, the percentage of salt and the quadratic term of disperser solvent volume had a significant effect on recovery of OTA, HT-2 and STE, while none of the studied variables had a significant effect on the recoveries of the other mycotoxins. Figure 3.14 shows the Pareto Chart for OTA, HT-2 and STE.

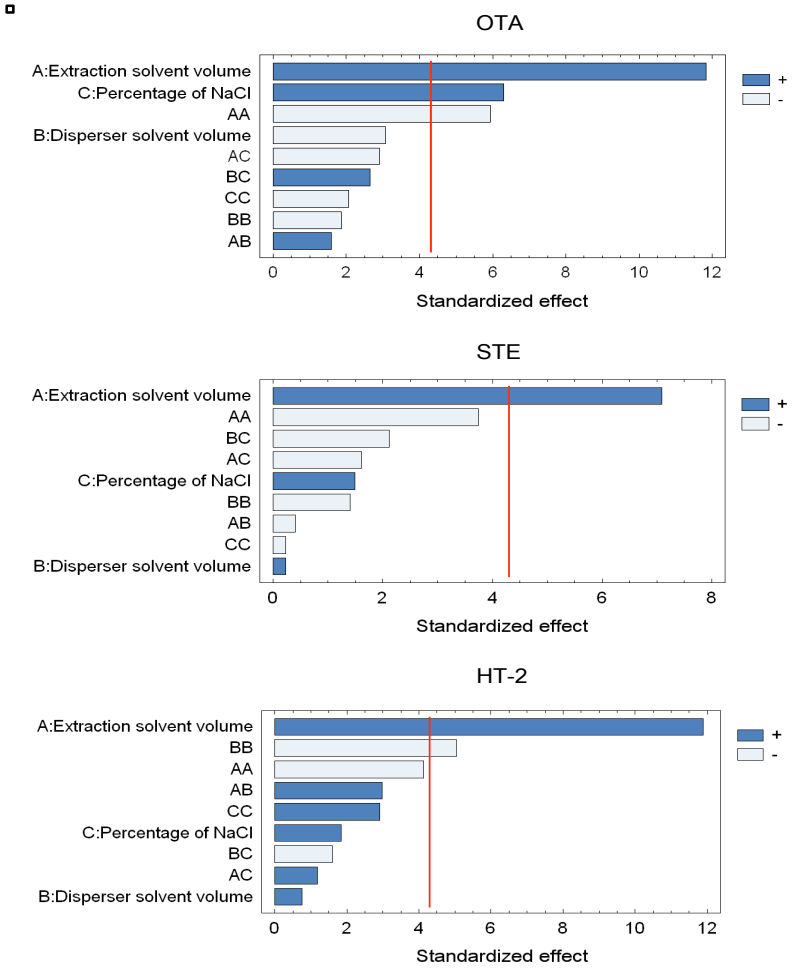


Figure 3.14. DLLME optimization: Pareto chart showing the effects of the studied variables on the recovery percentage: (+) Positive effects on the response; (-) negative effects on the response. Red line shows the limit of decision to consider the significance of the factors (based on the effect = estimated effect/standard error, P-value = 0.05 at 95% of confidence)

The response surface plot obtained after the multiple response optimisation using the desirability function is shown in Figure 3.15. It was obtained considering only the responses for OTA, HT-2 and STE, because only for these mycotoxins the variables had a significant effect on the recovery. The P-values of the lack of fit test were 0.071, 0.147 and 0.329, and the determination coefficient (R^2) were 82.1, 87.5 and 87.5% for OTA, HT-2 and STE, respectively, showing the suitability of the design. The optimum conditions for the maximum efficiency in the extraction using DLLME were as follows: extraction solvent = 620 µL; disperser solvent = 950 µL and percentage of salt = 4.2%.

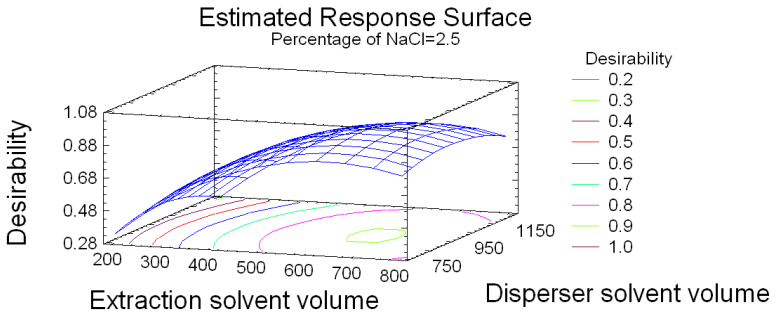
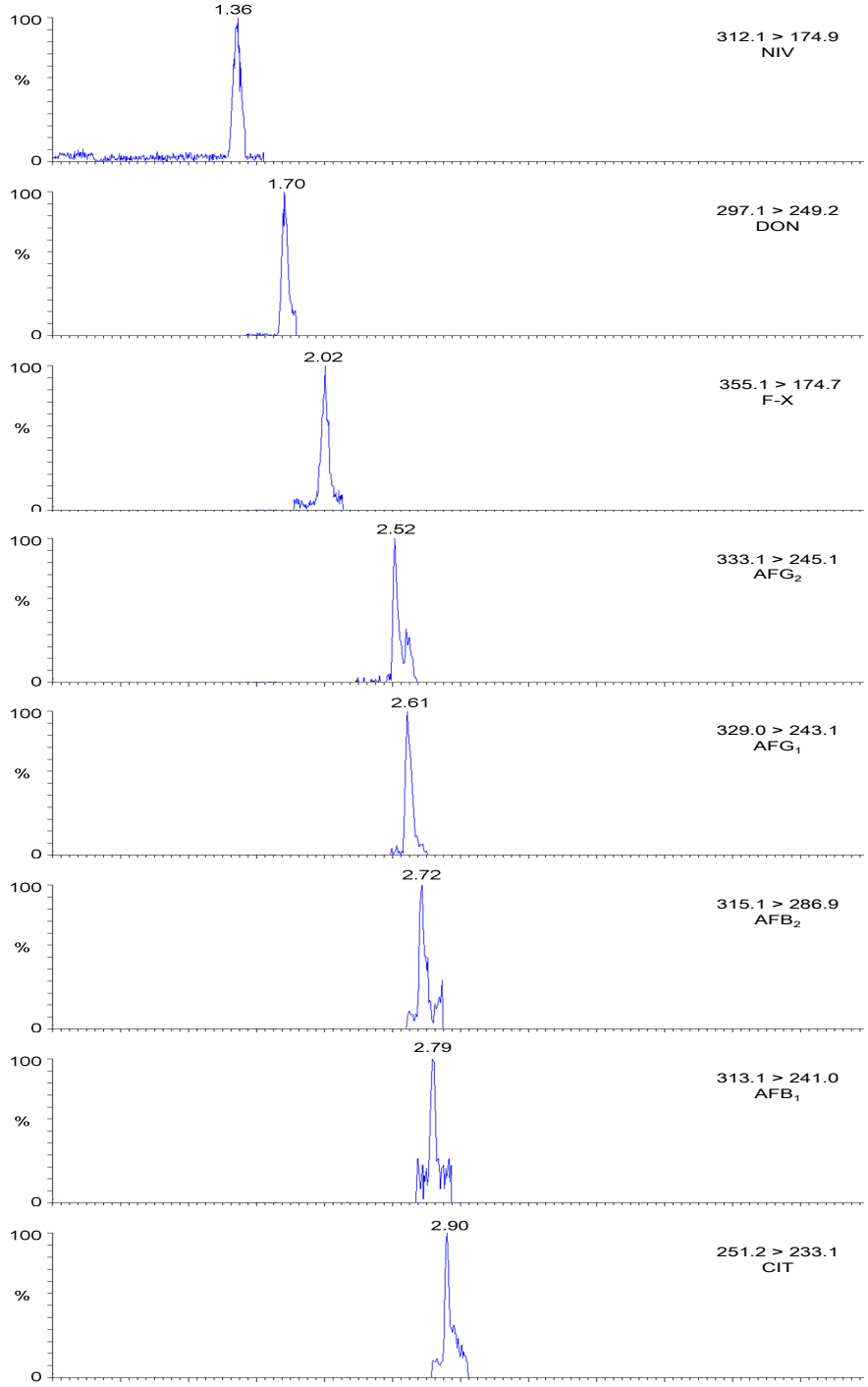


Figure 3.15. Estimated response surface obtained in the optimization procedure using a central composite design (2^3 + star, face centred), with three spaced central points

After the optimization of the DLLME parameters and the rapid injection of the mixture of solvents into the sample, the extraction was carried out by shaking the sample manually for a few seconds, as this step is not critical in DLLME, as explained in Chapter 2. Subsequently, the mixture was centrifuged and the sedimented phase was evaporated to near dryness using a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). After this step, the rest of mycotoxins were determined by UHPLC–MS/MS. A typical extracted chromatogram of a spiked milk thistle sample is shown in Figure 3.16.


1.36
312.1 > 174.9
NIV
1.70
297.1 > 249.2
DON
2.02
355.1 > 174.7
F-X
2.52
333.1 > 245.1
AFG2
2.61
329.0 > 243.1
AFG1
2.72
315.1 > 286.9
AFB2
2.79
313.1 > 241.0
AFB1
2.90
251.2 > 233.1
CIT
100
%
0

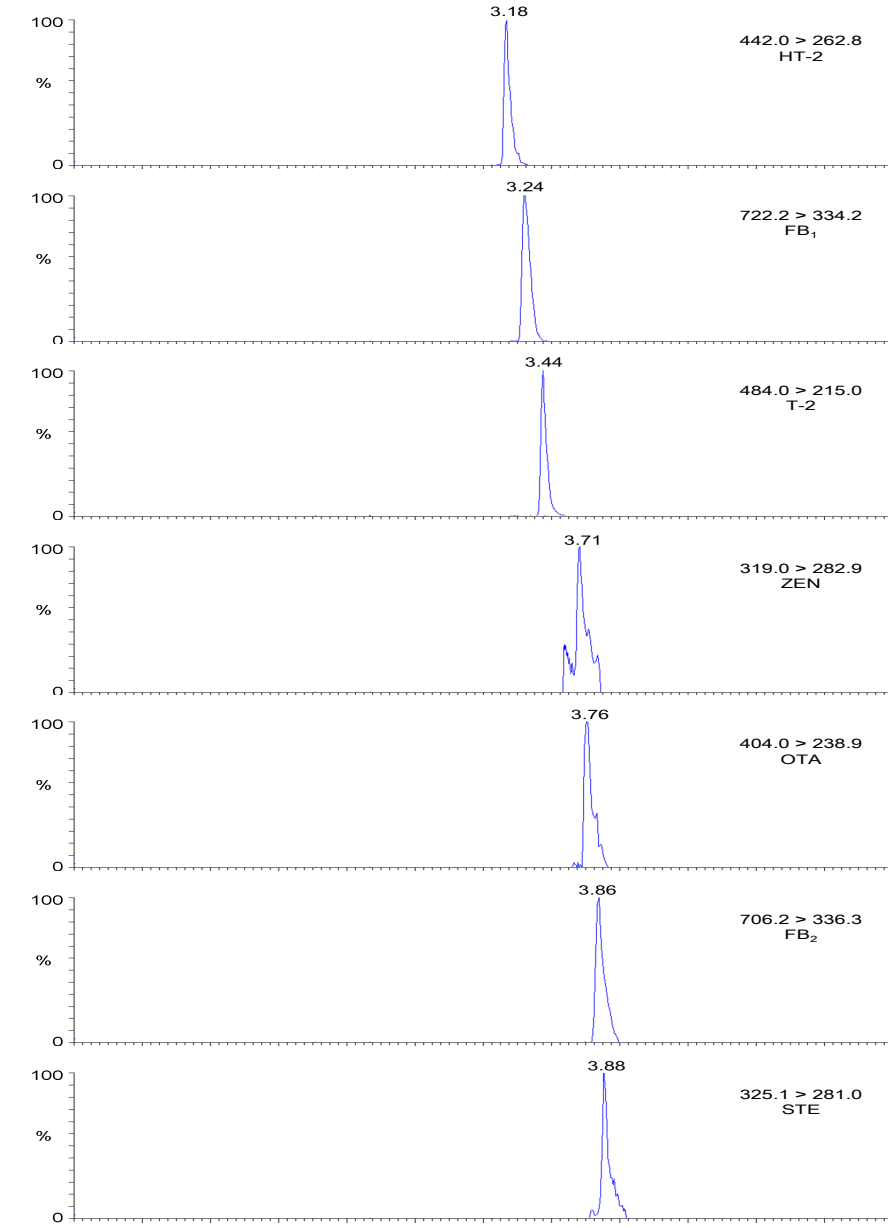


Figure 3.16. UHPLC–MS/MS extracted ion chromatogram of a spiked milk thistle sample (10 μg kg^{-1} for AFB_1, AFB_2, AFG_1, AFG_2 and OTA; 250 μg kg^{-1} for FB_1, FB_2, T-2, HT-2, CIT, ZEN and STE; 500 μg kg^{-1} for DON and F-X; 2000 μg kg^{-1} for NIV) analysed with the proposed method

Finally, a comparison of the recoveries (R) and matrix effect (ME) obtained after each step (QuEChERS-based extraction and DLLME) was performed. R was calculated as the percentage of the signal corresponding to a sample spiked with a mixture of mycotoxins and the signal of an extract (obtained at the end of each step) spiked with the same final concentration, considering the dilution carried out in each step. ME was calculated as the percentage of the signal corresponding to an extract (obtained at the end of each step) spiked with a mixture of mycotoxins and the signal of a standard solution of the same concentration. A value close to 0% indicates that there is not a significant matrix effect, while values >0% and <0% indicate signal enhancement and signal suppression, respectively. The results are shown in Table 3.2.

Table 3.2. Comparison of the matrix effect (ME) and recoveries (R) obtained after each step of the sample treatment.

Analyte	QuEChERS		DLLME	
	%ME [a]	%R[b]	%ME [a]	%R[b]
AFB_1	nd	nd	- 88.6	88.4
AFB_2	nd	nd	- 81.4	93.7
AFG_1	nd	nd	- 84.6	89.0
AFG_2	nd	nd	- 83.4	93.8
OTA	- 82.9	96.6	- 77.2	95.2
FB_1	- 49.7	74.9	- 48.5	0.2
FB_2	- 63.1	72.7	- 52.8	0.1
T-2	- 83.8	67.9	- 76.2	64.6
HT-2	- 89.1	78.6	- 73.9	73.2
STE	- 91.4	92.6	- 79.8	85.3
CIT	- 84.0	94.0	- 75.2	68.3
F-X	- 78.5	87.0	- 69.0	7.4
DON	- 84.1	72.4	- 76.2	0.1
NIV	- 71.4	39.8	- 64.6	0.8
ZEN	- 79.1	63.7	- 68.5	51.8

[a] % ME: matrix effect calculated as 100×[(signal of a spiked extract - signal of standard solution) / signal of standard solution]

[b] % R: recovery calculated as 100×(signal of a spiked sample / signal of a spiked extract)

nd: not detectable

Concentrations: 10 μg Kg^{-1} for AFB_1, AFB_2, AFG_1, AFG_2 and OTA; 100 μg Kg^{-1} for CIT and STE; 200 μg Kg^{-1} for T-2, HT-2, FB_1, FB_2 and ZEN; 500 μg Kg^{-1} for F-X and DON; 3000 μg Kg^{-1} for NIV.

It can be concluded that aflatoxins could not be measured after the QuEChERS-based extraction due to ME and interfering peaks; however, after DLLME these peaks were eliminated, allowing their determination. Moreover, NIV, DON, F-X, FB_1 and FB_2 showed very low recoveries after the DLLME step, indicating that they were not properly extracted by this procedure; consequently they must be determined directly after the first QuEChERS-based extraction step. For the rest of mycotoxins, significant differences were not observed for recoveries or matrix effects, showing that the first QuEChERS-based extraction step would allow their determination; nevertheless, their quantification after DLLME was preferred, as lower LODs were obtained due to the preconcentration achieved. It is also remarkable that a high matrix effect due to signal suppression (ME <80%) was observed for all mycotoxins, making mandatory to perform matrix-matched calibrations.

3.3.3. Characterization of the method

3.3.3.1 Calibration curves and performance characteristics

Performance characteristics of the optimized method were established with spiked milk thistle samples, studying linearity, LODs and LOQs.

In order to compensate matrix effect, matrix-matched calibration curves were established using milk thistle samples spiked with different concentrations of mycotoxins injected in triplicate and considering the peak areas as analytical signal. The studied concentrations were as follows: 2 to 100 μg kg^{-1} for AFB_1, AFB_2, AFG_1, AFG_2 and OTA; 25 to 1000 μg kg^{-1} for FB_1; 50 to 1000 μg kg^{-1} for FB_2, T-2 and HT-2 toxin; 25 to 500 μg kg^{-1} for STE; 50 to 500 μg kg^{-1} for CIT; 100 to 2500 μg kg^{-1} for DON; 2000 to 6000 μg kg^{-1} for NIV; 500 to 5000 μg kg^{-1} for F-X and 100 to 1000 μg kg^{-1} for ZEN.

The LODs and LOQs were calculated as 3 × S/N and 10 × S/N, respectively. The statistical parameters calculated by least-square regression, as well as LODs and LOQs, are shown in Table 3.3. The satisfactory determination coefficients confirm that mycotoxin analytical responses were linear over the studied ranges. As can be seen, low LOQs were obtained for most of the compounds. However, LODs for F-X and NIV were higher than those of the rest of compounds due to their lower sensitivity. This fact

has also been reported by other authors [26]. Thus, the method allows the quantification of aflatoxins at concentrations lower than their maximum levels established in botanicals by Pharmacopeia [9]. Also, the rest of mycotoxins can be determined at concentrations lower than their usual established limits in different foodstuff [7].

Table 3.3. Statistical and performance characteristics for the UHPLC-MS/MS method

Analyte	Linear dynamic range (µg kg^{-1})	R^2	LOD (µg kg^{-1})	LOQ (µg kg^{-1})
AFB_1	1.87 – 100	0.998	0.56	1.87
AFB_2	1.91 – 100	0.996	0.57	1.91
AFG_1	1.50 – 100	0.993	0.45	1.50
AFG_2	1.79 – 100	0.997	0.54	1.79
OTA	1.95 – 100	0.998	0.59	1.95
FB_1	13.5 – 1000	0.997	3.9	13.5
FB_2	45.7 – 1000	0.997	13.7	45.7
T-2	30.5 – 1000	0.997	9.1	30.5
HT-2	43.8 – 1000	0.994	13.0	43.8
STE	16.3 – 500	0.995	4.9	16.3
CIT	47.6 – 500	0.998	14.3	47.6
F-X	460 – 5000	0.997	138	460
DON	58.5 – 2500	0.995	17.6	58.5
NIV	1530 – 6000	0.990	459	1530
ZEN	98.7 – 1000	0.994	29.6	98.7

3.3.3.2. Precision study

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by application of the whole procedure on the same day to three milk thistle samples (experimental replicates) spiked at three concentration levels of mycotoxins. Each sample was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, with five samples analysed in different days. The results, expressed as %RSD of peak areas are shown in Table 3.4. In all cases, precisions lower than 10% were obtained, in agreement with current legislation [10].

Table 3.4. Precision study in milk thistle seed samples (%RSD of peak areas)

Analyte	Repeatability (n=9)			Intermediate precision (n=15)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	7.8	6.9	5.8	9.5	9.2	8.6
AFB_2	7.9	8.3	7.5	8.9	9.4	7.6
AFG_1	7.1	6.9	6.5	7.5	8.1	7.4
AFG_2	8.5	7.2	9.3	9.5	9.8	9.7
OTA	4.4	5.1	4.7	5.6	4.9	6.1
FB_1	4.6	5.2	5.3	6.8	5.3	5.5
FB_2	7.5	7.9	7.5	8.1	8.5	9.3
T-2	6.4	5.3	6.1	8.9	7.8	9.9
HT-2	8.2	8.4	7.2	9.6	8.5	9.1
STE	6.9	7.4	6.7	8.3	7.6	7.8
CIT	7.4	6.5	7.5	8.4	8.6	9.1
F-X	6.2	5.1	5.8	7.8	7.3	8.5
DON	9.1	7.4	8.5	9.1	7.4	8.7
NIV	8.5	8.7	6.7	8.7	9.4	9.3
ZEN	6.6	5.2	4.9	5.9	6.4	6.3

Level 1: AFB_1, AFB_2, AFG_1, AFG_2 and OTA: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 100 μg kg^{-1}; CIT and STE: 50 μg kg^{-1}; DON: 100 μg kg^{-1}; F-X: 500 μg kg-1; and NIV 2000 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2 and OTA: 5 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; CIT and STE: 100 μg kg^{-1}; DON: 500 μg kg^{-1}; F-X: 1000 μg kg^{-1}; and NIV: 4000 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2 and OTA: 25 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; CIT and STE: 500 μg kg^{-1}; DON: 2500 μg kg^{-1}; F-X: 5000 μg kg^{-1}; and NIV: 6000 μg kg^{-1}.

3.3.3.3. Recovery studies

In order to check the trueness of the proposed methodology, recovery experiments were carried out in seeds and extract of milk thistle samples, free of mycotoxins. Samples were spiked with mycotoxins at three levels, similar to those used in the precision study, and submitted to the whole method. Each level was prepared in triplicate, submitted to the proposed method, and injected in triplicate. The results are shown in Table 3.5, and, as can be seen, very good recoveries were obtained (between 62.3% and 98.9%, except for ZEN in seeds samples and CIT in extract), fulfilling the current legislation [10] for most mycotoxins.

Table 3.5. Recovery study (%RSD of peak areas)

Analyte	Seed (n=9)			Extract (n=9)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	86.6 (7.8)	87.6 (6.9)	85.7 (5.8)	89.8 (6.1)	89.6 (4.8)	88.3 (5.6)
AFB_2	94.6 (7.9)	93.5 (8.3)	95.6 (7.5)	98.9 (6.4)	97.3 (3.8)	95.8 (4.4)
AFG_1	93.0 (7.1)	90.2 (6.9)	92.8 (6.5)	96.0 (6.3)	95.4 (5.5)	96.1 (5.8)
AFG_2	90.6 (8.5)	91.6 (7.2)	90.6 (9.3)	95.4 (6.1)	95.1 (6.6)	96.4 (6.3)
OTA	98.4 (4.4)	95.8 (5.1)	96.7 (4.7)	96.6 (4.8)	92.9 (6.8)	95.6 (5.1)
FB_1	71.9 (4.6)	75.6 (5.2)	74.7 (5.3)	79.0 (5.0)	82.1 (3.6)	80.5 (4.3)
FB_2	77.2 (7.5)	70.3 (7.9)	78.6 (7.5)	71.3 (3.3)	77.7 (3.3)	75.6 (3.8)
T-2	63.3 (6.4)	65.8 (5.3)	64.3 (6.1)	73.7 (2.7)	74.5 (8.7)	72.9 (5.6)
HT-2	78.2 (8.2)	76.4 (8.4)	77.2 (7.2)	73.8 (2.8)	81.8 (9.3)	82.6 (7.7)
STE	86.2 (6.9)	85.9 (7.4)	84.9 (6.7)	67.6 (5.1)	70.1 (7.9)	68.7 (6.3)
CIT	73.4 (7.4)	72.5 (6.5)	73.8 (7.5)	60.0 (9.0)	57.3 (9.2)	57.2 (8.1)
F-X	97.4 (6.2)	95.5 (5.1)	95.9 (5.8)	93.2 (6.0)	91.7 (9.1)	94.3 (7.1)
DON	73.8 (9.1)	75.9 (7.4)	74.8 (8.5)	77.9 (9.3)	81.2 (11.1)	82.4 (8.9)
NIV	78.6 (8.5)	75.6 (8.7)	79.3 (6.7)	90.1 (6.0)	87.9 (2.5)	86.6 (5.4)
ZEN	46.1 (6.6)	47.4 (5.2)	48.5 (4.9)	65.8 (10.9)	62.3 (9.0)	63.2 (9.5)

% RSD of peak areas is given in parentheses

Level 1: AFB_1, AFB_2, AFG_1, AFG_2 and OTA: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 100 μg kg^{-1}; CIT and STE: 50 μg kg^{-1}; DON: 100 μg kg^{-1}; F-X: 500 μg kg-1; and NIV 2000 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2 and OTA: 5 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; CIT and STE: 100 μg kg^{-1}; DON: 500 μg kg^{-1}; F-X: 1000 μg kg^{-1}; and NIV: 4000 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2 and OTA: 25 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; CIT and STE: 500 μg kg^{-1}; DON: 2500 μg kg^{-1}; F-X: 5000 μg kg^{-1}; and NIV: 6000 μg kg^{-1}.

3.3.3.4. Analysis of commercial samples

Different samples of commercial milk thistle purchased in local markets from Granada were analysed in order to show the applicability of the method: six samples of seed (three of them purchased in bulk from a street vendor) and one natural extract of milk thistle in vegetable glycerine. Each one was analysed in triplicate and injected also in triplicate. Two of the samples (purchased from a street vendor) gave a positive result for T-2 (363.0 and 453.9 μg kg^{-1} with RSD of 7.9% and 9.2%, respectively) and HT-2 (826.9 and 943.7 μg kg^{-1} with RSD of 5.7% and 5.6%, respectively). Also, ZEN was

detected in one of them, but its concentration was lower that the LOQs. A total ion chromatogram (TIC) from one of these samples with the extracted ion chromatogram (XIC) of T-2 and HT-2 is shown in Figure 3.17.

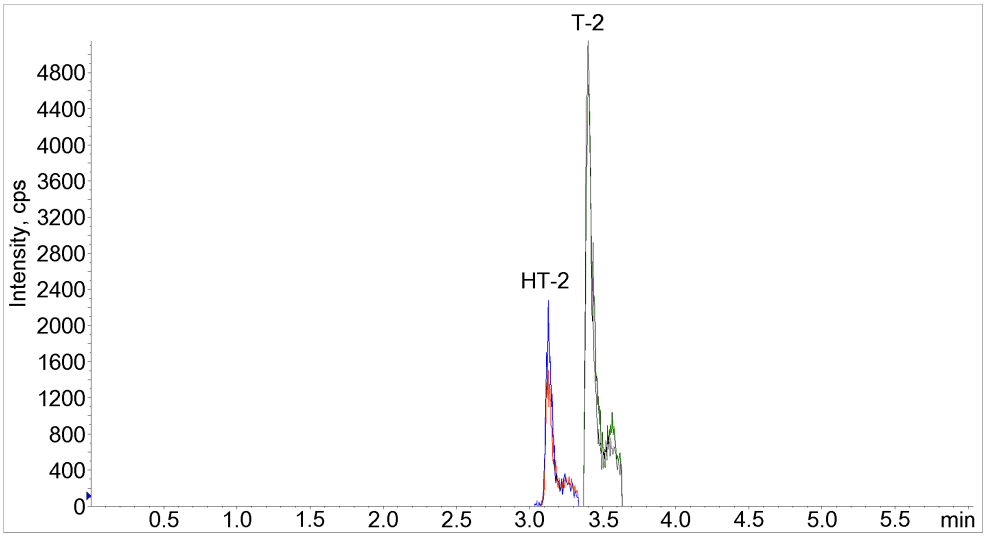


Figure 3.17. TIC from one of the positive samples of milk thistle seeds with the XIC of T-2 and HT-2

3.4. Conclusion

A new UHPLC–MS/MS method with a new sample treatment based on QuEChERS and DLLME has been proposed for the determination of 15 mycotoxins in milk thistle (a botanical). The method allows the determination of FB_1, FB_2, NIV, DON and F-X with only a QuEChERS-based extraction step. To determine aflatoxins an additional clean-up step using DLLME is required, as the strong matrix effect avoids their determination at this stage. The rest of mycotoxins (OTA, T-2, HT-2, STE, CIT and ZEN) could be also determined after QuEChERS-based extraction step, but DLLME significantly improved their LODs. To the best of our knowledge, this is the first time that QuEChERS and DLLME have been combined for the extraction of those analytes. Matrix-matched calibration curves were established and LODs and LOQs were in all cases below the usual maximum levels established by EU regulation in food and by Pharmacopeia in botanicals. The RSD was lower than 10%, and the recoveries

obtained were between 62.3% and 98.9%, except for ZEN in seed samples and CIT in extract samples, fulfilling with the requirements of EU legislation for the methods of determination of mycotoxins, except for ZEN in seed samples. Different samples obtained from local markets (seeds and extract) have been analysed. Two samples of seed purchased in street vendors gave a positive result for T-2 and HT-2; also, ZEN was detected in one of them. Taking advantage of the benefit of QuEChERS and DLLME, this method has shown to be a good alternative for the sample treatment of botanicals in multiclass mycotoxin determination, reducing extraction time, providing good recoveries and precision and being environmentally friendly. However, it shows the drawbacks that, due to the strong matrix effect of this sort of samples, two different runs are required for a proper quantification of all the analytes. The method could be adapted for other herbal products, although it would require an in-depth study and validation for each matrix, according to Pharmacopeia.

This work was published as:

"Multiclass mycotoxin analysis in *Silybum Marianum* by ultra-high performance liquid chromatography-tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid-liquid microextraction" N. Arroyo-Manzanares, A. M. García-Campaña, L. Gámiz-Gracia, J. Chromatogr. A 1282 (2013) 11.

CHAPTER 4

Nueva propuesta para la determinación de micotoxinas en cereales, pseudocerales y melazas mediante UHPLC-MS/MS

A new approach for the determination of mycotoxins in cereals, pseudocereals and cereal syrups by UHPLC-MS/MS

Resumen

Actualmente, el interés y el consumo de pseudocereales y melazas están aumentando debido a sus propiedades nutricionales. Sin embargo, como los cereales y semillas oleaginosas, las semillas de pseudocereales son susceptibles a la proliferación de hongos y la contaminación por micotoxinas. Por otro lado, las melazas de cereales se obtienen por aislamiento del almidón tras la molienda en húmedo de los granos, su hidrólisis y purificación. Debido a la utilización de materia prima contaminada o por crecimiento de hongos en el producto final, las melazas también pueden presentar contaminación por micotoxinas, sin embargo, en este sentido estas matrices han sido poco exploradas. En este capítulo se han desarrollado y caracterizado dos métodos sensibles, simples y rápidos para la determinación de 15 micotoxinas (aflatoxina B_1, aflatoxina B_2, aflatoxina G_1, aflatoxina G_2, ocratoxina A, fumonisina B_1, fumonisina B_2, nivalenol, deoxinivalenol, fusarenona-X, toxina T-2 y HT-2, citrinina, esterigmatocistina y zearalenona) en cereales (espelta, arroz blanco, arroz integral y arroz rojo) y pseudocereales (trigo sarraceno, quinoa y amaranto) y de 10 micotoxinas (ocratoxina A, fumonisina B_1, fumonisina B_2, deoxynivalenol, fusarenona-X, toxina T-2 y HT-2, citrinina, esterigmatocistina y zearalenona) en melazas (de arroz, trigo y cebada). Para la determinación se ha empleado la cromatografía de líquidos de ultra eficacia acoplada a la espectrometría de masas, proponiéndose un tratamiento de muestra basado en el procedimiento QuEChERS. Los límites de cuantificación obtenidos fueron inferiores a los contenidos máximos establecidos por la legislación europea en cereales. La precisión fue inferior al 12% en todos los casos, con recuperaciones comprendidas entre 60.0-103.5% para pseudocereales y cereales, y entre 70.2-100.6% para melazas, cumpliendo la legislación vigente. Finalmente, se confirmó el contenido de aflatoxina B_1 encontrado en una muestra de arroz rojo, comparando el resultado con el obtenido mediante el empleo de columnas de inmunoafinidad para la extracción y limpieza.

Abstract

Nowadays the interest and consumption of pseudocereals and cereal syrups is increasing due to its nutritional properties. However, like cereals and oilseeds, pseudocereal seeds are susceptible to fungal growth and mycotoxin contamination. On the other hand, cereal syrups are obtained by isolation of starch after wet milling of grains, its hydrolysis and further purification. Thus, mycotoxins may be found in cereal syrups resulting from the use of contaminated raw material or due to fungal growth in the syrup. Nevertheless, in these sense, these matrices have received little attention in literature. Two sensitive, simple and rapid methods for the determination of 15 mycotoxins (aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, ochratoxin A, fumonisin B_1, fumonisin B_2, nivalenol, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, sterigmatocystin and zearalenone) in cereals (spelt, white rice, brown rice and red rice) and pseudocereals (buckwheat, quinoe and amaranth), and for the determination of 10 mycotoxins (ochratoxin A, fumonisin B_1, fumonisin B_2, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxin, citrinin, sterigmatocystin and zearalenone) in cereal syrups (rice, wheat and barley) have been developed and characterized. For the determination, ultra-high performance liquid chromatography coupled with tandem mass spectrometry was proposed, using a sample treatment based on a QuEChERS procedure. Matrix-matched calibration curves were established and limits of quantification were below the maximum contents established by European Union regulation in cereals. The precision (repeatability and intermediate precision) was lower than 12% in all the cases, and recoveries were between 60.0% and 103.5% for pseudocereals and cereals and between 70.2% and 100.6 % for syrups, fulfilling the current legislation. Finally, the content of aflatoxin B_1 found in a red rice sample was confirmed by comparison of the result with this obtained by using immunoaffinity columns for extraction and clean-up.

4.1. Introduction

Regarding mycotoxin contamination, cereals are one of the most studied and controlled food matrices. Rice (*Oryza sativa*) is one of the most consumed cereals in the world and, as described in Chapter 1, it is highly prone to contamination by mycotoxins. Among the different varieties, brown rice is increasingly been chosen by customers because of its health benefits, whereas red rice (obtained by the fermentation of normal rice with fungal from genus *Monascus* [1] has been traditionally used in China due to its medicinal properties (anti-hypertensive, anti-diabetic and blood circulation regulator), and nowadays it is an important component of the Chinese diet. However, several species of the genus *Monascus* also produce CIT, thus this is a matrix of interest in the determination of mycotoxins [2].

Other cereal of interest is spelt (*Triticum spelta*), which was about to disappear due to its low yield; however, its nutritional properties, high resistance in unfavourable environments and lower fertilization requirement compared to wheat, have made it increasingly valuable for food product manufacturers and consumers [3]. Currently, spelt is one of the major cereals in isolated regions throughout Middle Europe [4]. Although mycotoxin contamination in spelt has not been extensively explored, spelt products has been included in several studies, such as the determination of aflatoxins, OTA, T-2 toxin, DON and ZEN by ELISA in spelt products of the Polish market [5]. Moreover, LC-MS was proposed for studying the occurrence of 14 mycotoxins in cereals of the Mediterranean area [6], trichothecenes, ZEN and its metabolites in grain cereal, flour and bread [7], mycotoxin profiles in the grain of several cereals after

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infection with *Fusarium culmorum* [8], multi-mycotoxin determination in raw cereals [9] and DON in various grains [10], all of them including spelt products.

Other matrices of great interest are pseudocereals, which are plants that produce fruits or seeds, used and consumed as grains. Though botanically they are neither grasses nor true cereal grains, as they produce starch-rich seeds like cereals they are called pseudocereals. Amaranth (*Amaranthus*), quinoa (*Chenopodium quinoa*) and buckwheat (*Fagopyrum esculentum*) are among the most consumed pseudocereals. Thus, the world total production of buckwheat was 1,516,250 tonnes in 2010, being the main producers China (38.9%), Russia (22.4%), Ukraine (8.8%) and France (8.3%); while quinoa world total production was 78,025 tonnes in 2010 and main producers were Peru (52.6%) and Bolivia (46.3%) [11]. Amaranth cultivation remains relatively low, but an appreciable commercial cultivation of this pseudocereal for human nutrition is currently taking place. Nowadays the interest and consumption of pseudocereals is increasing due to its nutritional properties, such as its protein quality and amino acid balance of amaranth and quinoa or the concentration of phytochemicals of buckwheat [12]. In addition, pseudocereals are currently emerging as healthy alternatives to gluten-containing grains in the gluten-free diet necessary for celiac disease.

Like cereals and oilseeds, pseudocereal seeds are susceptible to fungal growth and subsequent mycotoxin contamination; however this issue has received little attention in literature. The papers concerning this aspect include a study of the influence of water activity on the growing of aflatoxins and ZEN in amaranth grains [13], the isolation of strains of *Fusarium* from quinoa [14] and buckwheat [15] or the study of the incidence of fungal contamination of quinoa, where toxigenic strains of *A. flavus*, *A. parasticus*, *P.*

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citrinum, *P. grisefulvum* were found on this pseudoceral [16]. Moreover, strains of these fungi in addition to *A. versicolor*, *P. viridicatum*, *F. moniliforme*, *F. equiseti* and *F. semitectum* have been isolated from amaranth [17]. Therefore, there is an actual risk of pseudocereal contamination by mycotoxins as demonstrated in several studies. For example, a survey of *Fusarium* toxins in foodstuffs of plant origin, including pseudocereals, collected during 2000 and 2001 within the German market was carried out [18]; fortunately in this case pseudocereal products were free of these toxins. However, natural occurrence of ZEN has been found in amaranth [19]; AFB_1 [20] and DON [21] were found in buckwheat, and OTA in buckwheat flour and buckwheat noodles, among other products tested in several studies carried out to assess the exposure to mycotoxins in Japan [22, 23]. Moreover, ZEN, OTA and traces of roquefortin C [24] and STE were found in two samples of buckwheat in a survey carried out in Lativa [25]. The analytical methods employed for these determinations were based mainly on LC with fluorescence detection using a derivatization step [20], LC with MS [22,24,25], TLC [13,19,22] or, less commonly, GC-MS [18,21].

On the other hand, some foods that use cereals as feedstock are also interested for the control of mycotoxins, as is the case of syrup. Cereal syrups are obtained by isolation of starch after wet milling of grains, its hydrolysis and further purification. Different temperatures, pH, and kind of enzyme employed in the starch hydrolysis process, produce syrups with different characteristics due to different carbohydrate profiles

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(maltose, glucose or fructose) [26]. Cereal syrups are widely applied in food and pharmaceutical industry and its consumption is increasing nowadays. They are used as an alternative to refined cane or beet sugar due to its soft texture, adding volume and increasing flavour of food. For instance, the starch sweeteners are replacing the traditional cane sugar in USA and the consumption of high fructose corn syrup, containing either 42% or 52% of fructose, is almost similar to that of sucrose [27, 28]. Reasons for this trend are a cheaper production and preferences in food and soft drink industries, since starch sweeteners are easier to handle than crystalline sucrose, readily water-soluble and easier to incorporate into soft drinks, imparting a fruit flavour to beverages and foods [26,28]. Also, pure maltose crystallized from maltose syrup is employed in manufacture of antibiotics, vaccines, etc. [29].

Mycotoxins may also be found in cereal syrups resulting from the use of contaminated raw material. Depending on the properties of different mycotoxins (mainly stability and water solubility), carry over from contaminated grains to different fractions during cereal processing may take place [30], therefore increasing the chances of final food product contamination. Some studies carried out at laboratory scale to study the fate of mycotoxin contamination through the wet-milling of maize, concluded that very low levels of toxins were detected in the starch fractions [31]. However, a study carried out at plant scale demonstrated that about 30% of DON was transferred to the starch fraction in the commercial wet milling process of maize [32]. In addition to the starting cereals employed, another potential source of mycotoxin contamination of the final

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manufactured cereal syrups is the invasion by microorganisms. In the case of sugar cane molasses, a matrix with similar characteristics as cereal syrups, Scott *et al.* considered that prolonged storage could permit mould growth particularly if the package is opened [33]. These moulds exhibit the potential to produce toxic metabolites [34]. Moreover, El-Said [35] and Anfossi *et al.* [36] have already described the mycotoxin contamination of molasses. From these studies it is possible to suggest that accurate and reliable methods for the determination of mycotoxins in cereal syrups (a scarcely explored matrix) are required in order to ensure the quality of these products and to preserve the consumer health. To date, there are very few methods for the determination of mycotoxins in these matrices, usually based on TLC [37].

The analytical methods above mentioned have been mainly based on IACs [18,20,22], SPE [25,37] or extractions with different solvents [7,8,9,10,13,19,24]. However, some of these sample treatments, as IAC columns, are expensive and comprise complex purification; moreover, multiclass analysis is quite limited, due to their inherent selectiveness. As a consequence, simpler, more efficient, multiclass and environmentally friendly extraction systems are demanded.

In this paper we present an analytical method with an extraction based on the first step of QuEChERS methodology, followed by UHPLC-MS/MS determination. The method allowed the simultaneous quantification of 15 mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2, CIT, STE, F-X, NIV and DON) in different kinds of rice (an extensively consumed cereal), spelt and pseudocereals (as scarcely studied matrices). Slightly modified, the method was also suitable for the determination of 10 mycotoxins

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(OTA, FB_1, FB_2, T-2, HT-2, CIT, STE, F-X, NIV and DON) in several cereal syrups (wheat, barley and rice syrup).

The studied mycotoxins are regulated by the European legislation [38-42], and other not regulated but considered by the IARC [43]. The proposed methods were fully validated and characterized in terms of linear dynamic range, LODs and LOQs, matrix effect, precision and trueness. Moreover, when a rice sample was found to be contaminated with a high concentration of AFB_1, the obtained result was statistically confirmed by a standard method, based on extraction by IACs [44].

4.1. Experimental

4.1.1. Chemicals and solvents

All reagents were of analytical reagent grade, solvents were LC-MS grade and mycotoxins were analytical standard grade. Formic acid eluent additive for LC-MS, MeOH, ammonium formate and individual standards of each mycotoxin were obtained from Sigma Aldrich (St Louis, MO, USA). Formic acid (analysis grade) was supplied by Merck (Darmstadt, Germany); MeCN, potassium dihydrogen phosphate, disodium

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[39] Commission Regulation (EC) No. 1126/2007 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards Fusarium toxins in maize and maize products. Off. J. Eur. Commum. L255 (2007) 14.

[40] Commission Regulation (EU) No. 165/2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. Off. J. Eur. Commum. L50 (2010) 8.

[41] Commission Regulation (EU) No. 594/2012 amending Regulation (EC) 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs. Off. J. Eur. Commum. L173 (2012) 43.

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[44] UNE-EN 12955, ‘‘Foodstuffs. Determination of aflatoxin B_1 and the sum of aflatoxins B_1, B_2, G_1 and G_2 in cereals, shell-fruits and derived products. High performance liquid chromatographic method with post-column derivatisation and immunoaffinity column clean up’’

phosphate, potassium chloride and sodium chloride were supplied by Panreac (Madrid, Spain).

Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout the work.

SampliQ QuEChERS kits (supplied by Agilent Technologies Inc., Wilmington, DE, USA) consisted of buffered QuEChERS extraction packet (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate), or non-buffered QuEChERS extraction packet (4 g $MgSO_4$, 1 g NaCl).

The phosphate buffer used for purification of the samples in the IACs (AflaCLEAN, LC Tech, Dorfen, Germany) was prepared by dissolving 0.2 g KCl, 0.2 g KH_2PO_4, 1.16 g Na_2HPO_4 and 8 g NaCl in 1 L of water, and adjusting the pH at 7.4.

A 30 mM phosphate buffer solution pH 7.1 was prepared by dissolving 2.07 g of sodium dihydrogen phosphate monohydrate in 500 mL of water, adjusting the pH with NaOH solution.

Acrodisc 13 mm syringe filters with 0.2 µm nylon membrane (Pall Corp., MI, USA) were used for filtration of samples prior to the injection into the chromatographic system.

4.2.2. Instrumentation and software

All experiments were carried out using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump, online degasser, autosampler (5 µL loop), and a column thermostat. The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (AB SCIEX, Toronto, ON, Canada) with ESI. A Zorbax Eclipse Plus HHRD (50×2.1 mm, 1.8 µm) was used as chromatographic column.

An Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and an evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used.

The pH of the different buffers was adjusted with a pH meter (Crison model pH 2000, Barcelona, Spain) with a resolution of 0.01 pH unit. A vacuum manifold system from Supelco (Bellefonte, USA) was used for IAC procedure.

The instrumental data were collected using the Analyst® Software version 1.5 with Schedule MRM TM Algorithm (AB SCIEX).

4.2.3. Sample treatment

Pseudocereals (buckwheat, quinoe and amaranth), cereal (white rice, brown rice, red rice and spelt) and cereal syrup samples were purchased in local markets of Granada (Spain) and stored at room temperature. The pseudocereal and cereal samples were grinded, homogenised and subjected to sample treatment.

4.2.3.1. QuEChERS procedure for cereals and pseudocereals

A 2 g portion of sample and 8 mL of water were placed into a 50 mL screw cap test tube with conical bottom, which was shaken by vortex for 10 s. Subsequently, 10 mL of 5% formic acid in MeCN was added to the tube and shaken by vortex for 2 min. Agilent SampliQ EN QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the tube was shaken vigorously by hand for 1 min. After that, the sample was centrifuged at 4500 rpm for 5 min. Then, 2 mL of the upper MeCN layer was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2 μm filter before injection and the 15 mycotoxins were determined by UHPLC-MS/MS. The sample treatment is summarized in Figure 4.1.

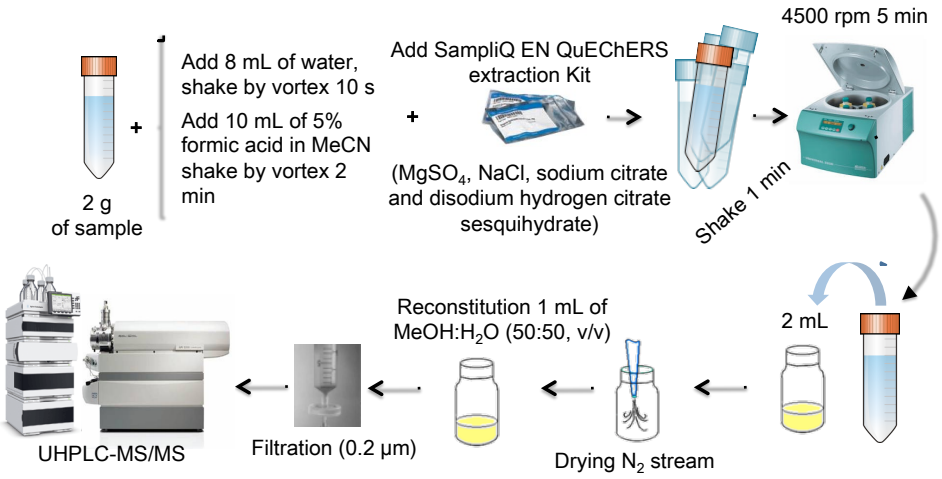

Figure 4.1. Sample treatment for the determination of mycotoxins in cereals and pseudocereals

4.2.3.2. QuEChERS procedure for cereal syrups

A 2 g portion of syrup and 8 mL of 30 mM NaH_2PO_4 buffer pH 7.1 were placed into a 50 mL screw cap test tube with conical bottom and shaken by vortex for 10 s. Subsequently, 10 mL of 5% formic acid in MeCN was added to the tube, which was shaken again by vortex for 2 min. Agilent SampliQ EN QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate and 0.5 g disodium hydrogen citrate sesquihydrate) was added and the tube was shaken vigorously for 1 min. After that, the sample was centrifuged at 4500 rpm for 5 min. Then, 2 mL of the upper MeCN layer was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2 µm filter before injection and the 10 mycotoxins were determined by UHPLC-MS/MS. The sample treatment is summarized in Figure 4.2.

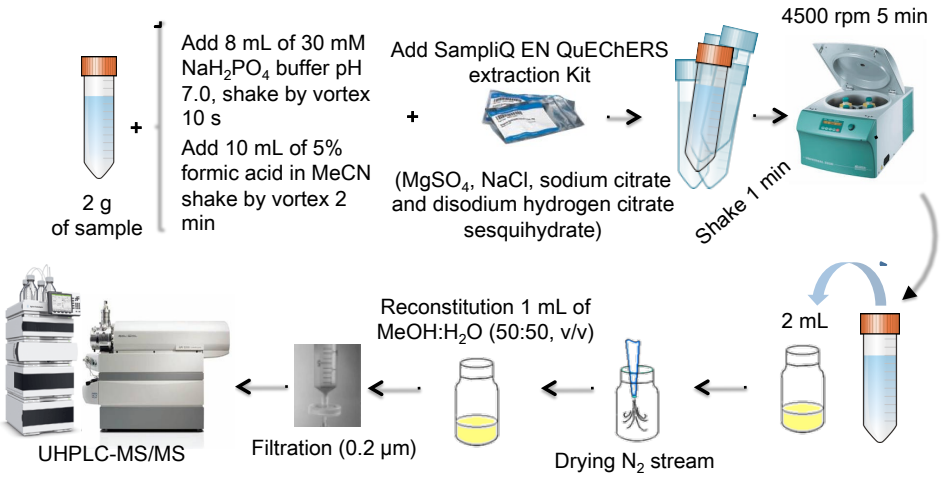

Figure 4.2. Sample treatment for the determination of mycotoxins in cereal syrups

4.2.3.3. Sample preparation with IAC for determination of aflatoxins

Following the recommendations of a standard method [44], described in Chapter 1, a portion of 5 g was accurately weighted and placed in a falcon tube (50 mL) and 25 mL of MeOH:H_2O (70:30, v/v) was added. The mixture was vortexed for 30 s twice. An additional volume of 25 mL of water was added and the solution was gently mixed. The tube was then centrifuged at 4500 rpm for 10 min. A 10 mL aliquot of the extract was taken for the clean-up step through an IAC, at a flow rate of 1 drop per second, approximately. After that, the column was washed twice with phosphate buffer (prepared as described in Section 4.2.1.) and subsequently dried. Finally, the aflatoxins were eluted with 1 mL of MeOH (three back flushing) followed by 1 mL of water. The eluate was collected in a vial and directly analysed by UHPLC-MS/MS.

4.2.4. UHPLC-MS/MS analysis

The chromatographic conditions and MS detection were described in Chapter 3. UHPLC separations were performed in a C_{18} column (Zorbax Eclipse Plus HHRD 50 × 2.1 mm, 1.8 µm) using a mobile phase consisting of 0.3% aqueous formic acid solution

with 5 mM ammonium formate (solvent A), and MeOH with 0.3% formic acid and 5 mM ammonium formate (solvent B) at a flow rate of 0.4 mL min^{-1}.

The eluent gradient profile was as follows: 0 min: 5% B; 1 min: 50% B; 2 min: 72% B; 4 min: 80% B; and 6 min: 90% B. Finally it was back to 5% B in 0.2 min and maintained for 1.8 min for column equilibration. The temperature of the column was 35°C and the injection volume was 5 µL (full loop).

The MS was working with ESI in positive mode under the MRM conditions shown in Table 4.1. The ionization source parameters were: source temperature 500°C; curtain gas (nitrogen) 30 psi; ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi. In all the cases a precursor ion and two product ions (one for quantification and the other one for confirmation) were studied, obtaining four identification points, as usually required by EU for confirmation of contaminants in foodstuff [45].

[45] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). Off. J. Eur. Commun. L221 (2002) 8.

Table 4.1. Monitored ions of the target analytes and MS/MS parameters

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CEn[a]	CXP[a]
NIV	1.08	313.1	$[M+H]^+$	41.0	5.0	14.0	174.9 (Q)	17.0	4.0
							128.0 (I)	75.0	6.0
DON	1.32	297.1	$[M+H]^+$	36.0	5.5	16.0	249.2 (Q)	17.0	4.0
							161.0 (I)	29.0	4.0
F-X	1.50	355.1	$[M+H]^+$	26.0	12.0	18.0	174.7 (Q)	23.0	4.0
							137.1 (I)	31.0	4.0
AFG_2	1.86	331.1	$[M+H]^+$	61.0	6.0	42.0	245.1 (Q)	39.0	4.0
							313.1 (I)	27.0	6.0
AFG_1	1.95	329.0	$[M+H]^+$	76.0	9.5	16.0	243.1 (Q)	39.0	6.0
							311.1 (I)	29.0	6.0
AFB_2	2.07	315.1	$[M+H]^+$	81.0	4.0	34.0	286.9 (Q)	33.0	6.0
							259.0 (I)	39.0	8.0
AFB_1	2.18	313.1	$[M+H]^+$	46.0	12.0	26.0	241.0 (Q)	41.0	4.0
							284.9 (I)	39.0	4.0
CIT	2.31	251.2	$[M+H]^+$	26.0	11.0	18.0	233.0 (Q)	23.0	23.0
							204.8 (I)	73.0	10.0
HT-2	2.70	442.0	$[M+NH_4]^+$	21.0	5.5	21.0	262.8 (Q)	22.0	8.0
							215.4 (I)	19.0	4.0
FB_1	2.93	722.2	$[M+H]^+$	71.0	10.0	30.0	334.2 (Q)	51.0	6.0
							352.2 (I)	47.0	6.0
T-2	3.11	484.0	$[M+NH_4]^+$	21.0	10.0	22.0	215.0 (Q)	22.0	4.0
							185.0 (I)	29.0	4.0
ZEN	3.53	319.0	$[M+H]^+$	26.0	8.0	20.0	282.9 (Q)	19.0	4.0
							301.0 (I)	15.0	10.0
OTA	3.59	404.0	$[M+H]^+$	41.0	7.5	16.0	238.9 (Q)	31.0	6.0
							102.1 (I)	91.0	6.0
STE	3.68	325.1	$[M+H]^+$	66.0	3.5	26.0	281.0 (Q)	43.0	4.0
							310.0 (I)	37.0	4.0
FB_2	3.76	706.2	$[M+H]^+$	71.0	10.5	20.0	336.3 (Q)	43.0	14.0
							318.3 (I)	45.0	12.0

[a] Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CEn). All expressed in voltage.

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

4.3. Results and discussion

4.3.1. Optimization of sample treatments

4.3.2.1. Optimization of the QuEChERS procedure for cereals and pseudocereals

The sample treatment was based on the extraction/partitioning process of the QuEChERS procedure, allowing the isolation of 15 mycotoxins, and was adapted from that described in Chapter 3 for the determination of mycotoxins in botanicals [46]. White rice was used as representative matrix during the optimization. Initially, a simple solid-liquid extraction was tested, using two different extraction solvents: a) 10 mL of MeOH with 5% formic acid; and b) 10 mL of MeCN with 5% formic acid. However, poor recoveries were obtained and then, different solvents with different extraction kits for extraction/partitioning process were tested: c) 8 mL of H_2O + 10 mL of MeCN with 5% formic acid + non-buffered QuEChERS extraction packet (4 g $MgSO_4$, 1 g NaCl); d) 8 mL of H_2O + 10 mL of MeCN with 5% formic acid + buffered QuEChERS extraction packet (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate); e) 8 mL of 30 mM NaH_2PO_4 buffer pH 7.1 + 10 mL of MeCN with 5% formic acid + buffered QuEChERS extraction packet. The best results were obtained with d) (see Figure 4.3). Therefore, those conditions were selected for further experiments.

[46] N. Arroyo-Manzanares, A. M. García-Campaña, L. Gámiz-Gracia, J. Chromatogr. A 1282 (2013) 11.

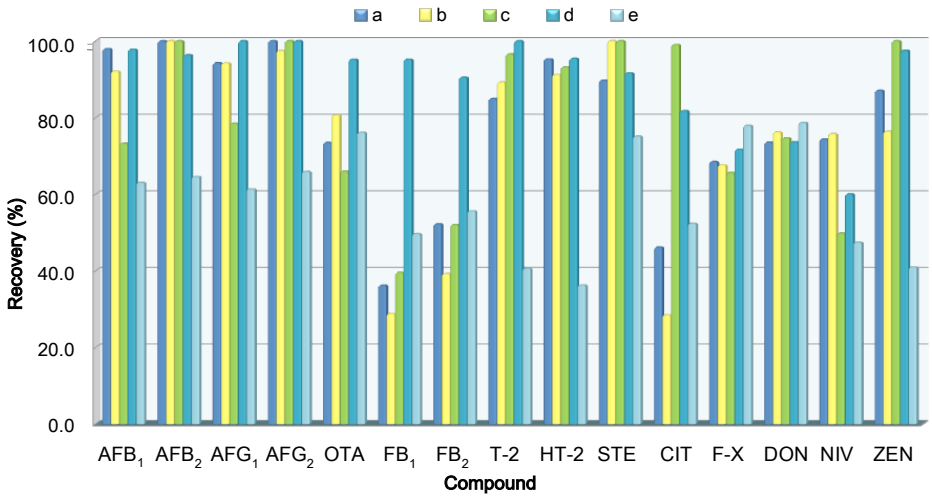

Figure 4.3. Study of different solvents and different QuEChERS extraction kits for mycotoxin extraction in white rice: (a), (b), (c), (d) and (e) extraction conditions as indicated in the text

Then, the volume of extraction solvent (MeCN with 5% formic acid) was studied between 5 and 10 mL, choosing 10 mL as optimum value, because recoveries considerably worsened when lower extraction volumes were used (see Figure 4.4), while using higher volumes the sample was more diluted and LODs and LOQs increased.

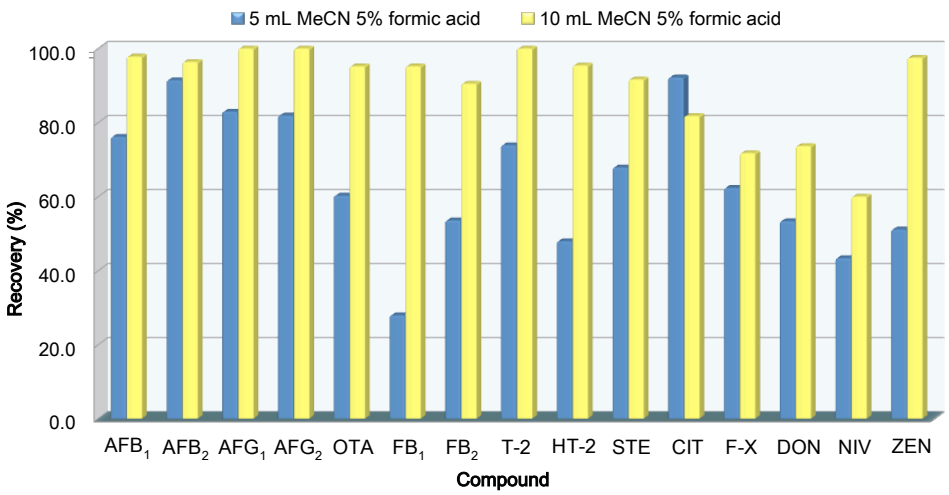

Figure 4.4. Study of the volume of the extraction solvent for mycotoxin extraction in white rice

Subsequently, the volume of dried organic phase extract was studied and 2 mL was selected as a compromise, allowing low LODs and a short time of sample treatment (Figure 4.5).

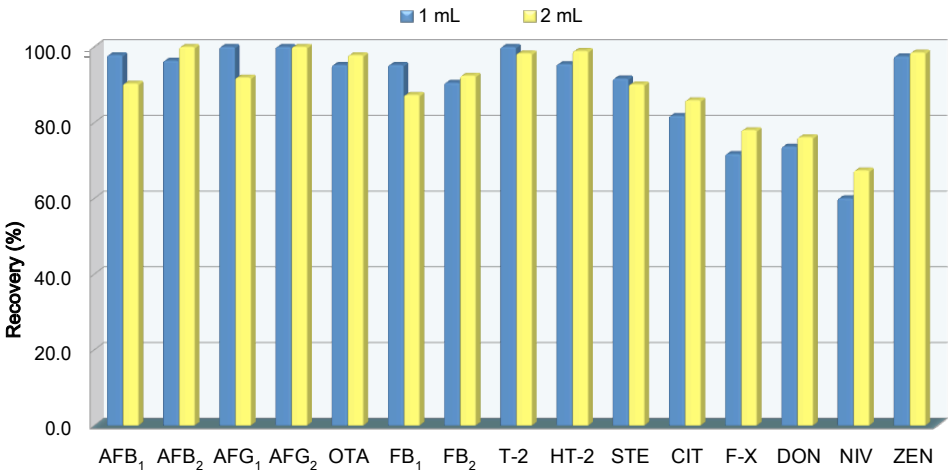

Figure 4.5. Study of the volume of dried organic phase for mycotoxin extraction in white rice

After this extraction, further clean-up by dSPE was not necessary, as extracts were clean enough for quantification purposes. This procedure is described in Figure 4.1.

A typical chromatogram corresponding to a spiked white rice sample submitted to the proposed method is shown in Figure 4.6.

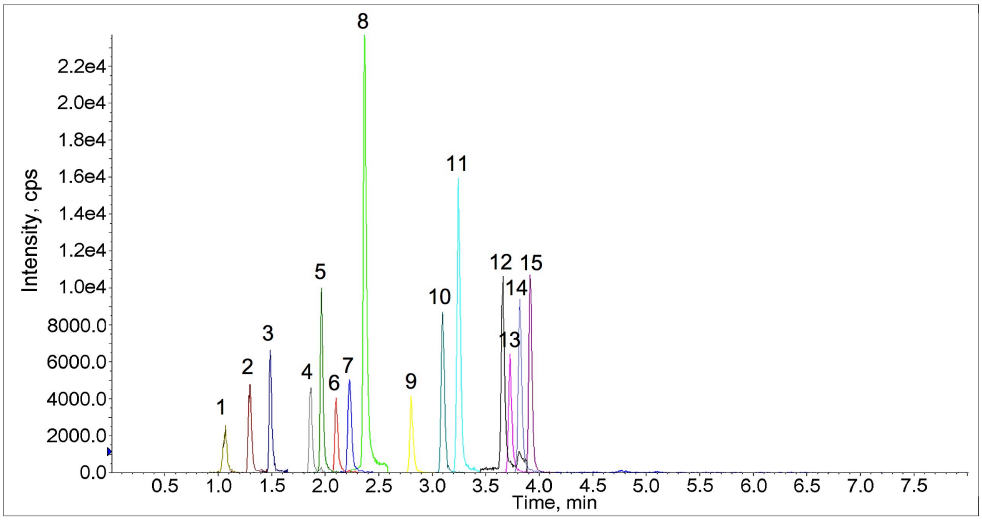

Figure 4.6. Chromatogram of a spiked white rice sample before sample treatment (AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X and NIV: 2500 μg kg^{-1}). 1: NIV; 2: DON; 3: F-X; 4: AFG_2; 5: AFG_1; 6: AFB_2: 7: AFB_1; 8: CIT; 9: HT-2; 10: FB_1; 11: T-2; 12: ZEN; 13: OTA; 14: FB_2; 15: STE

4.3.2.3. Optimization of the QuEChERS procedure for cereal syrups

The sample treatment is based on the extraction/partitioning process of the QuEChERS procedure, which allowed the determination of 10 mycotoxins. Initially, this procedure was optimized for barley syrups testing different solvents and different Agilent SampliQ EN QuEChERS extraction kits, following the same steps described in section 4.3.2.2. The dilution of the sample on less polar medium was not possible, so a) and b) were discarded. The best results were obtained with e) (see Figure 4.7), so those conditions were selected for further experiments.

Aflatoxins and NIV were also included in these studies but low recoveries were obtained for these compounds, which were finally discarded. The volume of extraction solvent was set at 10 mL as a compromise between matrix effect and LOQs. Likewise, the volume of dried organic phase extract was set at 2 mL, since higher or lower volumes involved an increase in the matrix effect or further dilution, respectively.

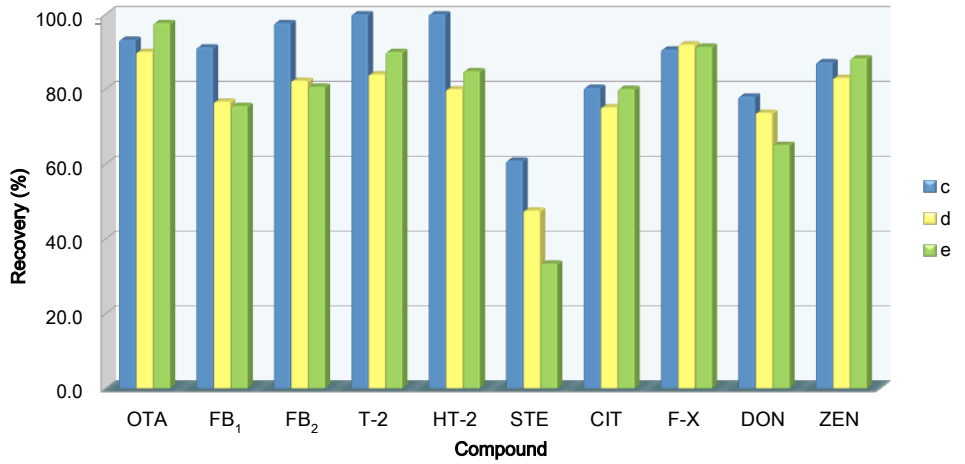

Figure 4.7. Study of different solvents and different QuEChERS extraction kits for mycotoxin extraction in barley syrup: (c), (d) and (e) extraction conditions as indicated in the text

This procedure is described in detail in Figure 4.2. A typical chromatogram corresponding to a spiked barley syrup sample submitted to the proposed method is shown in Figure 4.8.

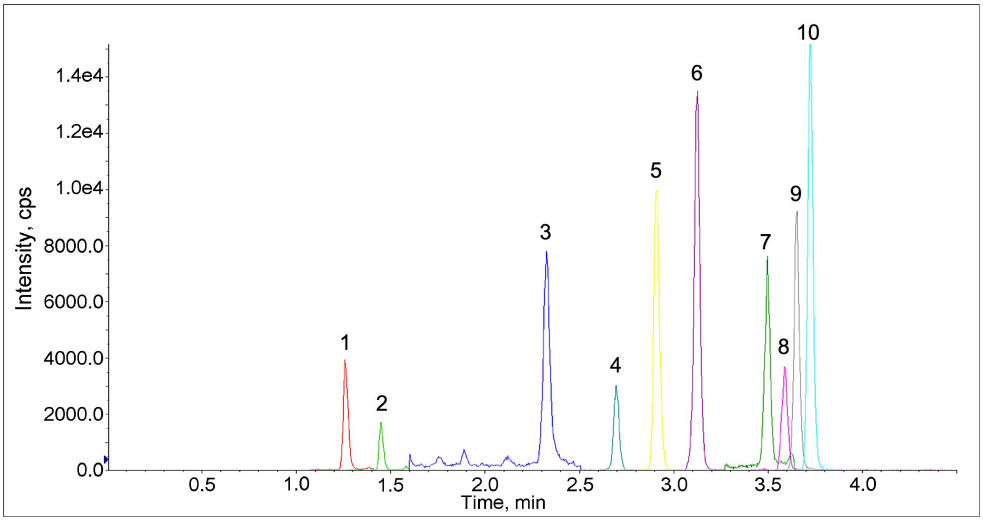

Figure 4.8. Chromatogram of a spiked barley syrup sample applying the QuEChERS-UHPLC-MS/MS proposed methodology (OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2

and ZEN: 250 µg kg^{-1}; DON: 1000 µg kg^{-1}; and F-X: 2500 µg kg^{-1}). 1: DON; 2: F-X; 3: CIT; 4: HT-2; 5: FB_1; 6: T-2; 7: ZEN; 8: OTA; 9: FB_2; 10: STE

4.3.2. Characterization of the proposed methods

In order to check the suitability of the proposed methods for the determination of mycotoxins in cereals, pseudocereals and cereals syrups, matrix effect, linear dynamic ranges, LODs and LOQs, precision and trueness were evaluated using white rice and barley syrup as representative matrices.

4.3.3.1. Matrix effect, calibration curves, limits of detection and quantification

First of all, matrix effect (ME,%) at three concentration levels was calculated as 100×[(signal of spiked extract - signal of standard solution)/signal of standard solution]. Subsequently, matrix-matched calibration curves were used for quantification purposes, established by spiking blank samples with different concentrations of mycotoxins, depending on the matrix. Each concentration level was prepared in duplicate and injected in triplicate. Peak areas of the most abundant product ions were considered as analytical signals. The statistical parameters were calculated by least-square regression, and LODs and LOQs were considered as 3×S/N ratio and 10×S/N ratio, respectively.

❖ **Cereals and pseudocereals**

Table 4.2 shows the values of the matrix effect and, as can be seen, for aflatoxins, DON and NIV matrix effect was higher than 20%, having a significant effect on the quantification of these compounds.

Table 4.2. ME (%) from white rice, calculated as 100×[(signal of a spiked extract - signal of standard solution) / signal of standard solution]

Analyte	Level 1	Level 2	Level 3
AFB_1	-23.6	-26.7	-20.5
AFB_2	-25.9	-24.7	-26.3
AFG_1	-27.5	-25.4	-23.6
AFG_2	-20.1	-17.1	-18.8
OTA	-3.1	-5.9	-6.9
FB_1	-13.2	-15.6	-14.3
FB_2	-15.7	-14.2	-17.5
T-2	-15.3	-19.8	-16.3
HT-2	-6.0	-6.3	-5.0
STE	-2.4	-4.1	-4.9
CIT	-5.2	-6.7	-6.2
F-X	-17.0	-15.3	-16.0
DON	-21.2	-26.4	-24.1
NIV	-20.6	-25.8	-21.9
ZEN	-16.5	-19.3	-17.8

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 5 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1}; F-X and NIV: 1000 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X and NIV: 2500 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X and NIV: 5000 μg kg^{-1}.

In order to compensate this matrix effect, matrix-matched calibration curves were established by spiking blank white rice samples with different concentrations of mycotoxins (1–50 μg kg^{-1} for AFB_1 and AFB_2; 0.5–50 μg kg^{-1} for AFG_1, AFG_2, OTA and STE; 5–100 μg kg^{-1} for CIT; 5–500 μg kg^{-1} for FB_1, FB_2, T-2 and HT-2 toxin; 25–2000 μg kg^{-1} for DON; 500–10000 μg kg^{-1} for NIV; 100–5000 μg kg^{-1} for F-X and 25–500 μg kg^{-1} for ZEN). Table 4.3 summarizes the results. The satisfactory determination coefficients confirm that mycotoxin analytical responses were linear over the studied ranges. The low LOQs obtained allow the quantification of legislated mycotoxins at concentrations lower than the maximum content established by legislation in cereals [38-42].

Table 4.3. Statistical and performance characteristics of the proposed method in white rice.

Analyte	Linear dynamic range (μg kg^{-1})	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Maximum content[a]
AFB_1	0.89 – 50	0.9953	0.27	0.89	2-5 μg kg^{-1} for AFB_1 and 4-10 μg kg^{-1} for sum of AFB_1, AFB_2, AFG_1 and AFG_2
AFB_2	0.67 – 50	0.9965	0.20	0.67	
AFG_1	0.23 – 50	0.9974	0.07	0.23	
AFG_2	0.30 – 50	0.9988	0.09	0.30	
OTA	0.48 – 50	0.9990	0.14	0.48	3-5 μg kg^{-1}
FB_1	0.65 – 500	0.9977	0.20	0.65	800-4000 μg kg^{-1} for sum of FB_1 and FB_2
FB_2	1.01 – 500	0.9993	0.30	1.01	
T-2	2.75 – 500	0.9990	0.82	2.75	50-1000 μg kg^{-1} for sum of T-2 and HT-2[b]
HT-2	3.45 – 500	0.9993	1.03	3.45	
STE	0.32 – 50	0.9982	0.10	0.32	-----
CIT	2.50 – 100	0.9983	0.75	2.50	-----
F-X	57.1 – 5000	0.9990	17.1	57.1	-----
DON	18.2 – 2000	0.9970	5.45	18.2	750-1750 μg kg^{-1}
NIV	233 – 10000	0.9978	69.8	233	-----
ZEN	22.7 – 500	0.9988	6.82	22.7	75-350 μg kg^{-1}

[a] Range of legislated maximum content [38-,39,40,41], including all unprocessed cereals and process cereals intended for direct human consumption with the exception of dietary foods for special medical purposes or for infants.

[b] The values given for T-2 and HT-2 are a recommendation [42].

❖ Cereal syrups

Table 4.4 shows the results and as can be seen, for FB_1, FB_2, HT-2, DON and ZEN ME was higher than 20%, involving a significant effect on the quantification of these compounds.

Table 4.4. ME (%) for barley syrup, calculated as 100×[(signal of a spiked extract - signal of standard solution) / signal of standard solution]

Analyte	Level 1	Level 2	Level 3
OTA	-7.5	-8.9	-8.5
FB_1	-28.4	-27.7	-30.7
FB_2	-35.5	-34.2	-31.4
T-2	-14.1	-17.6	-14.2
HT-2	-25.0	-27.1	-27.3
STE	-10.0	-9.2	-8.2
CIT	-12.7	-13.4	-12.6
F-X	-14.4	-14.7	-13.8
DON	-50.3	-58.3	-58.2
ZEN	-27.2	-24.5	-23.0

Level 1: OTA and STE: 1 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; and F-X: 2500 μg kg^{-1}.

Level 3: OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2500 μg kg^{-1}; and F-X: 5000 μg kg^{-1}.

Once again, the establishment of matrix-matched calibration curves were required for quantification purposes, which were established using barley syrup samples spiked at five different concentration levels of each mycotoxin (ranging from 1–50 μg kg^{-1} for OTA; 0.5–50 μg kg^{-1} for STE; 10–100 μg kg^{-1} for CIT; 5–500 μg kg^{-1} for FB_1, FB_2, T-2 and HT-2 toxin; 100–5000 μg kg^{-1} for F-X and DON and 50–500 μg kg^{-1} for ZEN). Table 4.5 summarizes the statistical and performance characteristics. The satisfactory determination coefficients confirm that mycotoxin analytical responses were linear over the studied ranges. Moreover, with the low LOQs obtained, the mycotoxins can be determined at concentrations lower than the maximum contents usually established by current legislation in different foodstuff [38], considering that there are not specific legislation for this kind of matrixes.

Table 4.5. Statistical and performance characteristics of the proposed method in barley syrup

Analyte	Linear dynamic range (μg kg^{-1})	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
OTA	0.65 – 50	0.9967	0.19	0.65
FB_1	2.89 – 500	0.9981	0.87	2.89
FB_2	2.36 – 500	0.9990	0.71	2.36
T-2	0.91 – 500	0.9986	0.27	0.91
HT-2	3.70 – 500	0.9983	1.11	3.70
STE	0.45 – 50	0.9987	0.14	0.45
CIT	5.81 – 100	0.9992	1.74	5.81
F-X	39.2 – 5000	0.9984	11.8	39.2
DON	75.2 – 5000	0.9985	22.6	75.2
ZEN	38.6 – 500	0.9963	11.5	38.6

4.3.3.2. Precision study

The precision of the methods was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by application of the whole procedure on the same day to three samples (experimental replicates) spiked at three concentration levels of mycotoxins. Each extract was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, with five samples analysed in different days.

- **Cereals and pseudocereals**

The results, expressed as RSD of peak areas, are shown in Table 4.6. In all cases, precisions lower than 12% were obtained, in agreement with current legislation [47].

[47] Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Off. J. Eur. Commun. L70 (2006) 12.

Table 4.6. Precision study (% RSD of peak areas) in white rice samples

Analyte	Repeatability (n=9)			Intermediate precision (n=15)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	7.3	6.0	2.5	9.8	10.2	6.8
AFB_2	7.5	4.4	4.8	9.2	9.1	9.9
AFG_1	7.2	5.3	2.7	9.3	9.5	9.9
AFG_2	6.9	2.6	3.9	9.8	8.0	7.0
OTA	4.2	2.9	4.1	7.3	9.6	7.5
FB_1	5.3	2.7	3.8	6.9	11.9	9.0
FB_2	5.2	1.3	1.8	6.7	9.8	7.0
T-2	8.4	2.5	2.6	9.9	8.1	8.9
HT-2	7.8	5.8	2.1	10.0	9.5	6.4
STE	6.8	4.7	5.5	8.8	8.4	7.1
CIT	5.7	4.5	6.4	11.6	8.3	10.7
F-X	8.5	6.3	3.9	11.9	9.4	10.2
DON	6.5	2.3	9.8	9.8	8.8	9.1
NIV	7.3	5.2	3.7	10.8	10.9	10.9
ZEN	8.8	6.9	1.5	9.7	9.6	7.3

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 5 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1}; F-X and NIV: 1000 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X and NIV: 2500 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X and NIV: 5000 μg kg^{-1}.

❖ Cereal syrups

The results, expressed as RSD of peak areas are shown in Table 4.7. In all cases, precisions lower than 12% were obtained, in agreement with current requirements for residue analyses [47].

Table 4.7. Precision study (% RSD of peak areas) in barley syrup

Analyte	Repeatability (n=9)			Intermediate precision (n=15)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
OTA	4.4	1.4	4.1	11.5	9.3	10.9
FB_1	3.0	2.1	1.6	7.0	6.1	6.5
FB_2	2.7	1.5	1.4	6.9	6.4	9.9
T-2	2.4	1.8	1.3	9.0	6.6	6.7
HT-2	5.7	2.6	1.6	8.4	9.3	5.9
STE	6.1	2.9	3.3	10.2	7.0	9.7
CIT	4.0	1.6	1.3	6.0	2.6	7.2
F-X	6.4	1.8	2.1	10.4	8.8	8.3
DON	8.4	3.6	3.0	9.2	9.4	9.3
ZEN	3.3	3.8	3.8	9.0	8.2	9.1

Level 1: OTA and STE: 1 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; and F-X: 2500 μg kg^{-1}.

Level 3: OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2500 μg kg^{-1}; and F-X: 5000 μg kg^{-1}.

4.3.2.3. Recovery studies

In order to check the trueness of the proposed methodology, recovery experiments were carried out in a representative number of compounds.

- **Cereals and pseudocereals**

Recovery experiments were carried out in three different pseudocereal matrices (buckwheat, quinoa and amaranth), three different kinds of rice (white, brown and red rice) and spelt. For this purpose two samples of each product (except white rice, where three samples were analysed, as white rice was used as representative matrix) were spiked at three different concentration levels, treated following the procedure described in section 4.2.3.1., and injected in triplicate in the UHPLC-MS/MS system. The results are shown in Table 4.8. As can be seen, recoveries between 60.0% and 103.5% were obtained, thus fulfilling the current legislation [47].

Table 4.8. Recovery (%) study in pseudocereals (buckwheat, quinoa and amaranth), rice (white rice, brown rice and red rice) and spelt samples (% RSD of peak areas)

Analyte	White rice (n=9)			Brown rice (n=6)			Red rice (n=6)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	100.5 (2.5)	94.7 (6.0)	90.6 (2.5)	81.8 (9.1)	77.6 (6.9)	75.5 (5.1)	95.5 (7.0)	91.4 (2.8)	80.2 (3.5)
AFB_2	99.4 (4.8)	96.1 (4.4)	102.6 (4.8)	93.4 (7.0)	94.9 (8.0)	99.5 (4.2)	86.9 (7.5)	90.1 (3.4)	97.3 (4.4)
AFG_1	92.9 (2.7)	93.6 (5.3)	99.9 (2.7)	78.5 (6.4)	77.2 (5.4)	70.5 (1.3)	94.1 (5.0)	89.2 (4.9)	85.4 (5.6)
AFG_2	99.8 (3.9)	99.2 (2.6)	103.5 (3.9)	83.4 (8.1)	89.3 (8.4)	86.1 (8.6)	92.8 (3.7)	99.4 (3.3)	94.8 (5.5)
OTA	99.8 (4.1)	94.6 (2.9)	101.5 (4.1)	94.6 (8.3)	102.8 (4.7)	94.2 (1.8)	99.7 (6.2)	98.5 (2.6)	99.8 (1.9)
FB_1	90.5 (3.8)	91.5 (2.7)	89.3 (3.8)	84.2 (7.3)	71.5 (2.9)	80.6 (1.4)	62.8 (4.7)	78.2 (2.2)	84.0 (0.7)
FB_2	97.8 (1.8)	97.2 (1.3)	96.8 (1.8)	88.6 (7.5)	71.0 (3.5)	84.3 (1.6)	70.9 (5.3)	81.7 (2.3)	88.8 (1.5)
T-2	97.2 (2.6)	100.1 (2.5)	98.8 (2.6)	93.9 (4.5)	68.7 (7.2)	75.6 (2.7)	70.7 (6.1)	90.2 (3.2)	95.0 (1.9)
HT-2	97.5 (2.1)	102.0 (5.8)	100.9 (2.1)	95.2 (9.2)	83.1 (3.3)	95.4 (2.4)	75.2 (6.7)	88.4 (2.4)	95.7 (3.8)
STE	98.5 (5.5)	88.7 (4.7)	85.9 (5.5)	64.0 (10.3)	62.1 (6.3)	65.4 (3.8)	92.9 (8.8)	79.9 (3.5)	84.1 (4.9)
CIT	80.8 (6.4)	83.2 (4.5)	90.5 (6.4)	88.0 (10.0)	90.7 (6.4)	85.7 (8.5)	88.8 (6.2)	92.0 (5.8)	99.1 (2.8)
F-X	77.9 (3.9)	78.5 (6.3)	75.0 (3.9)	72.2 (6.4)	76.7 (9.8)	76.4 (3.9)	96.0 (2.4)	94.6 (3.6)	100.2 (2.6)
DON	80.4 (6.5)	87.7 (2.3)	81.0 (9.8)	94.8 (5.4)	85.4 (5.0)	81.2 (4.7)	96.0 (5.0)	85.5 (2.5)	97.6 (3.2)
NIV	61.2 (3.7)	65.5 (5.2)	62.2 (3.7)	69.3 (6.1)	64.9 (3.3)	64.7 (6.5)	70.1 (7.5)	60.0 (5.3)	60.5 (4.3)
ZEN	98.2 (1.5)	97.3 (6.9)	95.1 (1.5)	82.2 (6.7)	72.3 (9.9)	84.5 (6.3)	81.1 (3.1)	85.6 (4.6)	95.8 (3.6)

% RSD of peak areas is given in parentheses

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 5 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1}; F-X and NIV: 1000 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X and NIV: 2500 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X and NIV: 5000 μg kg^{-1}.

Table 4.8. Recovery (%) study in pseudocereals (buckwheat, quinoa and amaranth), rice (white rice, brown rice and red rice) and spelt samples (% RSD of peak areas)

Analyte	Buckwheat (n=6)			Quinoa (n=6)			Amaranth (n=6)		
	Level 2	Level 3	Level 1	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	86.2 (6.4)	83.3 (4.4)	81.2 (2.6)	80.3 (9.6)	73.2 (2.6)	75.9 (5.4)	89.8 (8.8)	84.7 (8.5)	77.5 (6.6)
AFB_2	87.4 (8.1)	88.9 (4.8)	96.1 (3.2)	85.2 (7.6)	94.3 (8.9)	99.9 (5.3)	91.5 (6.7)	98.0 (1.9)	97.8 (3.0)
AFG_1	86.2 (7.4)	73.2 (7.1)	83.8 (2.0)	71.0 (7.8)	77.1 (3.2)	71.4 (2.6)	92.9 (7.4)	83.3 (8.4)	78.6 (2.0)
AFG_2	96.0 (6.5)	97.2 (10.9)	97.3 (8.1)	96.3 (6.4)	95.1 (7.6)	96.2 (4.0)	91.8 (9.1)	96.6 (10.0)	100.0 (5.7)
OTA	96.2 (2.5)	87.8 (2.4)	96.9 (1.3)	95.7 (3.9)	98.3 (3.6)	98.3 (2.8)	91.0 (4.4)	87.1 (7.2)	91.3 (3.1)
FB_1	88.5 (2.0)	83.5 (4.1)	99.7 (2.5)	74.6 (9.6)	70.0 (3.4)	78.3 (7.4)	75.7 (8.9)	75.0 (1.6)	84.0 (1.1)
FB_2	84.0 (1.7)	86.1 (5.0)	81.2 (1.1)	77.1 (9.3)	70.4 (2.5)	88.3 (5.1)	80.0 (4.2)	84.5 (3.7)	89.5 (2.6)
T-2	81.3 (1.3)	81.0 (1.1)	88.5 (2.3)	94.2 (5.8)	90.0 (2.4)	93.7 (4.1)	81.0 (8.5)	72.4 (6.8)	83.1 (6.3)
HT-2	82.0 (3.9)	88.4 (7.9)	91.3 (2.9)	92.3 (9.7)	93.2 (6.2)	99.3 (5.7)	76.6 (3.3)	85.4 (2.1)	83.1 (1.9)
STE	83.6 (8.8)	71.3 (8.4)	75.5 (3.3)	77.7 (8.9)	71.8 (5.2)	70.9 (3.6)	73.8 (10.6)	80.2 (11.4)	71.3 (5.4)
CIT	87.1 (6.9)	73.6 (2.7)	80.2 (1.5)	92.5 (9.8)	88.7 (6.9)	85.7 (5.3)	82.1 (5.9)	79.7 (1.8)	81.4 (2.4)
F-X	95.9 (11.1)	81.6 (1.0)	81.5 (3.1)	67.2 (8.7)	73.3 (4.5)	65.5 (6.7)	68.9 (10.1)	69.5 (2.0)	60.9 (4.5)
DON	82.3 (9.2)	83.7 (3.7)	88.3 (2.6)	73.2 (8.1)	68.3 (2.5)	73.2 (5.9)	79.3 (5.9)	80.4 (3.6)	85.6 (3.0)
NIV	71.8 (3.5)	65.2 (6.9)	60.9 (5.5)	92.8 (8.6)	89.8 (1.8)	88.2 (5.9)	67.4 (2.0)	64.5 (2.2)	73.0 (2.0)
ZEN	86.9 (6.4)	90.1 (6.9)	97.3 (0.9)	83.8 (10.4)	78.2 (5.4)	85.3 (7.7)	90.1 (7.7)	93.2 (2.3)	96.0 (3.2)

% RSD of peak areas is given in parentheses

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 5 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1}; F-X and NIV: 1000 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X and NIV: 2500 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X and NIV: 5000 μg kg^{-1}.

Table 4.8. Recovery (%) study in pseudocereals (buckwheat, quinoa and amaranth), rice (white rice, brown rice and red rice) and spelt samples (% RSD of peak areas)

Analyte	Spelt (n=6)		
	Level 1	Level 2	Level 3
AFB_1	94.7 (5.0)	86.8 (4.5)	87.1 (6.8)
AFB_2	95.1 (8.0)	89.2 (2.5)	92.2 (3.2)
AFG_1	90.5 (5.1)	82.5 (2.9)	81.6 (9.2)
AFG_2	97.2 (8.0)	89.7 (5.1)	92.5 (4.5)
OTA	99.0 (5.4)	92.9 (1.2)	96.4 (1.1)
FB_1	79.4 (5.6)	78.8 (2.4)	81.6 (1.6)
FB_2	82.5 (4.5)	84.0 (1.2)	89.6 (0.7)
T-2	73.7 (8.8)	71.5 (0.7)	74.6 (2.8)
HT-2	89.9 (4.1)	94.8 (1.2)	99.3 (1.2)
STE	80.1 (8.0)	88.7 (2.6)	86.4 (8.6)
CIT	69.2 (10.4)	66.2 (2.6)	61.4 (4.1)
F-X	75.8 (2.3)	88.2 (1.4)	83.5 (1.4)
DON	73.5 (4.6)	74.8 (4.6)	81.9 (7.9)
NIV	61.8 (6.7)	60.8 (9.1)	63.3 (1.9)
ZEN	92.1 (9.7)	90.2 (3.4)	98.1 (3.5)

% RSD of peak areas is given in parentheses

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 5 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1}; F-X and NIV: 1000 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; F-X and NIV: 2500 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1}; F-X and NIV: 5000 μg kg^{-1}.

❖ Cereals syrups

Recovery experiments were carried out in three different cereal syrups with different carbohydrates content: wheat syrup (28.5 % glucose, 10 % fructose and 25.5 % maltose in weight), barley syrup (8.5 % glucose and 39 % maltose in weight) and rice syrup (17% glucose and 39% maltose in weight). These samples were spiked at three different concentration levels, processed as described previously and injected in triplicate (n=9) into the UHPLC-MS/MS system. The results are shown in Table 4.9 and as can be seen, very good recoveries were obtained (between 70.2% and 100.6 %), fulfilling the current demands for residue analysis [47].

Table 4.9. Recovery (%) study in cereal syrups (barley, wheat and rice) (% RSD of peak areas)

Analyte	Barley syrup (n=9)			Wheat syrup (n=9)			Rice syrup (n=9)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
OTA	99.3 (4.4)	89.1 (1.4)	84.6 (4.1)	87.4 (9.5)	98.8 (2.6)	99.5 (2.8)	76.2 (8.2)	76.2 (4.9)	73.1 (2.5)
FB_1	76.9 (3.0)	71.3 (2.1)	72.4 (1.6)	81.3 (9.6)	89.8 (3.2)	91.3 (4.8)	71.3 (10.0)	74.4 (2.2)	77.0 (1.8)
FB_2	79.2 (2.7)	71.9 (1.5)	72.9 (1.4)	91.4 (6.8)	92.0 (1.4)	95.8 (3.1)	81.5 (4.6)	77.0 (1.8)	77.6 (1.8)
T-2	86.6 (2.4)	82.3 (1.8)	87.6 (1.3)	93.4 (9.3)	92.2 (1.6)	97.4 (5.6)	80.5 (7.3)	83.2 (2.6)	84.9 (1.7)
HT-2	81.3 (5.7)	79.3 (2.6)	84.1 (1.6)	85.3 (9.1)	93.2 (2.3)	92.4 (7.8)	75.8 (6.5)	80.0 (2.6)	84.1 (1.8)
STE	73.4 (6.1)	73.6 (2.9)	75.9 (3.3)	76.2 (7.9)	69.3 (3.7)	62.8 (5.1)	75.3 (8.9)	71.0 (7.5)	71.4 (3.1)
CIT	76.3 (4.0)	80.8 (1.6)	75.6 (1.3)	87.4 (4.4)	93.4 (3.6)	94.2 (1.3)	100.6 (3.1)	92.5 (3.4)	92.0 (1.7)
F-X	76.4 (6.4)	90.0 (1.8)	89.3 (2.1)	84.0 (7.0)	88.3 (2.6)	93.9 (3.5)	70.2 (5.2)	78.5 (2.9)	83.9 (7.6)
DON	83.4 (8.4)	71.7 (3.6)	72.3 (3.0)	98.6 (4.7)	83.7 (1.5)	89.3 (6.5)	83.3 (4.3)	85.4 (1.3)	74.1 (1.4)
ZEN	84.2 (3.3)	79.1 (3.8)	81.8 (3.8)	84.6 (7.2)	84.7 (2.2)	93.0 (2.6)	93.3 (7.2)	80.1 (2.8)	84.4 (2.1)

% RSD of peak areas is given in parentheses% RSD of peak areas is given in parentheses

Level 1: OTA and STE: 1 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 100 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: OTA and STE: 25 μg kg^{-1}; CIT: 50 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1}; and F-X: 2500 μg kg^{-1}.

Level 3: OTA and STE: 50 μg kg^{-1}; CIT: 100 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2500 μg kg^{-1}; and F-X: 5000 μg kg^{-1}.

4.3.2.4. Confirmation of AFB_1 content

Prior to recovery studies, preliminary analyses of all products were performed in order to check the absence of the mycotoxins under study in the selected samples. None of them gave a positive result above the LODs of the method, except a red rice sample for AFB_1 (see Figure 4.9). The concentration of AFB_1 was calculated with the proposed method, using standard addition calibration ($y = 480.8\ x + 4006.5$; $R^2 = 0.993$), and the found concentration was 8.3 μg kg^{-1}, high above of the regulated maximum content sets in legislation (2 μg kg^{-1}). In order to confirm this result, the positive red rice sample was analysed using the standard method based on IAC extraction and clean-up (described in detail in 4.2.3.3. section) [44], obtaining a concentration of 8.0 μg kg^{-1} (relative error = 1.9 %). Both results were statistically compared by means of the Student t-test, and the P-value obtained was 63.6%, confirming the trueness of the result and the suitability of the proposed method for the determination of mycotoxins.

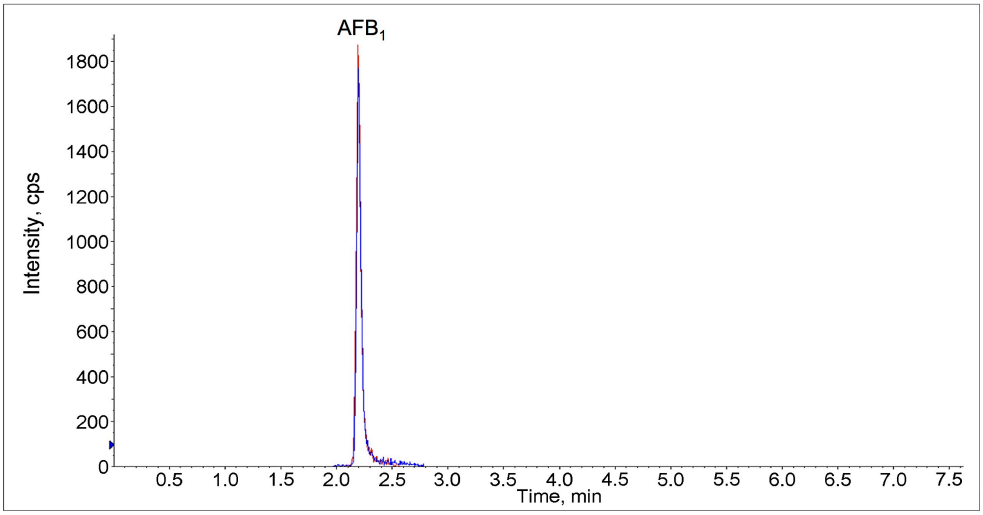

Figure 4.9. Total ion chromatogram of a positive red rice sample with the extracted ion chromatogram for AFB_1

4.4. Conclusions

An UHPLC-MS/MS method combined with sample treatments based on QuEChERS has been proposed for the determination of 15 mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2,

FB_1, FB_2, OTA, T-2, HT-2, STE, CIT, ZEN, NIV, DON and F-X) in cereals and pseudocereals, and 10 mycotoxins (OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, ZEN, DON and F-X) in cereal syrups. The proposed methodology has shown to be a suitable and efficient choice for the treatment of these samples in multiclass mycotoxin determination, reducing extraction time, providing good recoveries and precision, also being environmentally friendly. These simple treatments and the use of UHPLC-MS/MS technology, make possible a quick determination of the selected mycotoxins in a single run. Matrix-matched calibration curves were necessary to overcome matrix effect for some of the mycotoxins. LODs and LOQs were below the contents usually established by current legislation in cereals. The relative standard deviation (repeatability and intermediate precision) was lower than 12% in all cases, and recoveries were between 60.0% and 103.5% for cereals and pseudocereals and between 70.2% and 100.6% for cereal syrups, therefore fulfilling the current requirements for residue determination in foods. A red rice sample was positive for AFB_1 and the result was statistically confirmed by using IACs, as recommended by a standard method.

This work has been submitted for publication:

“Simple methodology for the determination of mycotoxins in pseudocereals, spelt and rice”. N. Arroyo-Manzanares, J. F. Huertas-Pérez, A. M. García-Campaña, L. Gámiz-Gracia, Food Control (accepted).

“Simple and efficient methodology to determine mycotoxins in cereal syrups”. N. Arroyo-Manzanares, J. F. Huertas-Pérez, L. Gámiz-Gracia, A. M. García-Campaña, Food Chem. (submitted for publication).

CHAPTER 5

Nuevo tratamiento de muestra combinado con UHPLC-MS/MS para la determinación de micotoxinas en frutos secos

A new approach in sample treatment combined with UHPLC-MS/MS for the determination of multiclass mycotoxins in edible nuts and seeds

Resumen

En este capítulo se propone un método sensible, simple y rápido para la determinación de 14 micotoxinas en frutos secos y semillas comestibles (incluyendo almendras, cacahuetes, semillas de girasol, semillas de calabaza, nueces, nueces de macadamia, pistachos, avellanas y piñones) utilizando cromatografía de líquidos de ultra resolución acoplada a espectrometría de masas en tándem. El tratamiento de muestra comprende una primera etapa basada en el procedimiento QuEChERS para la determinación de las fumonisinas B_1 y B_2, deoxinivalenol, fusarenona-X, toxinas T-2 y HT-2, citrinina, esterigmatocistina, zearalenona y ocratoxina A. Para la determinación de las aflatoxinas (B_1, B_2, G_1 y G_2) fue necesaria una posterior etapa de limpieza basada en la microextracción líquido-líquido dispersiva, ya que su determinación no era posible tras aplicar solamente la extracción basada en QuEChERS. El método fue caracterizado usando cacahuetes como matriz representativa y posteriormente se evaluó en las otras ocho matrices. Los límites de cuantificación obtenidos para las aflatoxinas, las únicas micotoxinas legisladas en estas matrices, fueron inferiores a los niveles máximos permitidos por la normativa vigente, mientras que los límites de cuantificación obtenidos para el resto de micotoxinas fueron inferiores a los permitidos en otras matrices alimentarias. La desviación estándar relativa del método fue inferior al 11% en todos los casos, y las recuperaciones oscilaron entre 60.7% y 104.3%.

Abstract

A sensitive, simple and rapid method for the determination of fourteen mycotoxins in nuts and seeds (including almonds, peanuts, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) has been developed using ultra-high performance liquid chromatography coupled with tandem mass spectrometry. The sample treatment comprises a first step based on QuEChERS procedure for the determination of fumonisin B_1, fumonisin B_2, deoxynivalenol, fusarenon-X, T-2 and HT-2 toxins, citrinin, sterigmatocystin, zearalenone and ochratoxin A. A subsequent clean-up step based on dispersive liquid-liquid microextraction was necessary for the determination of aflatoxins (B_1, B_2, G_1 and G_2), since their determination was not possible applying only the QuEChERS-based extraction. The method was characterized for peanuts as representative matrix and was subsequently evaluated for the other eight matrices. Quantification limits obtained for aflatoxins, the unique mycotoxins legislated on these matrices, were lower than the maximum levels allowed by the current legislation, while quantification limits obtained for the other mycotoxins were lower than the limits usually permitted by the legislation in other food matrices. Relative standard deviation of the method was always lower than 11%, and recoveries ranged between 60.7% and 104.3%.

5.1. Introduction

Nuts and seeds are susceptible to mould growth and consequently to mycotoxin contamination. In fact, one of the main entries of mycotoxins into the human and animal food chain is from agricultural products such as cereal grains and oil seeds or from products derived from these sources. In recent years, many studies have revealed a high mycotoxin and fungal contamination in these matrices [1,2,3]. Insect feeding-damage is a principal factor leading to pre-harvest fungal infection of nut kernels, and subsequent mycotoxins contamination, but infection may also occur after harvesting, storage and transition. Early splitting of the nuts, immature nuts and the shell splitting in early growth period causes fungal contamination [4,5]. Moreover, the difference in climate conditions, methods of handling during harvesting (tearing of the hull, remaining on the ground for an extended period), drying process and transferring leading to mechanical damages of nuts and inadequate drying after rewetting for dehulling are determinant for the final mycotoxins content [5]. In addition, long-term storage or marketing under non-hygienic conditions can produce mycotoxin contamination [6]. High humidity and high temperature within bulk bins provide ideal conditions for the infection of early split and cracked nuts, which dramatically increases the mycotoxin content [7].

These facts are reflected in the high number of alerts reported by the Rapid Alert System for Food and Feed (RASFF) in its annual reports; for instance, in 2010 about 500 alerts for contaminations of aflatoxins were reported in nut, nut products and seeds and up to 320 in 2011. Also, two alerts of OTA in pistachio were reported in 2010 [8].

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This significant reduction of notifications regarding aflatoxins in nuts and nut products between 2010 and 2011 is remarkable, and it is at least partially related to the change in the EU legislation, more than in a decrease in mycotoxin contamination levels. Thus, current EU food safety legislation only regulates the content of aflatoxins AFB_1, AFB_2, AFG_1 and AFG_2 in these matrices by means of the Regulation (CE) 1881/2006 [9]. However, this regulation was amended by Regulation (EU) 165/2010 [10], which increased the maximum levels for aflatoxins in almonds, hazelnuts and pistachios in agreement with the Codex Alimentarius. In summary, current maximum permitted levels are ranging between 2 and 8 μg kg^{-1} for AFB_1, and between 4 and 15 μg kg^{-1} for the sum of AFB_1, AFB_2, AFG_1 and AFG_2, depending on the kind of nuts or seeds for direct human consumption.

Different analytical techniques have been used for the control of mycotoxins in these foods, i.e. HPLC-FL detection in the determination of aflatoxins in nuts [1], pistachio [11], tiger nuts and beverages [12], TLC for peanuts and pistachios [11,13] or ELISA for the determination of AFB_1 in peanuts, pistachios, nuts, sesame and sunflowers seeds [14,15]. In recent years, multiresidue methods able to monitor a high number of compounds in a single run according to the established legislation [16] for many different matrices, have become one of the main target in residue analysis. In this sense, the use of LC-MS/MS has increased, as reported for the control of ochratoxins [17] or aflatoxins [18] in nuts. However, the number of multiclass analysis is still

[9] Regulation (EC) No. 1881/2006 setting maximum levels for certain contaminants in foodstuffs, Off. J. Eur. Commun. L364 (2006) 5.

[10] Commission Regulation (EU) No. 165/2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins, Off. J. Eur. Commum. L50 (2010) 8.

[11] A. M. Cheraghali, H. Yazdanpanah, N. Doraki, G. Abouhossain, M. Hassibid, S. Ali-abadi, M. Aliakbarpoor, M. Amirahmadi, A. Askarian, N. Fallah, T. Hashemi, M. Jalali, N. Kalantari, E. Khodadadi, B. Maddah, R. Mohit, M. Mohseny, Z. Phaghihy, A. Rahmani, L. Setoodeh, E. Soleimany, F. Zamanian, Food Chem. Toxicol. 45 (2007) 812.

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[15] K. R. Reddy, N. I. Farhana, B. Salleh, J. Food Sci. 76 (2011) T99.

[16] Commission Regulation (EC) No. 401/2006 of laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs, Off. J. Eur. Commum. L70 (2006) 12.

[17] K. Saito, R. Ikeuchi, H. Kataoka, J. Chromatogr. A 1220 (2012) 1.

reduced for this kind of matrices; some examples are the determination of 87 mycotoxins in chestnuts and other matrices [19] or 33 mycotoxins in peanuts and pistachios [20].

Until now, the most commonly sample treatment used for the determination of mycotoxins in nuts and seeds involved the use of solid-liquid extraction [14,15,19,20] usually followed by a clean-up step using IAC [11,13]. The disadvantages of IACs have been commented in previous chapters. Moreover, their high selectivity in the extraction would limit the multiclass analysis of these contaminants. Also, SPE with C_{18} sorbent has been proposed for the determination of aflatoxins [1,12,21] or OTA [17] in nuts and seeds, and SPME for aflatoxins in peanuts and tree seeds [18].

In the light of the good results obtained in the previous chapters, a multiclass method for the simultaneous determination of 14 mycotoxins in different nuts and seeds, based on an extraction using a QuEChERS procedure for determination of FB_1, FB_2, DON, F-X, HT-2, OTA, CIT, STE and ZEN with an additional DLLME clean-up step for determination of AFB_1, AFB_2, AFG_1 and AFG_2 is now proposed. Considering the advantages of UHPLC-MS/MS, this technique has been applied in this work for the analysis of 9 different nut and seed samples (almonds, peanuts, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts). To the best of our knowledge, this is the first time that the proposed sample treatment has been used for these food matrices, obtaining satisfactory results in combination with UHPLC-MS/MS.

5.1. Experimental

5.1.1. Chemicals and solvents

All reagents were of analytical reagent grade, solvents were LC-MS grade and mycotoxins were analytical standard grade. Formic acid, used as eluent additive for

[18] Y. Nonaka, K. Saito, N. Hanioka, S. Narimatsu, H. Kataoka, J. Chromatogr. A. 1216 (2009) 4416.
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[20] M. C. Spanjer, P. M. Rensen, J. M. Scholten, Food Addit. Contam. 25 (2008) 472.
[21] M. Vosough, M. Bayat, A. Salem, Anal. Chim. Acta 663 (2010) 11.

LC-MS, MeOH, ammonium formate and individual standards of each mycotoxin were obtained from Sigma Aldrich (St Louis, MO, USA). Formic acid (analysis grade) was supplied by Merck (Darmstadt, Germany). Sodium chloride and MeCN, were supplied by Panreac (Madrid, Spain); and chloroform was purchased from VWR BDH Prolabo (West Chester, Pensilvania, USA).

Ultrapure water (18.2 MΩ cm^{-1}, Milli–Q Plus system, Millipore Bedford, MA, USA) was used throughout all the work.

SampliQ QuEChERS Kits consisted of buffered QuEChERS extraction packet (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) and were supplied by Agilent Technologies Inc. (Wilmington, DE, USA).

Acrodisc 13 mm syringe filters with 0.2 µm nylon membrane (Pall Corp., MI, USA) were used for filtration of samples prior to the injection into the chromatographic system.

5.2.2. Instrumentation and software

All experiments were carried out using an Agilent 1290 Infinity LC (Agilent Technologies, Waldbron, Germany) equipped with a binary pump, online degasser, autosampler (5 µL loop), and a column thermostat. The mass-spectrometer measurements were performed on a triple quadrupole mass spectrometer API 3200 (AB SCIEX, Toronto, ON, Canada) with ESI. A Zorbax Eclipse Plus RRHD (50×2.1 mm, 1.8 µm) chromatographic column was used for the separation.

A Universal 320R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany), a vortex-2 Genie (Scientific Industries, Bohemia, NY, USA) and an evaporator System (System EVA-EC, from VLM GmbH, Bielefeld, Germany) were also used in the sample treatment procedure.

The instrumental data were collected using the Analyst® Software version 1.5 with Schedule MRM TM Algorithm (AB SCIEX).

5.2.3. Sample treatment

Nut and seed samples (peanuts, almonds, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) were purchased in local markets from Granada (Spain) and stored at room temperature. After milling and homogenisation of the sample, a 2 g portion was weighed into a 50 mL screw cap test tube with conical bottom. A volume of 8 mL of water was subsequently added and the tube was shaken by vortex for 10 s. Subsequently, 10 mL of 5% formic acid in MeCN was added to the tube, which was shaken by vortex for 2 min. QuEChERS extraction kit (4 g $MgSO_4$, 1 g NaCl, 1 g sodium citrate, 0.5 g disodium hydrogen citrate sesquihydrate) was added and the tube was vigorously hand-shaken for 1 min. After that, it was centrifuged at 4500 rpm for 5 min.

Then, 2 mL of the upper MeCN layer was transferred to a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The samples were filtered with a 0.2 µm filter before injection into the chromatographic system for the determination of DON, F-X, FB_1, FB_2, T-2, HT-2, STE, OTA, ZEN and CIT.

For the analysis of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) an additional clean up step based on DLLME was necessary. Thus, 2 mL of the upper MeCN layer (obtained after extraction/partitioning step) was transferred into a vial, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). This solution was placed into a 15 mL screw cap test tube with conical bottom. Subsequently, 4 mL of water and 0.21 g of NaCl (4.2%, w/v) were added. The mixture of the disperser solvent (950 µL of MeCN) and the extraction solvent (620 µL of chloroform) was rapidly injected into the test tube with a 2.0 mL syringe. The solution was vigorously shaken for a few seconds, creating a cloudy solution in the tube. In this step, the aflatoxins were extracted into the fine droplets of chloroform. The mixture was centrifuged at 6000 rpm for 3 min and the fine particles were sedimented at the bottom of the tube. The sedimented phase (approximately 400 µL) was removed using a 1 mL syringe, evaporated to near dryness under a gentle stream of N_2 and reconstituted with 1 mL of MeOH:H_2O (50:50, v/v). The solution was filtered and injected into the UHPLC-

MS/MS system for the analysis of AFB_1, AFB_2, AFG_1 and AFG_2. Figure 5.1 shows a diagram of the sample treatment.

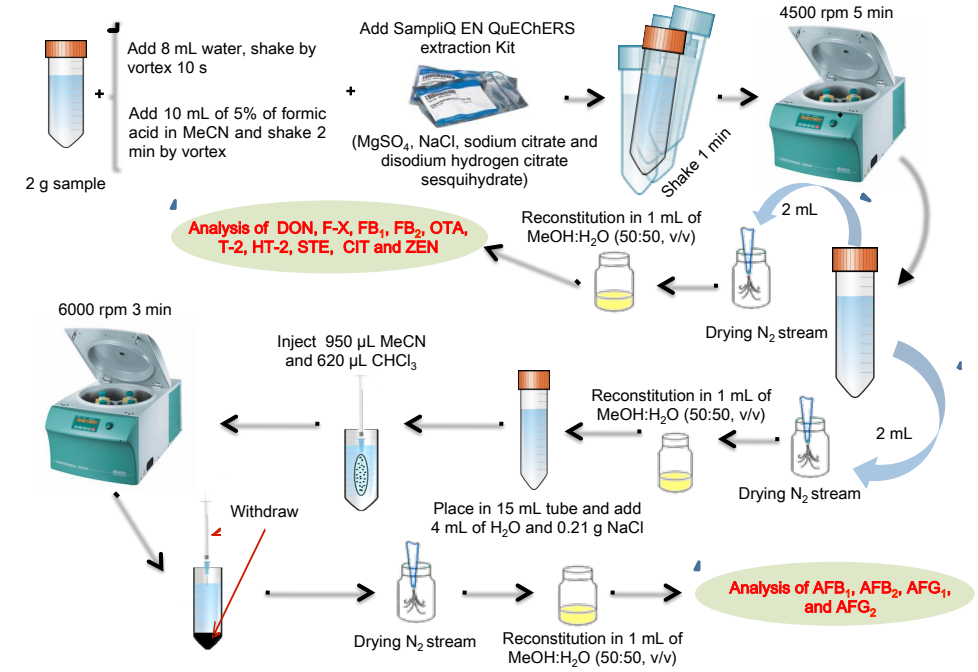

Figure 5.1. Sample treatment for the determination of mycotoxins in nuts and seeds

5.2.4. UHPLC-MS/MS analysis

UHPLC separations were performed in a C_{18} column (Zorbax Eclipse Plus RRHD 50 × 2.1 mm, 1.8 µm) using a mobile phase consisting of 0.3% aqueous formic acid solution with 5 mM ammonium formate (solvent A), and MeOH with 0.3% formic acid and 5 mM ammonium formate (solvent B) at a flow rate of 0.4 mL min^{-1}.

The eluent gradient profile was as follows: 0 min: 5% B; 1 min: 50% B; 2 min: 72% B; 4 min: 80% B; and 6 min: 90% B. Finally it was back to 5% B in 0.2 min and maintained for 1.8 min for column equilibration. The temperature of the column was 35°C and the injection volume was 5 µL (full loop).

The MS was working with ESI (+) under the MRM conditions shown in Table 5.1.

Table 5.1. Monitored ions of the target analytes and MS/MS parameters

	Retention time (min)	Precursor ion (m/z)	Molecular ion	DP[a]	EP[a]	CEP[a]	Product ions[b]	CEn[a]	CXP[a]
DON	1.32	297.1	$[M+H]^+$	36.0	5.5	16.0	249.2 (Q)	17.0	4.0
							161.0 (I)	29.0	4.0
F-X	1.50	355.1	$[M+H]^+$	26.0	12.0	18.0	174.7 (Q)	23.0	4.0
							137.1 (I)	31.0	4.0
AFG_2	1.86	331.1	$[M+H]^+$	61.0	6.0	42.0	245.1 (Q)	39.0	4.0
							313.1 (I)	27.0	6.0
AFG_1	1.95	329.0	$[M+H]^+$	76.0	9.5	16.0	243.1 (Q)	39.0	6.0
							311.1 (I)	29.0	6.0
AFB_2	2.07	315.1	$[M+H]^+$	81.0	4.0	34.0	286.9 (Q)	33.0	6.0
							259.0 (I)	39.0	8.0
AFB_1	2.18	313.1	$[M+H]^+$	46.0	12.0	26.0	241.0 (Q)	41.0	4.0
							284.9 (I)	39.0	4.0
CIT	2.31	251.2	$[M+H]^+$	26.0	11.0	18.0	233.0 (Q)	23.0	23.0
							204.8 (I)	73.0	10.0
HT-2	2.70	442.0	$[M+NH_4]^+$	21.0	5.5	21.0	262.8 (Q)	22.0	8.0
							215.4 (I)	19.0	4.0
FB_1	2.93	722.2	$[M+H]^+$	71.0	10.0	30.0	334.2 (Q)	51.0	6.0
							352.2 (I)	47.0	6.0
T-2	3.11	484.0	$[M+NH_4]^+$	21.0	10.0	22.0	215.0 (Q)	22.0	4.0
							185.0 (I)	29.0	4.0
ZEN	3.53	319.0	$[M+H]^+$	26.0	8.0	20.0	282.9 (Q)	19.0	4.0
							301.0 (I)	15.0	10.0
OTA	3.59	404.0	$[M+H]^+$	41.0	7.5	16.0	238.9 (Q)	31.0	6.0
							102.1 (I)	91.0	6.0
STE	3.68	325.1	$[M+H]^+$	66.0	3.5	26.0	281.0 (Q)	43.0	4.0
							310.0 (I)	37.0	4.0
FB_2	3.76	706.2	$[M+H]^+$	71.0	10.5	20.0	336.3 (Q)	43.0	14.0
							318.3 (I)	45.0	12.0

[a] Declustering potential (DP), Entrance potential (EP), Collision cell entrance potential (CEP), Collision cell exit potential (CXP) and Collision energy (CEn). All expressed in voltage.

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

The ionization source parameters were: source temperature 500°C; curtain gas (nitrogen) 30 psi; ion spray voltage 5000 V; and GAS 1 and GAS 2 (both of them nitrogen) were set to 50 psi. In all the cases a precursor ion and two product ions (one

for quantification and the other one for confirmation) were studied, obtaining four identification points, as usually required by EU for confirmation of contaminants in foodstuff [22].

5.3. Results and discussion

5.3.1. Optimization of sample treatment

As described in Section 5.2.3., the first step of sample treatment was based on the extraction/partitioning process of the QuEChERS procedure previously optimized in our laboratory for the determination of mycotoxins in cereals, described in Chapter 4. This simple extraction allowed the determination of DON, F-X, FB_1, FB_2, T-2, HT-2, STE, OTA, ZEN and CIT. However, aflatoxins could not be determined at low concentrations due to lack of sensitivity produced by strong ion suppression; thus a subsequent clean-up step was required in order to enable their quantification. For this purpose DLLME clean-up step, previously optimized for the determination of targeted mycotoxins in botanicals, as described in Chapter 3 [23], was selected. Combining these two treatments, satisfactory results were obtained; thus, further optimization was not carried out.

A typical chromatogram corresponding to a spiked peanut sample under developed conditions is shown in Figure 5.2, showing the effectiveness of the DLLME step in the determination of aflatoxins.

[22] Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). Off. J. Eur. Commun. L221 (2002) 8.

[23] N. Arroyo-Manzanares, A. M. García-Campaña, L. Gámiz-Gracia, J. Chromatogr. A 1282 (2013) 11.

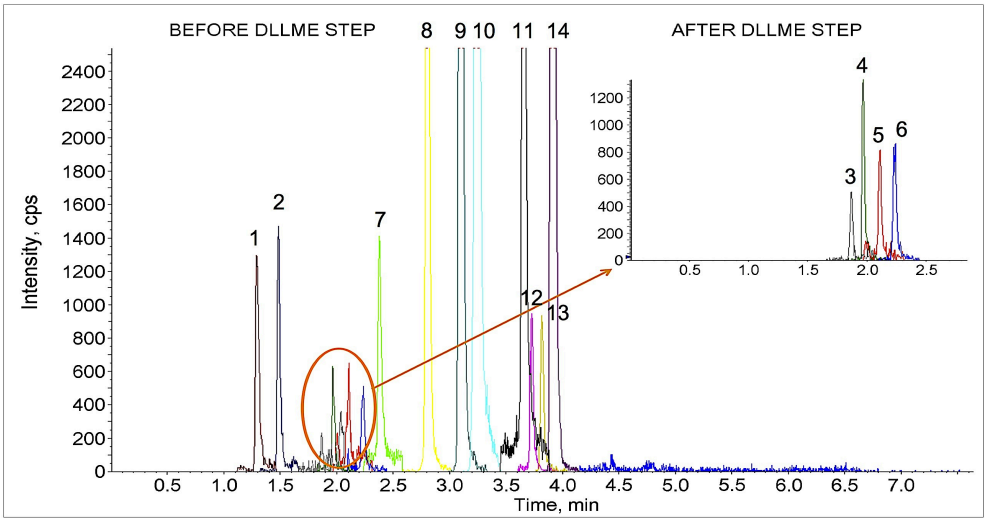

Figure 5.2. Chromatogram of a spiked peanut sample at optimum conditions (AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1} and F-X: 2500 μg kg^{-1}). 1: DON; 2: F-X; 3: AFG_2; 4: AFG_1; 5: AFB_2; 6: AFB_1; 7: CIT; 8: HT-2; 9: FB_1; 10: T-2; 11: ZEN; 12: OTA; 13: STE; 14: FB_2

5.3.2. Characterization of the method

In order to check the suitability of the method for the determination of the target mycotoxins in nuts and seeds, a full validation was carried out in peanut, which, together with pistachios, are responsible of most of the mycotoxin contamination alerts. Moreover, most of the proposed official methods for determination of aflatoxins in this kind of foods have been validated for this matrix [24].

ME, linear dynamic ranges, LODs and LOQs, and precision were evaluated. Moreover, recovery studies were performed in all the selected matrices to check the trueness of the method.

[24] AOAC Official Method, W. Horwitz (Ed.) Official Methods of Analysis of AOAC International, 17th Edition, Gaithersburg, MD, USA 2002.

5.3.2.1. Matrix effect, calibration curves and performance characteristics

Table 5.2 shows the values of ME at three concentration levels for each mycotoxin, calculated as 100 × [(signal of a spiked extract – signal of standard solution) / signal of standard solution]. As can be seen, ME was significant for all the compounds (even for aflatoxins after the clean-up step) and therefore matrix-matched calibration curves were necessary for quantification purposes.

Table 5.2. ME (%) from peanuts, calculated as 100 × [(signal of a spiked extract - signal of standard solution) / signal of standard solution]

Analyte	Level 1	Level 2	Level 3
AFB_1	-56.0	-57.8	-59.3
AFB_2	-54.8	-50.3	-52.7
AFG_1	-55.1	-52.1	-57.4
AFG_2	-54.1	-85.0	-56.2
OTA	-60.7	-68.7	-67.6
FB_1	-12.1	-15.7	-17.3
FB_2	-11.9	-19.3	15.6
T-2	-16.1	-19.5	-16.3
HT-2	-20.2	-27.4	-26.4
STE	-54.4	-40.7	-55.4
CIT	-17.7	-13.6	-19.9
F-X	-55.8	-59.7	-50.9
DON	-54.8	-50.9	-51.7
ZEN	-56.1	-58.3	-61.3

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1} and F-X: 2500 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1} and F-X: 5000 μg kg^{-1}.

As a consequence, in order to compensate ME, matrix-matched calibration curves were established by spiking peanut blank samples at 5 different concentrations of each mycotoxin, and considering the peak areas as analytical signal. Each concentration

level was prepared in duplicate and injected in triplicate. Spiked concentrations ranged from 1–50 μg kg^{-1} for AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE; 2–50 μg kg^{-1} for CIT; 10–500 μg kg^{-1} for FB_1, FB_2, T-2 and HT-2 toxin; 50–2000 μg kg^{-1} for DON; 200–5000 μg kg^{-1} for F-X; and 25–500 μg kg^{-1} for ZEN. The statistical parameters were calculated by least-square regression and are presented in Table 5.3. The determination coefficients (R^2) were higher than 0.99, showing that mycotoxin analytical responses were linear over the studied ranges.

LODs and LOQs were estimated as 3×S/N ratio and 10×S/N ratio, respectively and are presented in Table 5.3. The low LOQs obtained by this methodology for aflatoxins, the only mycotoxins regulated in nut and seed matrices, allowed their quantification at concentrations lower than the maximum level established by current legislation [9,10]. In the same way, the rest of mycotoxins can be determined at concentrations lower than the maximum levels established for these contaminants in different foodstuff [9].

Table 5.3. Statistical and performance characteristics of the proposed method in peanut

Analyte	Linear dynamic range (μg kg^{-1})	R^2	LOD (μg kg^{-1})	LOQ (μg kg^{-1})
AFB_1	0.71 – 50	0.9988	0.21	0.71
AFB_2	0.97 – 50	0.9994	0.29	0.97
AFG_1	0.61 – 50	0.9986	0.18	0.61
AFG_2	0.95 – 50	0.9975	0.29	0.95
OTA	0.57 – 50	0.9989	0.17	0.57
FB_1	1.57 – 500	0.9994	0.47	1.57
FB_2	0.65 – 500	0.9977	0.19	0.65
T-2	4.74 – 500	0.9980	1.42	4.74
HT-2	3.77 – 500	0.9997	1.13	3.77
STE	0.61 – 50	0.9959	0.18	0.61
CIT	1.72 – 50	0.9993	0.52	1.72
F-X	150 – 5000	0.9978	45.1	150
DON	32.6 – 2000	0.9975	9.68	32.6
ZEN	22.7 – 500	0.9988	6.82	22.7

5.3.2.2. Precision study

The precision of the whole method was evaluated in terms of repeatability (intraday precision) and intermediate precision (interday precision). Repeatability was assessed by application of the whole procedure on the same day to three peanut samples (experimental replicates) spiked at three concentration levels of mycotoxins. Each sample was injected in triplicate (instrumental replicates). Intermediate precision was evaluated with a similar procedure, with five samples analysed in different days. The results, expressed as RSD of peak areas, are shown in Table 5.4. As can be seen RSD lower than 11% were obtained in all cases, in agreement with current legislation [16].

Table 5.4. Precision study (% RSD of peak areas) in peanut samples

Analyte	Repeatability (n=9)			Intermediate precision (n=15)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	5.9	5.4	5.1	6.3	9.6	8.1
AFB_2	6.6	6.4	4.6	7.2	9.5	8.4
AFG_1	3.0	7.2	4.1	6.4	9.1	8.9
AFG_2	8.8	6.5	6.3	8.1	8.6	10.4
OTA	8.6	1.4	3.3	9.2	9.9	9.7
FB_1	8.3	4.0	5.0	6.3	8.6	9.9
FB_2	7.7	2.9	2.7	7.5	9.3	9.8
T-2	7.2	3.6	3.5	9.8	6.0	9.6
HT-2	7.8	2.9	5.0	9.6	8.5	6.4
STE	7.4	7.3	5.9	8.0	7.0	9.7
CIT	8.9	2.9	4.9	8.1	7.8	9.8
F-X	5.3	5.2	4.2	9.5	8.4	9.6
DON	6.9	3.9	3.7	10.6	10.1	10.2
ZEN	8.5	2.2	0.6	10.5	7.8	10.4

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1} and F-X: 2500 μg kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1} and F-X: 5000 μg kg^{-1}.

5.3.2.3. Sample analysis and recovery studies

It must be highlighted that, before performing the recovery studies to check the trueness of the method, all the samples (peanuts, almonds, sunflower seeds, pumpkin seeds, walnuts, macadamia nuts, pistachios, hazelnuts and pine nuts) were previously analysed and three of them gave positive results for several mycotoxins. In these cases, the concentration of each mycotoxin was assessed by the standard addition methodology. Specifically, a sunflower seed sample gave a positive results in STE (3.7 μg kg^{-1}; y = 300.56 x + 1122.6, R^2 = 0.9929), a walnut sample in ZEN (222 μg kg^{-1}; y = 57.898 x + 12844.0, R^2 = 0.996) and DON (346 μg kg^{-1}; y = 43.471 x + 15018.0, R^2 = 0.994) and a macadamia nut sample in F-X (2466 μg kg^{-1}; y = 1.346 x + 3318.6, R^2 = 0.990). The total ion chromatograms of these positive samples are shown in Figure 5.3.

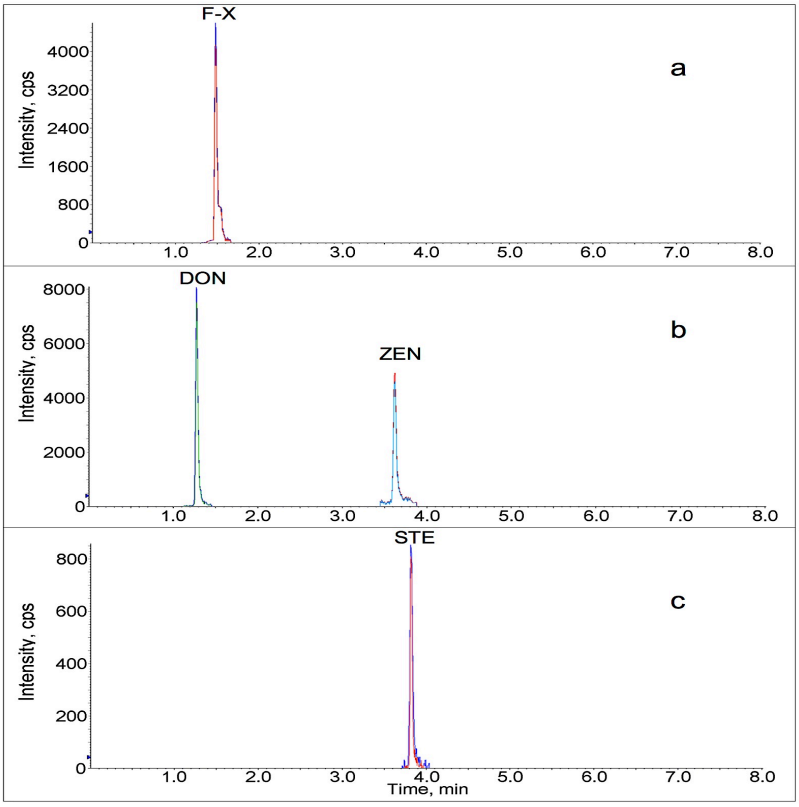

Figure 5.3. Total ion chromatogram of positive samples: sunflower seed sample (a); walnut sample sample (b); macadamia nut sample (c)

Subsequently, recovery experiments were carried out in all the selected matrices. With this purpose, two samples of each product (except peanuts, where three samples were analysed, as it was selected as representative matrix) were spiked at three different concentration levels, processed and injected in triplicate in the UHPLC-MS/MS system. The spiked concentration levels as well as the obtained results are summarized in Table 5.5. As can be seen, recoveries ranging from 60.7% to 104.3% were obtained for all the analytes. Thus, current legal requirements for the determination of legislated mycotoxins were fulfilled [16].

Table 5.5. Recovery (%) study in the selected foods (% RSD of peak areas)

Analyte	Peanut (n=9)			Pumpkin Seed (n=6)			Pistachio (n=6)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	71.8 (5.9)	75.4 (5.4)	74.9 (5.1)	75.7 (3.7)	77.2 (1.0)	80.4 (8.9)	95.0 (9.1)	89.1 (9.3)	72.9 (2.4)
AFB_2	91.6 (6.6)	80.3 (6.4)	84.9 (4.6)	99.3 (4.3)	80.1 (3.6)	84.9 (4.8)	94.1 (6.5)	88.1 (5.7)	85.3 (4.3)
AFG_1	71.4 (3.0)	74.4 (7.2)	74.0 (4.1)	91.7 (6.1)	82.8 (1.5)	79.3 (7.5)	84.9 (8.3)	82.7 (3.4)	72.8 (4.0)
AFG_2	86.2 (8.8)	84.3 (6.5)	94.0 (6.3)	98.9 (6.0)	73.3 (6.5)	80.7 (2.4)	96.5 (6.7)	87.3 (7.5)	85.8 (4.8)
OTA	83.6 (8.6)	88.6 (1.4)	89.0 (3.3)	84.3 (7.7)	82.6 (9.5)	84.5 (7.7)	81.4 (6.8)	89.3 (3.9)	94.6 (2.6)
FB_1	66.9 (8.3)	67.5 (4.0)	77.1 (5.0)	83.9 (2.1)	86.1 (0.5)	83.5 (5.5)	83.9 (3.1)	76.9 (2.6)	92.1 (2.2)
FB_2	83.1 (7.7)	78.1 (2.9)	91.2 (2.7)	75.5 (1.1)	85.1 (1.4)	90.8 (4.5)	95.5 (4.6)	101.3 (2.9)	100.8 (1.2)
T-2	96.1 (7.2)	93.1 (3.6)	94.2 (3.5)	92.0 (1.3)	99.3 (1.8)	94.4 (7.7)	99.5 (3.1)	93.0 (2.1)	94.7 (1.4)
HT-2	83.4 (7.8)	79.0 (2.9)	81.7 (5.0)	79.0 (2.6)	78.8 (0.7)	77.6 (5.3)	98.9 (4.6)	70.3 (2.4)	75.5 (1.3)
STE	85.1 (7.4)	76.6 (7.3)	70.0 (5.9)	80.0 (5.6)	76.9 (4.7)	74.0 (8.1)	68.5 (4.9)	71.4 (9.9)	74.5 (9.0)
CIT	80.1 (8.9)	69.5 (2.9)	72.5 (4.9)	77.7 (4.4)	80.4 (2.6)	87.3 (7.3)	80.8 (8.5)	95.7 (4.4)	88.3 (4.7)
F-X	89.3 (5.3)	73.0 (5.2)	81.8 (4.2)	69.9 (9.8)	75.2 (1.8)	72.3 (3.2)	90.3 (6.9)	92.5 (1.4)	85.5 (3.2)
DON	93.0 (6.9)	85.0 (3.9)	82.8 (3.7)	102.5 (3.7)	100.4 (1.7)	86.4 (1.5)	80.5 (9.2)	71.6 (5.4)	77.5 (2.3)
ZEN	94.2 (8.5)	89.5 (2.2)	81.2 (0.6)	84.4 (6.4)	89.5 (3.5)	79.4 (5.5)	98.6 (7.6)	79.1 (3.0)	73.7 (2.8)

% RSD of peak areas is given in parentheses

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg·kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1} and F-X: 2500 μg·kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg·kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1} and F-X: 5000 μg kg^{-1}.

Table 5.5. Recovery (%) study in the selected foods (% RSD of peak areas)

Analyte	Hazelnut (n=6)			Walnut (n=6)			Macadamia Nut (n=6)		
	Level 2	Level 3	Level 1	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	82.1 (8.5)	83.0 (9.3)	82.1 (8.4)	86.8 (7.0)	80.2 (5.8)	76.2 (4.1)	73.0 (8.4)	80.6 (8.7)	86.0 (8.4)
AFB_2	94.8 (6.5)	89.8 (5.2)	82.2 (6.2)	93.4 (9.0)	86.2 (7.1)	81.5 (5.9)	73.3 (8.2)	65.8 (5.1)	71.0 (2.0)
AFG_1	91.5 (9.8)	79.1 (9.4)	76.2 (8.0)	73.0 (8.3)	78.2 (8.2)	76.2 (9.2)	71.0 (4.3)	76.0 (6.1)	83.9 (9.8)
AFG_2	92.1 (7.0)	74.4 (5.6)	87.5 (5.1)	80.4 (7.6)	80.8 (9.4)	88.1 (2.2)	80.7 (6.7)	71.6 (9.0)	71.6 (2.4)
OTA	86.8 (9.7)	90.3 (7.8)	93.1 (5.0)	88.1 (7.2)	82.6 (6.9)	93.6 (5.9)	83.1 (7.9)	78.5 (5.8)	84.8 (7.7)
FB_1	93.0 (3.5)	84.3 (1.9)	87.4 (1.9)	81.8 (5.4)	88.5 (1.0)	86.9 (6.8)	85.6 (8.0)	78.1 (2.6)	94.8 (1.8)
FB_2	100.5 (1.8)	90.3 (6.6)	83.7 (2.1)	86.7 (4.6)	93.7 (1.9)	97.5 (4.1)	98.7 (5.4)	88.2 (2.8)	81.4 (0.7)
T-2	104.3 (1.7)	99.1 (1.8)	101.4 (4.1)	99.6 (5.6)	95.3 (5.3)	99.4 (3.4)	99.9 (4.9)	100.4 (3.8)	90.4 (8.7)
HT-2	101.9 (3.5)	92.0 (2.4)	97.8 (2.3)	90.2 (6.6)	95.0 (1.1)	97.6 (4.3)	98.1 (6.2)	99.0 (3.3)	86.4 (7.2)
STE	89.3 (8.6)	70.9 (8.1)	81.1 (5.1)	81.8 (5.1)	77.1 (8.5)	73.4 (3.1)	77.9 (9.0)	69.5 (6.6)	70.8 (5.6)
CIT	85.6 (8.0)	77.5 (3.7)	87.8 (3.1)	92.8 (7.7)	89.9 (4.6)	98.5 (6.3)	87.6 (7.3)	94.2 (3.3)	93.0 (6.9)
F-X	86.1 (7.5)	75.1 (2.0)	78.9 (2.6)	93.6 (6.6)	98.6 (2.5)	87.6 (4.1)	95.6 (8.7)	91.0 (5.6)	88.6 (2.5)
DON	88.0 (5.4)	80.6 (3.0)	76.0 (2.2)	92.6 (7.6)	85.3 (7.5)	82.5 (5.3)	100.0 (7.4)	95.1 (4.1)	92.1 (2.6)
ZEN	89.0 (8.2)	84.1 (5.1)	79.9 (1.9)	81.7 (7.6)	92.5 (2.8)	94.9 (5.7)	87.8 (7.3)	86.3 (3.1)	76.8 (2.4)

% RSD of peak areas is given in parentheses

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg·kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1} and F-X: 2500 μg·kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg·kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1} and F-X: 5000 μg kg^{-1}.

Table 5.5. Recovery (%) study in the selected foods (% RSD of peak areas)

Analyte	Pine nuts (n=6)			Sunflower Seed (n=6)			Almond (n=6)		
	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3	Level 1	Level 2	Level 3
AFB_1	74.7 (3.8)	77.5 (4.5)	85.7 (8.7)	77.7 (8.2)	84.2 (4.2)	72.5 (6.7)	75.2 (6.3)	82.6 (8.1)	71.3 (2.7)
AFB_2	85.3 (4.7)	77.7 (5.6)	74.9 (2.9)	79.4 (6.7)	78.2 (6.2)	87.3 (9.6)	87.0 (3.8)	78.2 (8.1)	80.9 (4.7)
AFG_1	72.5 (9.5)	71.1 (9.6)	79.3 (6.4)	77.7 (3.3)	72.9 (5.5)	69.4 (4.7)	86.5 (7.6)	80.3 (7.1)	77.4 (4.9)
AFG_2	86.0 (7.8)	88.7 (6.1)	73.7 (7.8)	84.4 (6.3)	78.1 (8.3)	73.3 (6.9)	97.9 (8.1)	91.3 (8.3)	89.1 (9.2)
OTA	87.6 (8.4)	81.1 (7.9)	89.4 (5.5)	97.0 (8.0)	90.6 (4.5)	93.4 (3.5)	91.7 (9.8)	97.8 (8.0)	88.4 (3.0)
FB_1	96.2 (9.4)	85.0 (8.6)	88.0 (2.3)	74.8 (8.6)	88.3 (2.3)	88.2 (1.3)	78.1 (5.0)	78.7 (2.7)	76.0 (2.2)
FB_2	96.3 (5.0)	86.6 (5.0)	92.0 (1.2)	94.8 (7.8)	89.9 (4.3)	91.5 (1.9)	86.2 (3.2)	84.7 (3.3)	87.5 (1.0)
T-2	72.4 (8.5)	63.2 (6.6)	61.9 (2.1)	94.2 (3.4)	98.8 (5.2)	98.3 (1.3)	90.7 (4.2)	97.4 (6.5)	91.6 (2.9)
HT-2	88.2 (3.2)	78.6 (5.1)	92.7 (4.8)	96.7 (5.2)	96.8 (2.7)	101.3 (2.0)	92.6 (4.2)	95.9 (2.1)	99.0 (2.6)
STE	81.6 (7.0)	74.8 (7.1)	78.3 (4.1)	96.8 (6.5)	92.4 (3.6)	89.3 (5.6)	61.7 (8.9)	60.7 (5.4)	65.6 (9.9)
CIT	77.6 (8.2)	86.9 (9.0)	84.4 (5.5)	91.9 (10.2)	87.9 (6.6)	101.6 (4.6)	95.0 (7.4)	100.0 (3.0)	92.1 (4.5)
F-X	86.1 (8.0)	94.1 (1.4)	92.0 (3.2)	93.7 (9.6)	82.5 (8.5)	78.4 (3.2)	92.6 (9.1)	88.0 (3.5)	91.5 (1.3)
DON	93.5 (8.5)	91.6 (2.8)	80.3 (3.9)	83.5 (9.8)	89.2 (7.6)	80.6 (4.3)	86.1 (7.5)	81.7 (2.3)	84.3 (2.1)
ZEN	94.6 (7.6)	97.0 (5.1)	85.3 (3.1)	92.7 (5.3)	95.8 (6.9)	87.5 (2.3)	89.7 (2.2)	99.3 (3.8)	81.8 (1.3)

% RSD of peak areas is given in parentheses

Level 1: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 1 μg kg^{-1}; CIT: 2 μg kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 25 μg kg^{-1}; DON: 50 μg kg^{-1} and F-X: 200 μg kg^{-1}.

Level 2: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 5 μg kg^{-1}; CIT: 10 μg·kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 250 μg kg^{-1}; DON: 1000 μg kg^{-1} and F-X: 2500 μg·kg^{-1}.

Level 3: AFB_1, AFB_2, AFG_1, AFG_2, OTA and STE: 10 μg kg^{-1}; CIT: 20 μg·kg^{-1}; FB_1, FB_2, T-2, HT-2 and ZEN: 500 μg kg^{-1}; DON: 2000 μg kg^{-1} and F-X: 5000 μg kg^{-1}.

5.4. Conclusions

A sample treatment based on QuEChERS and DLLME has been proposed for the determination of 14 mycotoxins in a great variety of nuts and edible seeds. The first step of the sample treatment, based on an extraction/partitioning, allows the determination of FB_1, FB_2, OTA, T-2, HT-2, STE, CIT, ZEN, DON and F-X. In order to determine the aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2) an additional clean-up step using DLLME (proposed as an alternative to dSPE in order to avoid losses of mycotoxins in terms of recovery) is required. This method has shown to be a suitable choice for the sample treatment of this kind of matrices in multiclass mycotoxin determination, reducing extraction time, providing good recoveries and precisions and always fulfilling the current EU legislation. This treatment and the use of UHPLC-MS/MS technology, make possible the determination of the most important mycotoxins in nine different matrices. Furthermore, the use of expensive and tedious IACs is avoided. Matrix-matched calibration curves were established and LODs and LOQs were below the usual maximum limits established by EU regulation in food. The RSD (repeatability and intermediate precision) was lower than 11% in all cases, and recoveries were between 60.7% and 104.3%. Some of the analysed samples were positive for some mycotoxins; STE was found in sunflower seeds, F-X in macadamia nuts and ZEN and DON were found in walnuts (all purchased in local markets).

This work was published as:

“A new approach in sample treatment combined with UHPLC-MS/MS for the determination of multiclass mycotoxins in edible nuts and seeds”. N. Arroyo-Manzanares, J. F. Huertas-Pérez, L. Gámiz-Gracia, A. M. García-Campaña. Talanta 115 (2013) 61.

CHAPTER 6

Nuevo enfoque para la investigación de alcaloides ergóticos en cereales basado en espectrometría de masas de alta resolución y de múltiples etapas

Holistic approach based on high resolution and multiple stage mass spectrometry to investigate ergot alkaloids in cereals

Resumen

Se ha desarrollado un nuevo enfoque basado en espectrometría de masas de alta resolución y de múltiples etapas, para la identificación de alcaloides ergóticos (alcaloides del cornezuelo de centeno) nuevos o poco explorados. Inicialmente se llevó a cabo la fragmentación de nueve alcaloides ergóticos conocidos, con el fin de establecer una estrategia para la identificación de nuevos alcaloides ergóticos. Los iones *m/z* 223 y *m/z* 251 fueron fragmentos comunes en todos las ergopeptinas, ergoamidas y ergopeptamas. A continuación, se realizaron espectros del ion precursor, utilizando estos iones, para examinar en diferentes muestras de cereales la presencia de posibles derivados de alcaloides ergóticos. Además de los seis alcaloides del cornezuelo de centeno más comunes y sus correspondientes epímeros (de los que se disponía patrones), se identificaron otros diez alcaloides ergóticos siguiendo la estrategia propuesta.

Abstract

A holistic approach based on high resolution and multiple stage mass spectrometry was developed for identification of less studied or novel ergot alkaloid derivatives. Initially, the fragmentation of nine known ergot alkaloids was studied to establish a strategy for the identification of novel ergot alkaloids. Ions with *m/z* 223 and *m/z* 251 were found to be common for all ergopeptines, ergoamides and ergopeptams. Subsequently, parent scan experiments using these ions were performed to screen grain samples for the presence of possible ergot alkaloid derivatives. Besides the six most common ergot alkaloids and their corresponding epimers (for which reference standards were available), ten other ergot alkaloid derivatives were identified following the proposed strategy.

6.1. Introduction

Ergot alkaloids are secondary metabolites produced by *Claviceps spp*. which mostly infect grains and grasses [1,2]. Ingestion of food and feed contaminated with ergot alkaloids might cause adverse health effects in humans and animals (e.g., ergotism). The poisoning is characterized by such symptoms as abdominal pain, vomiting, burning sensation of the skin, insomnia and hallucinations [3]. In 2012 the European Commission has been established a recommendation on the monitoring of the presence of ergot alkaloids, in cereals and cereal products intended for human consumption or animal feeding [4].

The majority of ergot alkaloids are commonly comprised of ergoamides, ergopeptines (or ergopeptides), also called cyclol ergot alkaloids, and the lactam ergot alkaloids, also named ergopeptams [1,5]. The ergoamides are D-lysergic acid amides (Figure 6.1a), whereas the ergopeptines are D-lysergic acid peptides containing lysergic acid and three amino acids in their structure (Figure 6.1b). The ergopeptams are tripeptidic non-cyclol ergot alkaloids (Figure 6.1c). Their structure is similar to that of ergopeptines except that L-proline is exchanged by D-proline, and the tripeptide chain is a non-cyclol lactam [1].

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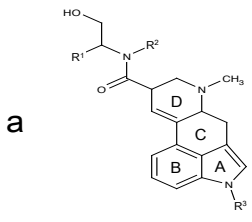
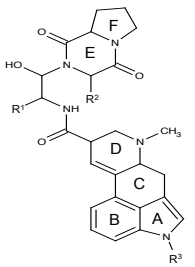


a

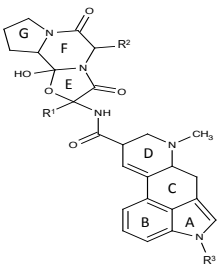
	R¹	R²	R³
Methylergometrine (M=339 Da)	$-CH_2CH_3$	-H	-H
Ergometrine (M= 325 Da)	$-CH_3$	-H	-H
Methysergide (M=352 Da)	$-CH_2CH_3$	-H	$-CH_3$



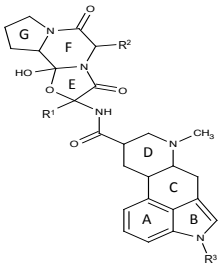
c

	R¹	R²	R³
Ergocristam (M=593 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Ergocryptam (M=559 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H
Ergocornam (M=545 Da)	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H
Ergostam (M=579 Da)	$-C_2H_5$	$-CH_2Ph$	-H
Ergoptam (M=546 Da)	$-C_2H_5$	$-CH_2CH(CH_3)_2$	-H
Ergonam (M=531 Da)	$-C_2H_5$	$-CH(CH_3)_2$	-H
Ergotamam (M=565 Da)	$-CH_3$	$-CH_2Ph$	-H
Ergosam (M=531 Da)	$-CH_3$	$-CH_2CH(CH_3)_2$	-H
Ergovalam (M=517 Da)	$-CH_3$	$-CH(CH_3)_2$	-H

b



	R¹	R²	R³
Ergosine (M=547 Da)	$-CH_3$	$-CH_2CH(CH_3)_2$	-H
Ergotamine (M=581 Da)	$-CH_3$	$-CH_2Ph$	-H
Ergocristine (M=609 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Ergokryptine (M=575 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H
Ergocornine (M=561 Da)	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H



	R¹	R²	R³
Dihydroergotamine (M=583 Da)	$-CH_3$	$-CH_2Ph$	-H
Dihydroergocristine (M=611 Da)	$-CH(CH_3)_2$	$-CH_2Ph$	-H
Dihydroergocryptine (M=577 Da)	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H

Figure 6.1. Common structures of ergoamides (a); ergopeptines (b); and ergopeptams (c) and representative ergot alkaloids for each class. M: molecular weight

To date, more than 40 ergot alkaloids are known. Several analytical techniques have been used to study the fragmentation of these compounds and attempts have been made to identify novel derivatives. In the last years, a number of new ergot alkaloids has been discovered [6-9]. Mohamed et al. [10] used triple quadrupole and multiple

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stage MS to characterize six ergot alkaloids belonging to lysergic acid and peptide-type derivatives and could confirm the presence of ergosine in a rye flour extract at trace levels. Lehner *et al.* [8,11] using HPLC coupled to tandem quadrupole MS and ion trap (IT) MS were able to establish the fragmentation patterns of eight ergot alkaloids and subsequently elucidate a new ergot alkaloid-related compound. Moreover, Uhlig and Petersen [12] obtained structural information of four ergopeptams using HPLC-IT-MS.

In the above-mentioned studies, fragment assignment was supported by hydrogen/deuterium exchange [10] and/or comparison of the fragmentation behaviour of known ergot alkaloids, using unit mass resolution data acquired by triple quadrupole and IT instruments. Along with fragmentation trees, accurate mass measurement is a highly important feature for correct structure elucidation [13]. In this sense, high-resolution mass spectrometry (HRMS), as Orbitrap MS, is becoming a more and more popular platform for identification purposes in natural product analysis. Regarding the secondary fungal metabolites and more specifically ergot alkaloids, Orbitrap MS has been utilized only in targeted analysis [14] or in pre-selected screening using a limited database [15]. The full-scan accurate mass capability of Orbitrap MS remains to be exploited in untargeted screening of ergot alkaloid derivatives.

In this work, a method based on HRMS and IT-MS technology is proposed for the study of the fragmentation pattern of ergot alkaloids and the identification of less studied or novel ergot alkaloid derivatives. In particular, Orbitrap MS was used, which allows to achieve high mass resolution (up to 100000 full width at half maximum (FWHM)) and high mass accuracy (mass error < 2 ppm), thereby leading to higher sensitivity,

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[13] T. Kind, O. Fiehn, Bioanalytical Reviews 2 (2010) 23.

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[15] S. M. Lehner, N. K. N. Neumann, M. Sulyok, M. Lemmens, R. Krska, R. Schuhmacher, Food Addit. Contam. 28 (2011) 1457.

dynamic range and selectivity for the analysis of complex matrices [16]. The fragmentation of twelve ergot alkaloids, namely ergometrine (Em), ergosine (Es), ergotamine (Et), ergocornine (Eco), ergokryptine (Ekr), ergocristine (Ecr), methylergometrine (MeEm), methysergide (MeErgi), dihydroergotamine (DHEt), ergocornam (Ecom), ergocryptam (Ecrypm) and ergocristam (Ecrism), was studied with the aim of establishing a simple strategy for identification of novel ergot alkaloid derivatives. Subsequently, this strategy was applied in screening of ergot alkaloid derivatives in a set of cereal samples.

6.2. Experimental

6.2.1. Chemicals and solvents

MeOH and MeCN (both of LC-MS grade) were supplied by Biosolve (Valkenswaard, the Netherlands). Ammonium bicarbonate was obtained from Sigma-Aldrich. Ammonium sulphate and ammonia (25%) were supplied by Merck (Darmstadt, Germany). MeCN and MeOH (both of HPLC grade), and *n*-hexane were purchased from VWR International (Zaventem, Belgium). Ethyl acetate (EtOAc) was obtained from Acros Organics (Geel, Belgium).

A Milli-Q purification system (Millipore, Brussels, Belgium) was used to purify demineralized water. Ultrafree®-MC centrifugal filter units (0.22 µm) were purchased from Millipore (Darmstadt, Germany).

Fine film dried ergot alkaloid standards Em, Es, Et, Eco, Ekr, Ecr, ergometrinine (Emn), ergosinine (Esn), ergotaminine (Etn), ergocorninine (Econ), ergokryptinine (Ekrn) and ergocristinine (Ecrn), were purchased from Coring System Diagnostix GmbH (Gernsheim, Germany). The film-dried standards were, as indicated by the manufacturer, reconstituted in 5 mL of solvent (MeCN), to give concentrations of 100.0 µg mL^{-1} (uncertainty: ± 5.0 µg mL^{-1}) and of 25.0 µg mL^{-1} (uncertainty: ± 1.5 µg mL^{-1}) for the main ergot alkaloids and for the -inine isomers, respectively. Definite ergot alkaloids in solution readily undergo epimerization. Therefore, from the freshly prepared standard

[16] Q. Hu, R. J. Noll, H. Li, A. Makarov, M. Hardman, R. G. Cooks, J. Mass Spectrom. 40 (2005) 430.

solutions, deep frozen standard residues were prepared as follows: defined volumes of individual or mixed standard solutions were pipetted into dark brown or aluminium covered glass tubes, evaporated to dryness at 40 °C under a stream of nitrogen, and deep frozen at -20 °C. Lauber *et al.* [17] reported that the ergot alkaloids stored under these conditions are stable for at least one year. The deep frozen standards were reconstituted in the required amount of solvent immediately before use. MeEm (as methylergometrine maleate, purity: 98 %), DHEt (as dihydroergotamine tartrate salt, purity: 99 %) and MeErgi (as methysergide maleate) were purchased from Sigma Aldrich (Bornem, Belgium). From the crystalline standards, individual stock solutions were prepared respectively in MeOH:MeCN (10:90, v/v) (MeEm, MeErgi) or in MeCN (DHEt) at a concentration of 1 mg mL^{-1}. These fresh solutions were used to prepare deep frozen standard residues as described above. The residues were reconstituted in the required amount of solvent immediately before use.

6.2.3. Sample preparation

Sample preparation procedure was as described by Diana Di Mavungu *et al.* [18]. Briefly, 5 g of sample were extracted with 40 mL EtOAc:MeOH:0.2 M ammonium bicarbonate pH 8.5 (62.5:25:12.5, v/v/v) during 30 min on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France) and the sample extract was centrifuged. Then, 15 mL of the extract layer was transferred to a falcon tube and phase separation was induced by adding 5 mL of a 0.2 M ammonium bicarbonate buffer pH 10, and 5 mL of a saturated solution of ammonium sulphate. 5 mL of the EtOAc-phase was evaporated until dryness, and the residue was reconstituted in 200 µL of MeOH:MeCN:H_2O (20:40:40, v/v/v). Subsequently, 200 µL of *n*-hexane were added and the resulting mixture was vortexed and centrifuged in an Ultrafree®-MC centrifugal (Millipore) device for 10 min at 14000 g. The *n*-hexane was discarded and the aqueous phase was analysed by LC-MS/MS.

[17] U. Lauber, R. Schnaufer, M. Gredziak, Y. Kiesswetter, Mycotoxin Research 21 (2005) 258.

[18] J. Diana Di Mavungu, S. V. Malysheva, M. Sanders, D. Larionova, J. Robbens, P. Dubruel, C. Van Peteghem, S. De Saeger, Food Chem. 135 (2012) 292.

6.2.4. HPLC-MS/MS analysis

The HPLC-MS/MS analyses were performed on an Alliance HPLC 2695 (Waters, Milford, MA, USA) platform coupled to a Micromass Quattro triple quadrupole mass spectrometer (Waters) equipped with a Z-spray ESI interface. Chromatographic separation was achieved using an XBridge MS C_{18} column (150 x 2.1 mm, 3.5 µm) with an XBridge Sentry guard column (10 x 2.1 mm, 3.5 µm) both supplied by Waters. The column temperature was set at 30 °C. A mobile phase consisting of solvent A [H_2O:0.2 M ammonium bicarbonate pH 10:MeOH (85:5:10, v/v/v)] and solvent B [H_2O:0.2 M ammonium bicarbonate pH 10:MeOH (5:5:90, v/v/v)] was used at a flow rate of 0.15 mL min^{-1}. A gradient elution was applied as follows: 0-3 min: 30-15 % A; 3-7 min: 15 % A; 7-10 min: 15-0 % A; 10-13 min: 0 % A; 13-14 min: 0-30 % A; 14-23 min: 30 % A. The injection volume was 20 µL.

The mass spectrometer was operated in the ESI (+) mode. MS parameters for the analysis were as follows: ESI source block and desolvation temperatures: 150 °C and 300 °C, respectively; capillary voltage: 3.5 kV; argon collision gas: 1.2 x 10^{-3} mbar; cone nitrogen and desolvation gas flows: 100 and 830 L h^{-1}, respectively. Masslynx and Quanlynx software (Micromass, Manchester, UK) were used for data acquisition and processing.

6.2.5. HPLC-MS^n analysis

LC analyses were performed on a Surveyor Plus HPLC System (Thermo Fisher Scientific, San Jose, CA, USA). The separation was performed on a XBridge MS C_{18} column (150 x 2.1 mm, 3.5 µm) with an XBridge Sentry guard column (10 x 2.1 mm, 3.5 µm) both supplied by Waters. The mobile phases were as described in section 6.2.4. and the gradient elution profile was as follows: 0-10 min: 10-35 % B; 10-30 min: 35 % B; 30-45 min: 35-60 % B; 45-50 min: 60-99 % B; 50-55 min: 99 % B; 55-57 min: 99-10 % B; 57-65 min: 10 % B. Flow rate was 0.25 mL min^{-1}. The temperature of the column was 40 °C and the injection volume was 5 µL.

Mass spectra were acquired using an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with a Heated Electrospray Ionization (HESI) source. The mass spectrometer was operated in positive mode (HESI (+)). The MS parameters were as follows: spray needle voltage 5 kV, capillary voltage 17 V, capillary temperature 200 °C, vaporizer temperature 125 ºC, nitrogen sheath gas flow 35 a.u., auxiliary gas flow 10 a.u. When performing MS^n experiments, the precursor ion was isolated in the ion trap with an isolation width of 3 Da and activated at different collision energy levels (CELs) to find the optimal conditions for distinct fragmentation. Xcalibur™ 2.0.7 software (Thermo Fisher Scientific) was used for instrument control, data acquisition and processing.

6.2.6. UHPLC-HRMS analysis

LC analyses were performed on a Thermo Accela UHPLC system (Thermo Fisher Scientific). The column used was a Zorbax Eclipse Plus RRHD C_{18} (2.1 x 100 mm, 1.8 µm) from Agilent Technologies (Diegem, Belgium). The mobile phases were as described in section 6.2.4. and the gradient elution program was set as follows: 0-1 min: 0 % B; 1-5 min: 0-25 % B; 5-15 min: 25-35 % B; 15-25 min: 35-40 % B; 25-40 min: 40-70 % B; 40-47 min: 70-98 % B; 47-50 min: 98 % B; 50-51 min: 98-0 % B; 51-55 min: 0 % B. The mobile phase flow rate was 0.4 mL min^{-1}, the temperature of the column was 30°C and the injection volume was 5 µL.

Accurate mass measurements of the precursor and product ions were carried out on an Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific) equipped with a HESI-II interface. The mass spectrometer was operated in the HESI (+) mode. The MS parameters were the following: spray voltage 4.5 kV, capillary temperature 250 °C, heater temperature 250 °C, sheath gas flow rate 45 a.u., and auxiliary gas flow rate 10 a.u. The data were processed using Xcalibur™ 2.1 (Thermo Fisher Scientific). The instrument was operated in full scan mode with a resolution of 100000 FWHM. The maximum injection time was 200 ms and the number of microscans per scan was 1. Each full scan was followed by a same-polarity “all ion fragmentation” higher energy collisional dissociation scan.

6.3. Results and discussion

6.3.1. Fragmentation pattern of ergot alkaloids

6.3.1.1. Ergopeptine and ergoamide alkaloids

The ergopeptines and ergoamides are structurally related compounds that share a common skeleton (see Figure 6.1). This characteristic was exploited to establish a strategy for the screening and identification of unknown ergot alkaloid derivatives. This was achieved through a careful study of the fragmentation pattern of known derivatives, including six ergopeptines (Es, Et, Eco, Ekr, Ecr and DHEt) and three ergoamides (Em, MeEm and MeErgi).

MS^n fragmentation data of the ergopeptine alkaloids are summarized in Table 6.1. It was observed that all the studied ergopeptines initially underwent a loss of a water molecule (-18 Da). Subsequently, in MS^3 and MS^4 experiments, neutral losses of 28 and 18 Da (corresponding to CO and H_2O) were observed. In further MS^5 experiments, losses of 91 Da for Ecr, Et and DHEt, 56 Da for Es and Ekr and 42 Da for Eco occurred (Figure 6.2).

Table 6.1. MSn fragmentation data for Et, Eco, Ecr, Ekr, Es and DHEt

Es	Ekr	Ecr	Eco	Et	DHet	Fragment assignment [a]
548	576	610	562	582	584	$[M+H]^+$
530	558	592	544	564	566	$[M+H]^+ - H_2O$
502	530	564	516	536	538	$[M+H]^+ - H_2O - CO$
484	512	546	498	518	520	$[M+H]^+ - H_2O - CO - H_2O$
428	456	455	466	427	429	$[M+H] - H_2O - CO - H_2O - R^2$
320	348	348	348	320	322	Cleavage within and loss of most of peptide ring system $[M+H]^+ - C_7H_{11}N_2O_3-R^2$
292	320	320	320	292	294	Loss of CO from 320, 348 or 322
277	305	305	305	277		$[M+H]^+ - C_7H_{11}N_2O_3 - R^2 - CO$
		268			270	Loss of R^1 from 292, 320 or 294
		251			253	Loss of NH_3 from 268 or 270
		223			225	Loss of CO from 251 or 253
		208			210	Loss of $-CH_3$ from 223 or 225
		208				Cleavage within the lysergic D ring and loss of R^1-C(CO)-NH
		197			197	Cleavage within and loss of D ring from 251 or 253
		194				Cleavage within the lysergic D ring and loss of N-CH_3 from 223
		182			182	Cleavage within lysergic D ring and loss of –CH-N-CH_3 from 223
		182			182	Loss of $-CH_3$ from 197
		180				Cleavage within lysergic D ring and loss of N-CH_2- from 208 or 210

[a] R^1 and R^2 are as defined in Figure 6.1

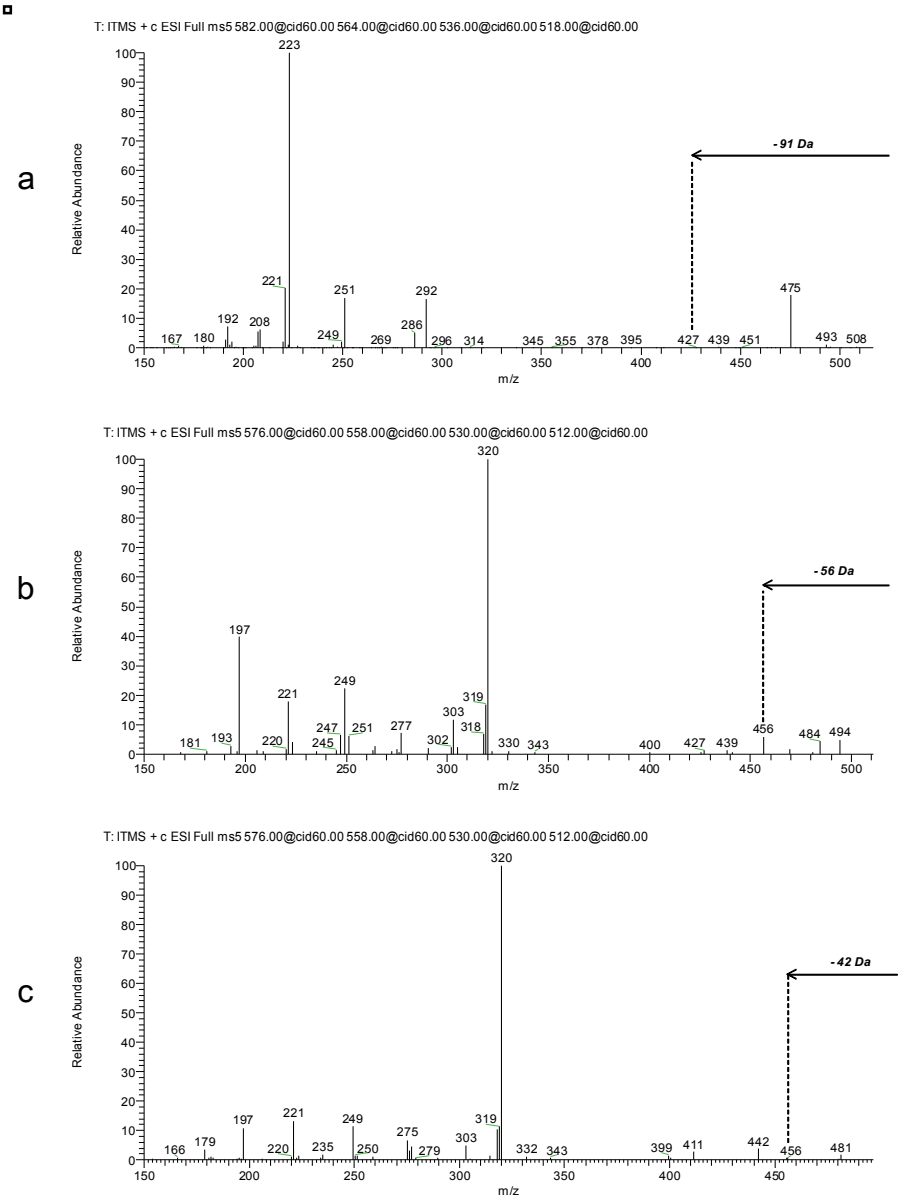


Figure 6.2. Fourth-generation collision-induced dissociation (CID) spectra acquired for Et (a), Ekr (b) and Eco (c), demonstrating the loss of the R^2 substituent from the main skeleton. R^2: $-CH_2Ph$ (for Et), $-CH_2CH(CH)_3$ (for Ekr) and $-CH(CH_3)_2$ (for Eco)

Taking into consideration the structural differences between the ergopeptines studied and the accurate mass data, these losses of 91, 56 and 42 Da were attributed to the radical R^2. The observed losses are consistent with a homolytic cleavage of the implied C-C bond. This would represent a violation of the even-electron fragmentation rule; however such a phenomenon has been previously described [154]. While the loss of 91 Da (Ecr, Et and DHEt) could be inferred from the homolytic cleavage in a straightforward fashion, the fragmentation mechanism for Es, Ekr and Eco implied the transfer of one hydrogen atom to the leaving fragment, leading to the formation of a double bond in the lost entity (Figure 6.3).

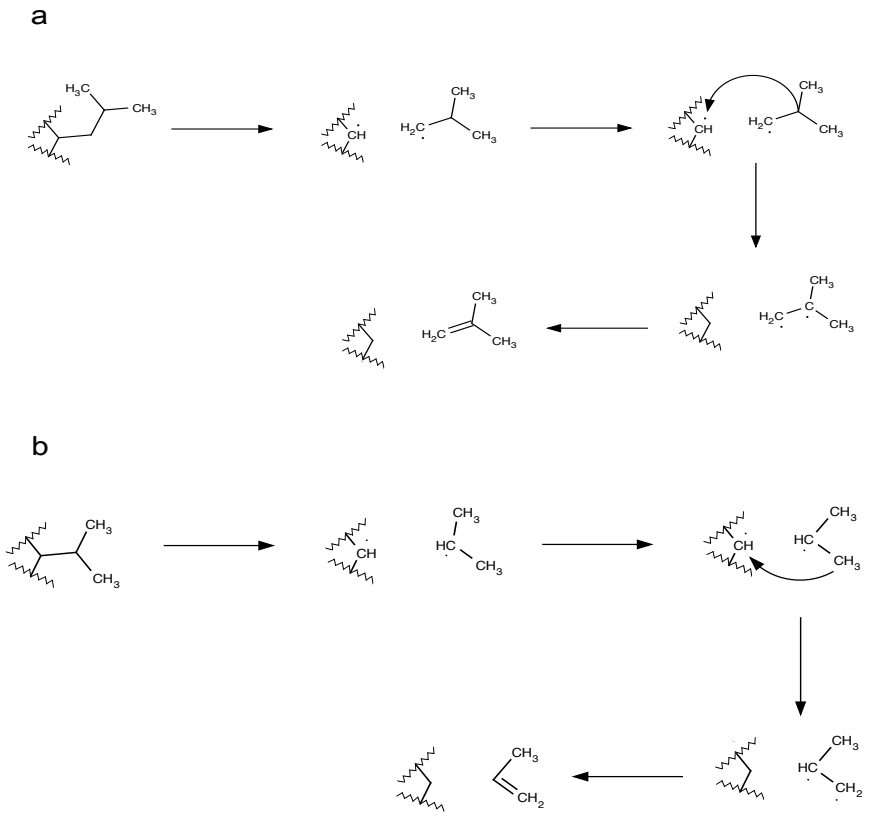


Figure 6.3. Proposed fragmentation mechanism for the loss of R^2 substituent from the main skeleton of Es and Ekr (a), and Eco (b)

Besides the loss of the R^2 radical, the ergopeptines underwent, subsequent to the losses of H_2O, CO and H_2O, a loss of most of the peptide ring system (Figure 6.4). For Ekr, Ecr and Eco (where R^1 is an isopropyl radical), an ion with an *m/z* of 348.1698 Da was observed, corresponding to $C_{21}H_{22}N_3O_2$ (mass error: -2.4 ppm). The ergopeptines having a methyl group at the R^1 position (*i.e.* Et, Es) produced a fragment with *m/z* 320.1388 Da ($C_{19}H_{18}N_3O_2$; -1.7 ppm). DHEt (an ergotamine-derivative having a saturated C9-C10 bond) rather produced an ion at *m/z* 322.1549 assigned as $C_{19}H_{20}N_3O_2$ (-0.5 ppm). The resulting fragments further underwent a loss of CO (-28 Da), yielding *m/z* 320.1750 ($C_{20}H_{22}N_3O$, -2.2 ppm) and *m/z* 292.1439 ($C_{18}H_{18}N_3O$, -1.8 ppm) for the derivatives with an isopropyl and methyl R^1 radicals, respectively (Figure 6.5). A fragment at *m/z* 294.1597 (assigned as $C_{18}H_{20}N_3O$; -0.4 ppm) was obtained for DHEt. Subsequent loss of the R^1-C-NH moiety gave an ion at *m/z* 251.1175 corresponding to $C_{16}H_{15}N_2O$ (-1.5 ppm) for all ergopeptines, except for DHEt, for which the corresponding ion at *m/z* 253.1332 ($C_{16}H_{17}N_2O$; -0.3 ppm) was obtained. Fragmentation of the ion at *m/z* 251.1175 yielded, among others, an ion at *m/z* 223.1227 ($C_{15}H_{15}N_2$; -1.4 ppm) through the loss of CO (- 28 Da). The corresponding ion for the dihydro-derivatives (*m/z* 253.1332) yielded a fragment at *m/z* 225.1384 ($C_{15}H_{17}N_2$; -0.2 ppm). The product ion at *m/z* 223.1227 (*m/z* 225.1384 for DHEt) proved to be the most abundant common fragment of the different ergopeptines. The product ion spectrum (Figure 6.5) of this common ion indicated that subsequently, a homolytic cleavage of the N–CH_3 bond in the D ring took place, giving rise to the radical cation at *m/z* 208.0993 ($C_{14}H_{12}N_2$; -0.8 ppm) for the ergopeptines and *m/z* 210.1151 ($C_{14}H_{14}N_2$; -0.1 ppm) for the dihydroergopeptines, as also reported by Mohamed *et al.* [154].

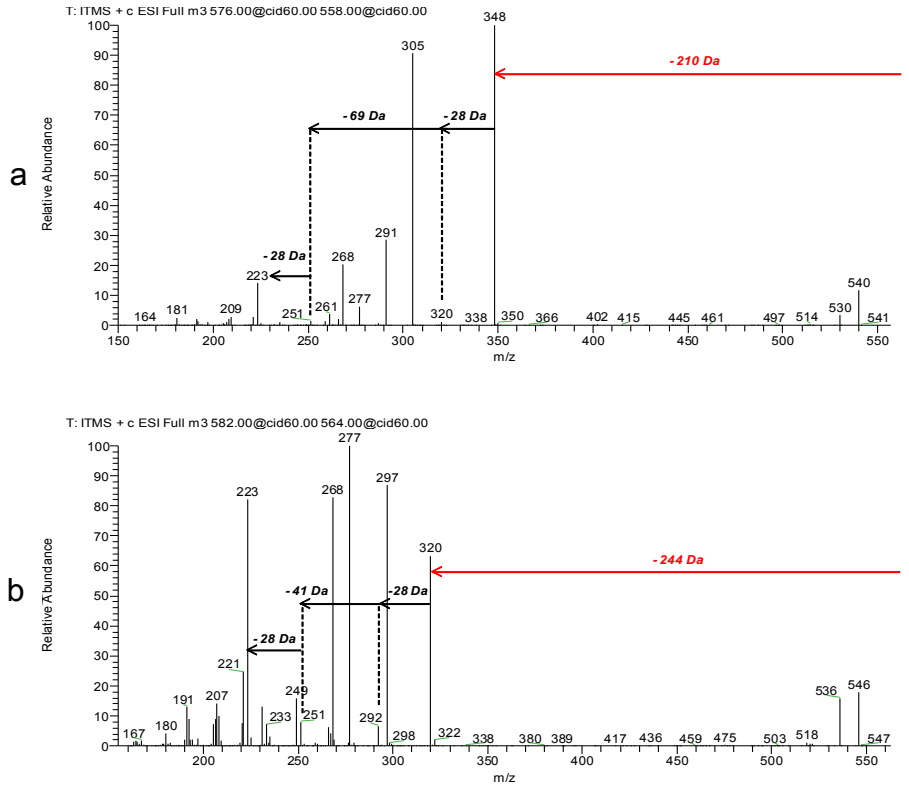


Figure 6.4. Second-generation CID spectra acquired for Ekr (a) and Et (b), demonstrating loss of most of the peptide ring system (shown in red). The resulting fragments, i.e. m/z 348 (Ekr) and m/z 320 (Et) underwent a loss of CO (-28 Da) followed by the loss of the R^1-C-NH moiety (69 Da for Ekr and 41 Da for Et), yielding a common ion at m/z 251

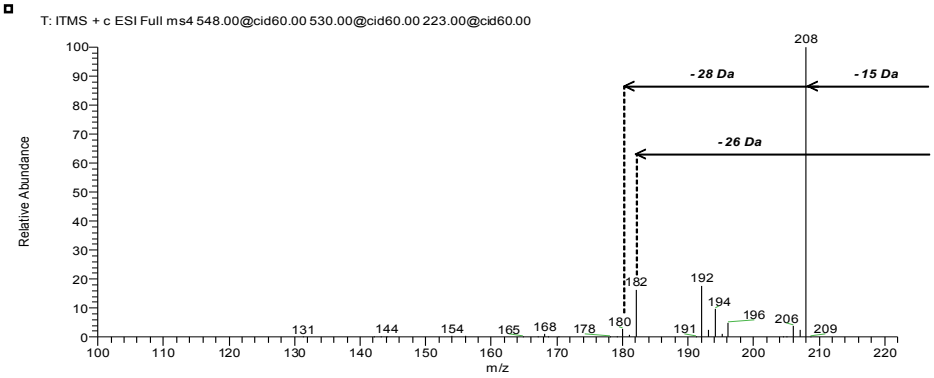


Figure 6.5. Third-generation CID spectrum acquired for Es shown as an example of the fragmentation pattern of the common ion at *m/z* 223. This product ion at *m/z* 223 was isolated and collisionally activated at 60 % CEL

It was observed that the ions at *m/z* of 348.1698 (Ekr, Ecr) and *m/z* 320.1388 (Eco, Et and Es), described above, followed an alternative fragmentation pathway. A fragment at *m/z* 305.1280 ($C_{19}H_{17}N_2O_2$; -0.4 ppm) or *m/z* 277.0969 ($C_{17}H_{13}N_2O_2$; -0.2 ppm) was obtained by cleavage within the lysergic D ring system for derivatives with isopropyl R^1 radical (Ekr, Ecr) or methyl R^1 radical (Eco, Et, Es), respectively. A subsequent loss of CO (- 28 Da) gave *m/z* 277.1332 (Ekr, Ecr) and *m/z* 249.1020 (Eco, Et, Es) (assigned as $C_{18}H_{17}N_2O$; -0.3 ppm and $C_{16}H_{13}NO$; -0.3 ppm, respectively). A loss of the R^1-C(CO)-NH moiety from these fragments yielded a common ion at *m/z* 208.0756 ($C_{14}H_{10}NO$; -0.1 ppm).

Loss of the lysergic ring-$CONH_2$ moiety from protonated molecule was also observed. The resulting fragment (Figure 6.6) subsequently lost a CO moiety or the radical R^2. Interestingly, the fragment obtained upon loss of R^2 confirmed the homolytic cleavage fragmentation mechanism described above for Ekr, Es and Eco, i.e., the transfer of one hydrogen atom to the leaving fragment (Figure 6.3).

	Measured *m/z*	Formula	Error (ppm)
Ecr	297.1603	$C_{18}H_{21}N_2O_2^+$	1.8
Ekr	263.1752	$C_{15}H_{23}N_2O_2^+$	- 0.9
Es	235.1447	$C_{13}H_{19}N_2O_2^+$	2.3
Eco	249.1603	$C_{14}H_{21}N_2O_2^+$	2.2
Et and DHEt	269.1282	$C_{16}H_{17}N_2O_2^+$	-1.0

	Measured *m/z*	Formula	Error (ppm)
Ecr	325.1552	$C_{19}H_{21}N_2O_3^+$	1.7
Ekr	291.1697	$C_{16}H_{23}N_2O_3^+$	-2.1
Es	263.1396	$C_{14}H_{19}N_2O_3^+$	2.1
Eco	277.1541	$C_{15}H_{21}N_2O_3^+$	- 2.1
Et and DHEt	297.1228	$C_{17}H_{17}N_2O_3^+$	- 0.5

	R^1	R^2	R^3	Formula	[M + H]$^+$
Ekr	-CH(CH$_3$)$_2$	CH$_2$CH(CH$_3$)$_2$	-H	$C_{32}H_{42}N_5O_5^+$	576
Ecr	-CH(CH$_3$)$_2$	-CH$_2$Ph	-H	$C_{35}H_{40}N_5O_5^+$	610
Eco	-CH(CH$_3$)$_2$	-CH(CH$_3$)$_2$	-H	$C_{31}H_{40}N_5O_5^+$	562
Et	-CH$_3$	-CH$_2$Ph	-H	$C_{33}H_{40}N_5O_5^+$	582
Es	-CH$_3$	CH$_2$CH(CH$_3$)$_2$	-H	$C_{30}H_{38}N_5O_5^+$	548

	R^1	R^2	R^3	Formula	[M + H]$^+$
DHEt	-CH$_3$	-CH$_2$Ph	-H	$C_{33}H_{42}N_5O_5^+$	584

	Measured *m/z*	Formula	Error (ppm)
Ecr	234.1004	$C_{12}H_{14}N_2O_3^{+\bullet}$	2.3
Et and DHEt	206.0691	$C_{10}H_{10}N_2O_3^{+\bullet}$	2.7

	Measured *m/z*	Formula	Error (ppm)
Ekr and Eco	235.1082	$C_{12}H_{15}N_2O_3^+$	2.3
Et and DHEt	207.0770	$C_{10}H_{11}N_2O_3^+$	2.6

Figure 6.6. Fragmentation mechanism of ergopeptines showing the loss of the lysergic acid moiety

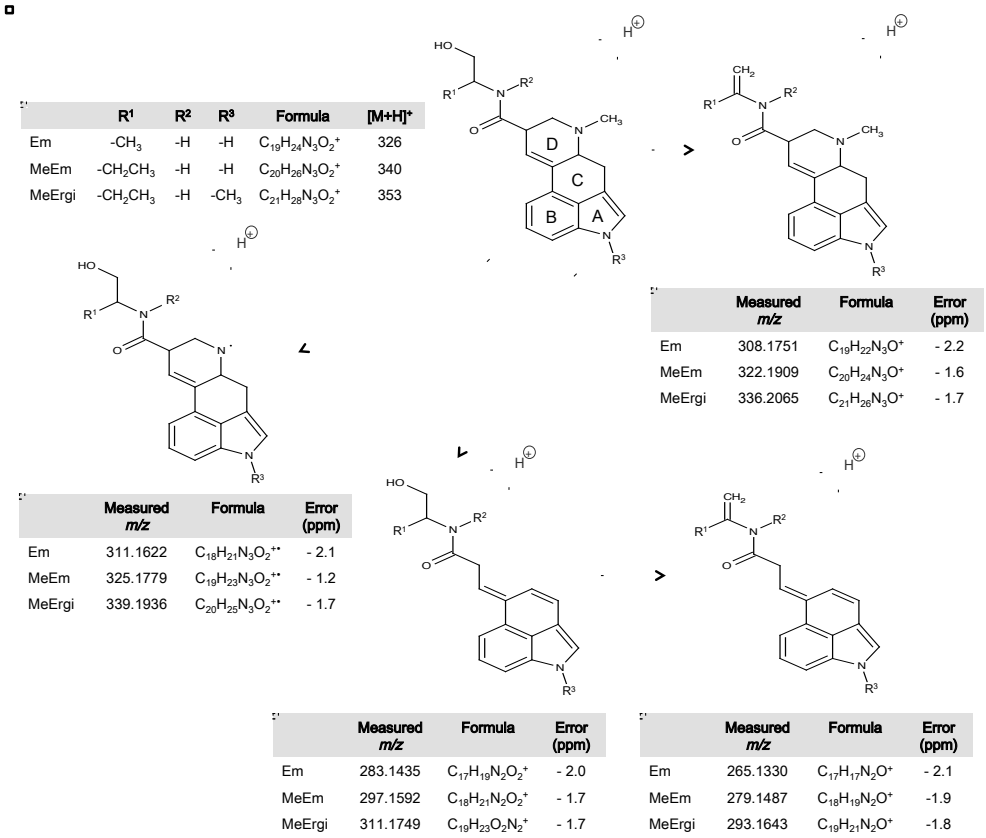
MS^n fragmentation data of the ergoamide alkaloids are summarized in Table 6.2. A fragmentation pattern similar to that of ergopeptines was observed for this group of ergot alkaloids. Initially, all the studied ergoamides lost a water molecule or $–CH_3$ by homolytic cleavage. The fragment at *m/z* 251.1175 (*m/z* 265.1334 for MeErgi) was obtained through a loss of R^1-$CH(CH_2OH)$-NH_2 from a protonated ion $[M+H]^+$, and followed a common pathway with the ergopeptines (Figure 6.7). The ergoamides also underwent a cleavage within the lysergic D ring as shown in Figure 6.8.

Table 6.2. MS^n fragmentation data for Em, MeEm and MeErgi

Em	MeEm	MeErgi	Fragment assignment [a]
326	340	354	$[M+H]^+$
311	325	339	$[M+H]^+$- CH_3
308	322	336	$[M+H]^+$- H_2O
283	297	311	Cleavage within the lysergic D ring and loss of C_2H_5N
265	279	293	Loss of H_2O from 283, 297 or 311
251		265	Loss of R^1-$CH(CH_2OH)$-NH-R^2 from $[M+H]^+$
223		237	Loss of CO from 251 or 265
208		222	Loss of -CH_3 from 223 or 237
197		211	Cleavage within the lysergic D ring from 251 or 265
194		208	Cleavage within the lysergic D ring and loss of N-CH_3 from 223 or 237
182		196	Cleavage within the lysergic D ring and loss of -CH-N-CH_3 from 223 or 237
182		196	Loss of -CH_3 from 197 or 211
180		194	Cleavage within the lysergic D ring and loss of N-CH_2- from 208 or 222

[a] R^1 and R^2 are as defined in Figure 6.1

Figure 6.7. Proposed common fragmentation pathway of ergopeptines and ergoamides



	R¹	R²	R³	Formula	[M+H]⁺
Em	-CH_3	-H	-H	$C_{19}H_{24}N_3O_2^+$	326
MeEm	-CH_2CH_3	-H	-H	$C_{20}H_{26}N_3O_2^+$	340
MeErgi	-CH_2CH_3	-H	-CH_3	$C_{21}H_{28}N_3O_2^+$	353

	Measured *m/z*	Formula	Error (ppm)
Em	308.1751	$C_{19}H_{22}N_3O^+$	- 2.2
MeEm	322.1909	$C_{20}H_{24}N_3O^+$	- 1.6
MeErgi	336.2065	$C_{21}H_{26}N_3O^+$	- 1.7

	Measured *m/z*	Formula	Error (ppm)
Em	311.1622	$C_{18}H_{21}N_3O_2^{+\bullet}$	- 2.1
MeEm	325.1779	$C_{19}H_{23}N_3O_2^{+\bullet}$	- 1.2
MeErgi	339.1936	$C_{20}H_{25}N_3O_2^{+\bullet}$	- 1.7

	Measured *m/z*	Formula	Error (ppm)
Em	283.1435	$C_{17}H_{19}N_2O_2^+$	- 2.0
MeEm	297.1592	$C_{18}H_{21}N_2O_2^+$	- 1.7
MeErgi	311.1749	$C_{19}H_{23}O_2N_2^+$	- 1.7

	Measured *m/z*	Formula	Error (ppm)
Em	265.1330	$C_{17}H_{17}N_2O^+$	- 2.1
MeEm	279.1487	$C_{18}H_{19}N_2O^+$	-1.9
MeErgi	293.1643	$C_{19}H_{21}N_2O^+$	-1.8

Figure 6.8. Fragmentation mechanism of ergoamides showing cleavage within the lysergic D ring

6.3.1.2. Ergopeptam alkaloids

Reference standards for the ergopeptams were not available during the course of this work. The study of their fragmentation pattern was achieved using a sample that was presumed to contain these ergot alkaloids based on the levels and patterns of ergot alkaloids for which commercial standards were available. Using accurate mass measurements and fragmentation data, three known ergopeptams [19,20,21], namely Ecom ($C_{31}H_{40}N_5O_4$, *m/z* 546.3072; -0.5 ppm), Ecrypm ($C_{32}H_{42}N_5O_4$, *m/z* 560.3234; 0.5

[19] E. L. Komarova, S. S. Shain, V. I. Sheichenko, Appl. Biochem. Micro. 38 (2002) 567.

[20] J. Olsovska, M. Sulca, P. Nováka, S. Pazoutová, M. Flieger, J. Chromatogr. B 873 (2008)165.

[21] S. Uhlig, D. Petersen, Toxicon 52 (2008) 175.

ppm) and Ecrism ($C_{35}H_{40}N_5O_4$, *m/z* 594.3078; 0.5 ppm) were detected in this sample. The fragmentation of these compounds is summarized in Table 6.3. As expected from their structures, the initial loss of 18 Da (corresponding to the elimination of a water molecule) observed for ergopeptines did not occur for the ergopeptam derivatives. The studied compounds underwent a loss of the dipeptide ring system in MS^2 experiments of the protonated ions, resulting in a common and abundant fragment at *m/z* 350 (Figure 6.9). This ion at *m/z* 350 was attributed to the fragment depicted in Figure 6.10, where the radical R^1, in accordance with the structure of the studied compounds, is $-CH(CH_3)_2$. A loss of CO from this fragment yielded an ion at *m/z* 322. A subsequent fragmentation yielded the ion at *m/z* 251, identical to that obtained for ergopeptines (Figure 6.5), as revealed by the further fragmentation. Based on the above-mentioned data, a fragmentation pathway of the ergopeptams was proposed (Figure 6.10).

Table 6.3. MS^n fragmentation data for ergopeptam alkaloids

Ecom	Ecrypm	Ecrism	Fragment assignment [a]
546	560	594	$[M+H]^+$
	350		Loss of the dipeptide ring system
	322		Loss of CO from 350
	307		Cleavage within the lysergic D ring system
	279		Cleavage within the lysergic D ring and loss of CO
	251		Loss of NH-CH-R^1 from 322
	223		Loss of CO from 251
	208		Loss of -CH_3 from 223
	208		Cleavage within the lysergic D ring and loss of R^1-C(CO)-NH
	197		Cleavage within and loss of D ring from 251
	194		Cleavage within the lysergic D ring and loss of N-CH_3 from 223
	182		Cleavage within the lysergic D ring and loss of -CH-N-CH_3 from 223
	182		Loss of -CH_3 from 197
	180		Cleavage within the lysergic D ring and loss of N-CH_2- from 208

[a] R^1 is as defined in Figure 6.1

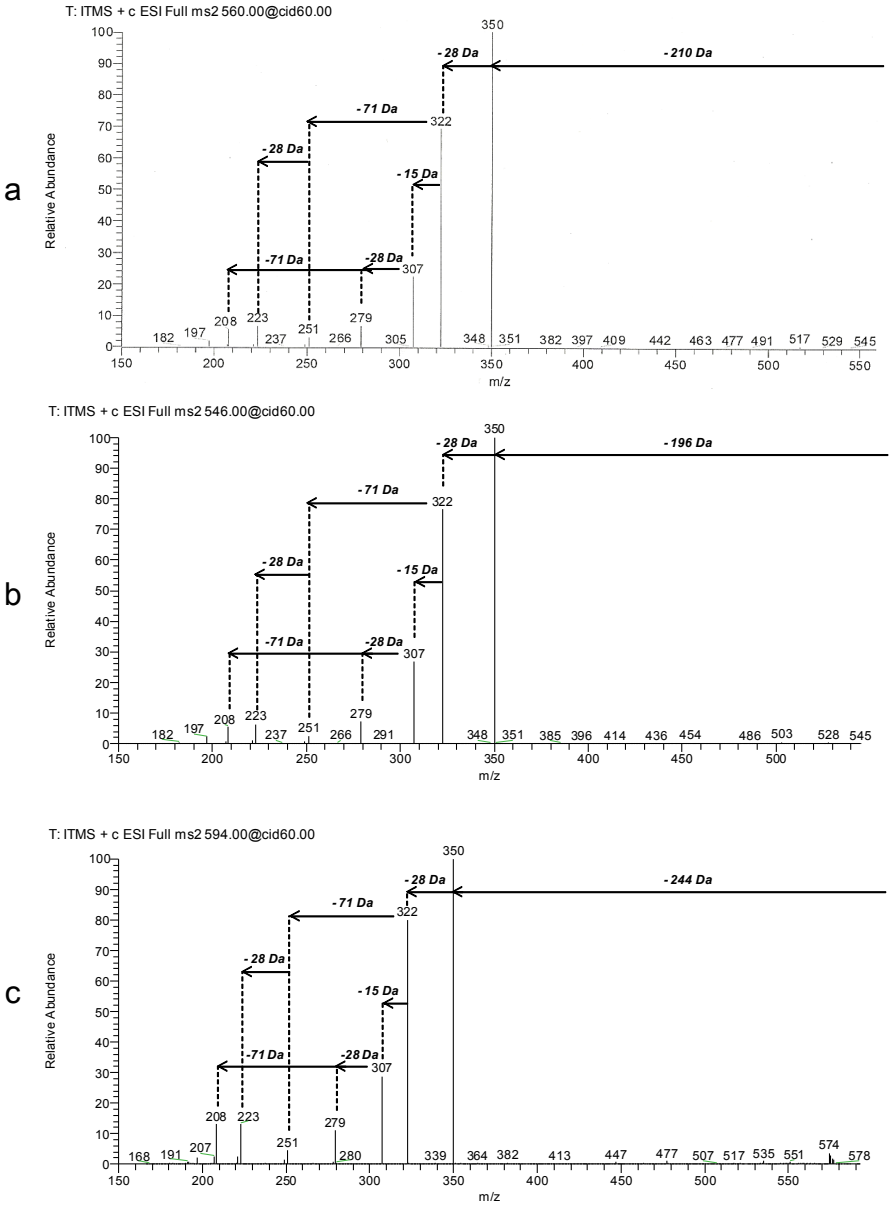


Figure 6.9. First-generation CID spectra acquired for Ecrypm (a), Ecom (b) and Ecrism (c)

	R^1	R^2	R^3	Formula	$[M+H]^+$
Ecom	$-CH(CH_3)_2$	$-CH(CH_3)_2$	-H	$C_{31}H_{40}N_5O_4^+$	546
Ecrypm	$-CH(CH_3)_2$	$-CH_2CH(CH_3)_2$	-H	$C_{32}H_{42}N_5O_4^+$	560
Ecrism	$-CH(CH_3)_2$	$-CH_2Ph$	-H	$C_{35}H_{40}N_5O_4^+$	594

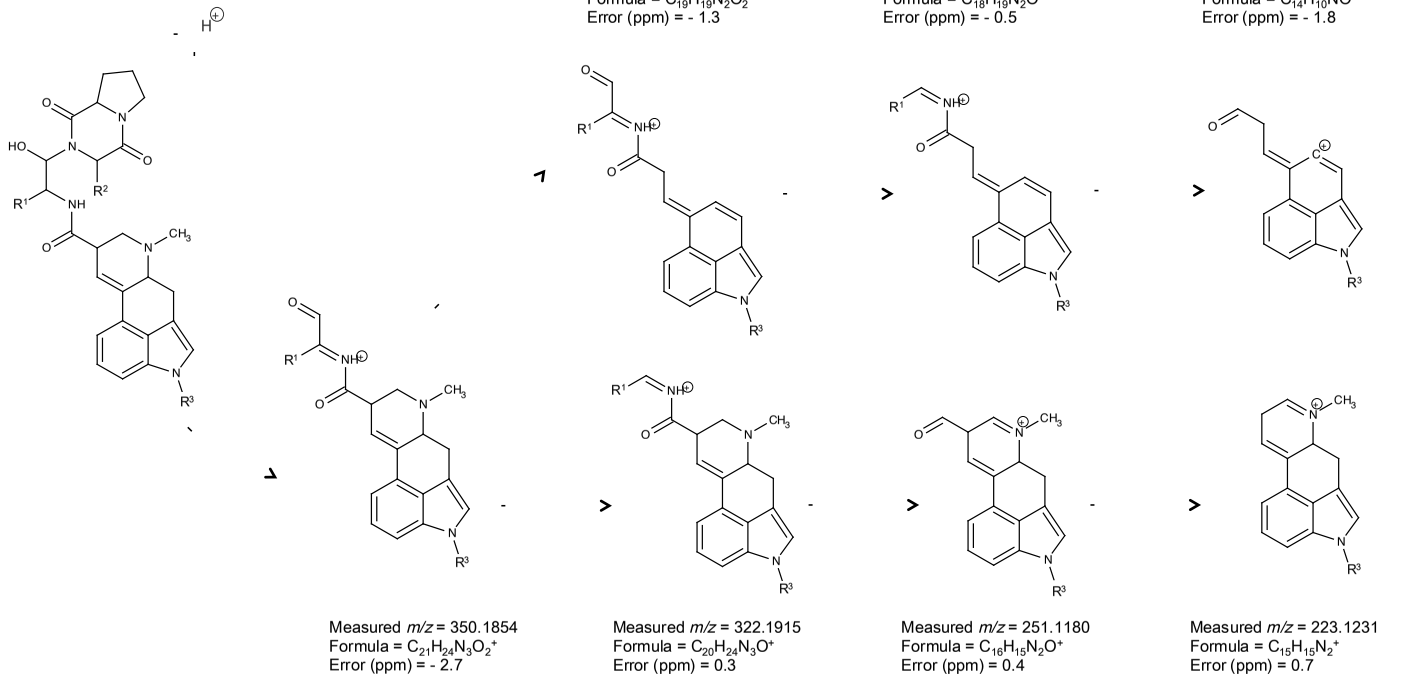


Figure 6.10. Proposed fragmentation pathway of ergopeptam ergot alkaloids. The fragment at m/z 223 was identical to that shown in Figure 6.5, thereby following the same pathway

6.3.2. Strategy for identification of novel ergopeptams and/or ergopeptines

A strategy for the identification of novel or less studied ergot alkaloids was proposed, as shown in Figure 6.11.

Based on fragmentation pathways described in section 6.3.1, the ions at *m/z* 223 and *m/z* 251 were found to be common for all the ergopeptines, ergoamides and ergopeptams, while for the dihydroergopeptines, ions *m/z* 225 and *m/z* 253 were characteristic. Therefore, it was proposed to monitor these ions as a first step to screen for ergot alkaloids. Initially, the samples with possible ergot alkaloid derivatives were analysed using non-selective fragmentation HRMS to screen for the fragment at *m/z* 223.1230 ($C_{15}H_{15}N_2$) or *m/z* 225.1386 ($C_{15}H_{17}N_2$). In practice, this screening can be performed by applying a parent scan of the ions at *m/z* 223.1230 and *m/z* 225.1386. The fragmentation of the possible ergot alkaloid derivatives was further studied by LC-MS^n. Compound identification was performed according to the scheme indicated in Figure 6.11, and is supported by accurate mass data.

If the ions at *m/z* 223 or *m/z* 225 were observed in the spectrum, the next step is to check whether the molecule of interest loses water upon fragmentation of the protonated ion. Formation of a dehydrate will indicate that the studied compound belongs to the group of ergopeptines or ergoamides. Noteworthy, if the cations at *m/z* 223 or *m/z* 225 were formed upon HRMS analysis, but the loss of H_2O from the protonated ion did not occur, the compound can be a possible ergopeptam.

Assignment of R^1 and R^2 radicals can be performed following the steps shown in Figure 6.11. As described under section 6.3.1, losses of 28 Da and 18 Da were characteristic features for ergopeptines during MS^3 and MS^4 fragmentation, respectively. Therefore, from the fragmentation spectrum of the ion corresponding to ($[M+H]^+$-64), the radical R^2 can be deduced (Figure 6.11, **step 1**). Subsequently, R^1 can be determined (see **step 2** in Figure 6.11), based on its mass calculated using Equation 6.1:

$$R^1 = [M+H]^+ - 448 - R^2 \qquad \text{(Eq. 6.1)}$$

in which 448 corresponds to the ergopeptine structure without radicals

For ergopeptams, the ion with the greatest *m/z* value (Mg), originating from MS^2 of the protonated ion, should be further fragmented. At this stage, a loss of 28 Da should be noted. Considering that the fragment at m/z 251 is obtained from consecutive losses of a 28 Da-moiety, R^1 and another 28 Da-moiety from the greatest fragment, R^1 can be calculated as follows (Equation 6.2):

$$R^1 = Mg - 307 \quad \text{(Eq. 6.2)}$$

in which Mg is the greatest fragment

R^2 is in turn calculated according to Equation 6.3:

$$R^2 = [M+H]^+ - Mg - 153 \quad \text{(Eq. 6.3)}$$

in which 153 corresponds to the ergopeptam dipeptide ring without R^2

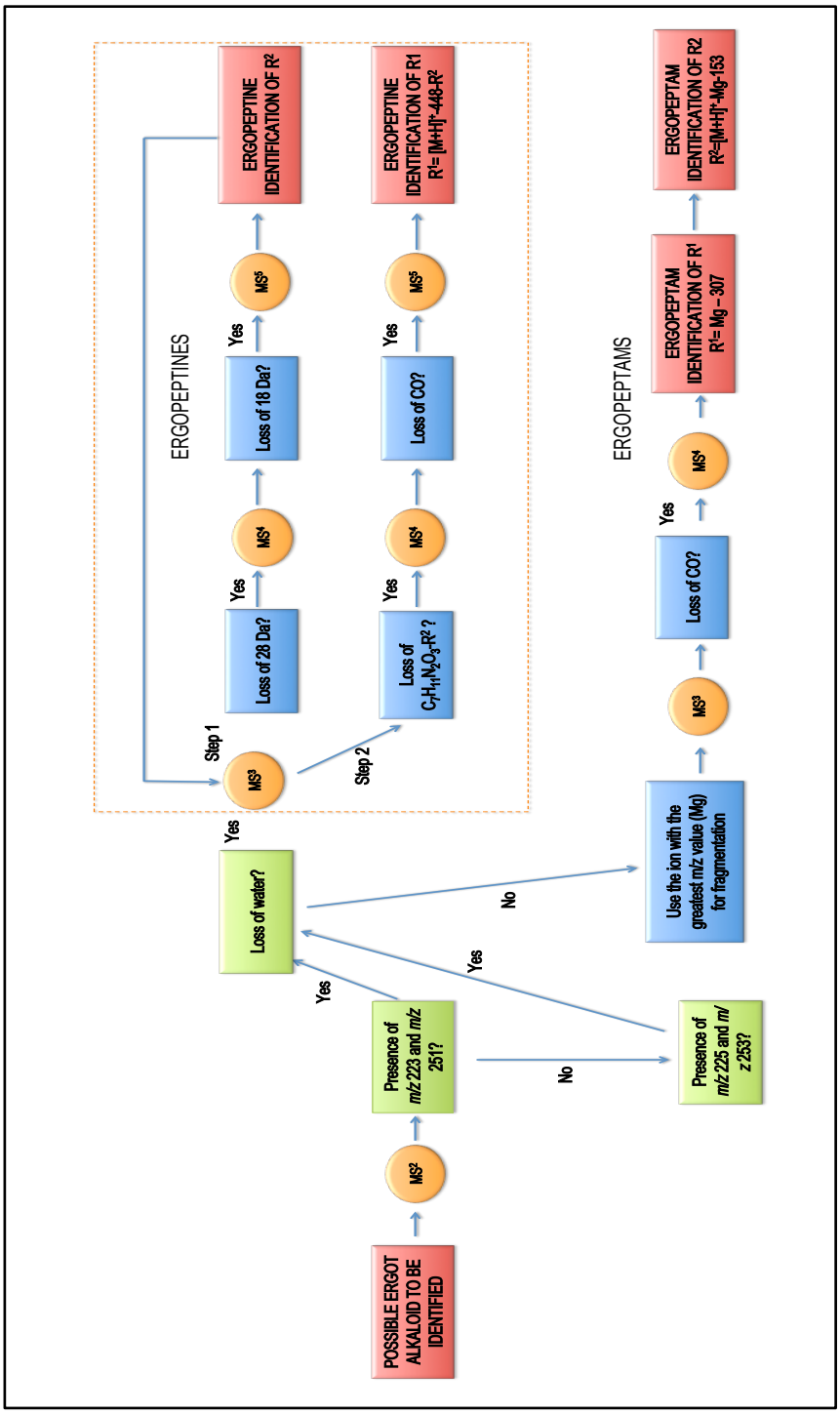


Figure 6.11. Proposed strategy for identification of novel ergopeptines and ergopeptams

6.3.3. Screening and identification of ergot alkaloid derivatives in grain samples

The fragmentation study of ergot alkaloids standards showed that the ion at *m/z* 223 was common for all the ergopeptines, ergoamides and ergopeptams. This observation has been previously described [154,155]. Therefore, detection of MS signals using *m/z* 223-parent scan experiments could point to possible occurrence of ergot alkaloids in real samples. The proposed identification strategy was applied for the screening of ergot alkaloid derivatives in grain samples (wheat and rye). Parent scan monitoring of the *m/z* 223 ion revealed the presence of possible ergot alkaloid derivatives in several feed samples. Among 19 analysed wheat and rye samples, four were free of ergot alkaloids. Em, Es, Et, Eco, Ekr, Ecr and their corresponding -inine epimers were identified in the remaining 15 samples by comparison with reference standards. Other presumed ergot alkaloid derivatives could not be identified at this stage. These unknowns were studied by fitting their mass spectral data into the proposed fragmentation pathway (described in section 6.3.1). Firstly, the authenticity of the ion at *m/z* 223 was checked through accurate mass measurements. Then, the IT fragmentation study was carried out for each unknown ergot alkaloid derivative. The obtained information was coupled with the exact mass data obtained with the Orbitrap instrument.

A TIC of a rye feed sample (sample 1) is given as example (Figure 6.12). Besides the known ergot alkaloids, ten presumed ergot alkaloid derivatives were detected in this sample. Among these unknown derivatives, four (**Unk**s **1**, **3**, **4** and **5**) followed the same fragmentation pathway as observed for the ergopeptams, while the others (**Unk**s **2**, **6**, **7**, **8, 9** and **10**) showed similarity with the fragmentation pathway of ergopeptines. Eventually, these compounds were identified as described in Table 6.4. These compounds, namely Ecom, ergovaline, Ecrypm, Ecrism, ergostine, ergoptine and ergogaline, have been previously reported in grain and grass samples [7,6,22]. An overview of the ergot alkaloid derivatives identified in other grain samples is presented in Table 6.4.

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Among the identified ergot alkaloids, some were accompanied by their respective epimers. Epimerization, with respect to the centre of symmetry at C8, is a characteristic feature of ergot alkaloids that have a double bond between C9 and C10, resulting in rotating (C8-(S) configuration) isomers [1,3]. Since both forms are found together in naturally contaminated samples [1], this feature was used as additional confirmation of the identification.

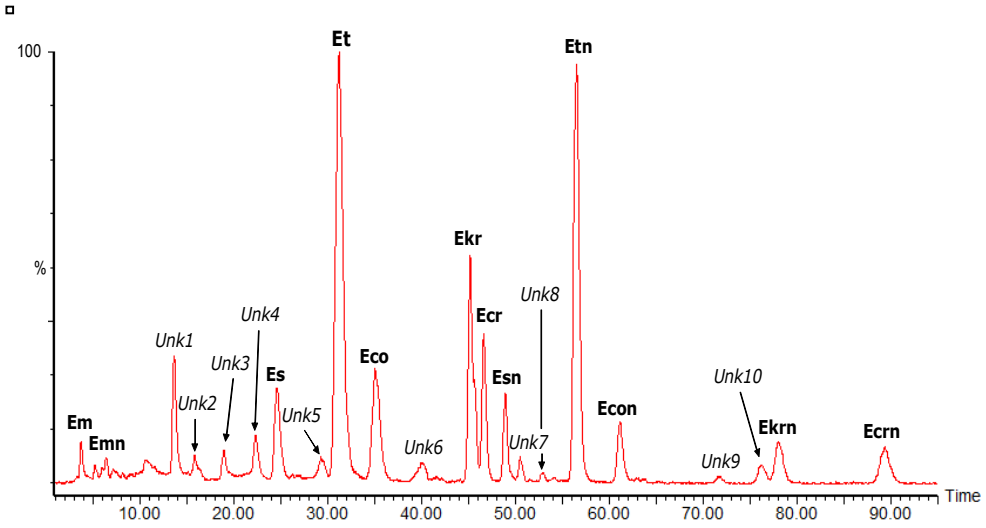

Figure 6.12. Parent scan total ion chromatogram of a rye sample

Table 6.4. Ergot alkaloid derivatives identified in grain samples (only positive samples are shown)

Metabolite	Elemental composition	Measured *m/z*	Calculated *m/z*	Error (ppm)	Sample[b] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Ergometrine	$C_{19}H_{24}N_3O_2$	326.1858	326.1863	-1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergometrinine	$C_{19}H_{24}N_3O_2$	326.1857	326.1863	-1.8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergosine	$C_{30}H_{38}N_5O_5$	548.2863	548.2867	-0.7	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergosinine	$C_{30}H_{38}N_5O_5$	548.2862	548.2867	-0.9	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergotamine	$C_{33}H_{36}N_5O_5$	582.2702	582.2711	-1.5	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Ergotaminine	$C_{33}H_{36}N_5O_5$	582.2702	582.2711	-1.5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ergocornine	$C_{31}H_{40}N_5O_5$	562.3019	562.3024	-0.9	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergocorninine	$C_{31}H_{40}N_5O_5$	562.3019	562.3024	-0.9	+	-	-	+	-	+	+	+	+	+	+	+	+	+	+
Ergokryptine	$C_{32}H_{42}N_5O_5$	576.3174	576.3180	-1.0	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergokryptinine	$C_{32}H_{42}N_5O_5$	576.3175	576.3180	-0.9	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergocristine	$C_{35}H_{40}N_5O_5$	610.3016	610.3024	-1.3	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergocristinine	$C_{35}H_{40}N_5O_5$	610.3017	610.3024	-1.1	+	+	-	+	-	+	+	+	+	+	+	+	-	+	+
Ergocornam (= Unk[a] 1)	$C_{31}H_{40}N_5O_4$	546.3072	546.3075	-0.5	+	-	-	+	-	+	+	+	+	+	+	-	-	-	+
Ergovaline (= Unk 2)	$C_{29}H_{36}N_5O_5$	534.2708	534.2716	-1.5	+	-	-	+	-	-	+	+	+	+	+	-	-	+	+
Ergocryptam (= Unk 3)	$C_{32}H_{42}N_5O_4$	560.3233	560.3231	0.4	+	-	-	-	-	-	+	+	+	+	+	-	-	-	+
Ergocryptam isomer (= Unk 4)	$C_{32}H_{42}N_5O_4$	560.3231	560.3231	0.0	+	-	-	-	-	-	+	+	+	+	+	-	-	-	+
Ergocristam (= Unk 5)	$C_{35}H_{40}N_5O_4$	594.3078	594.3075	0.5	+	-	-	+	-	-	+	+	+	+	-	-	-	-	+
Ergostine (= Unk 6)	$C_{34}H_{38}N_5O_5$	596.2886	596.2873	2.2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ergoptine (= Unk 7)	$C_{31}H_{40}N_5O_5$	562.3036	562.3029	1.2	+	-	-	+	-	-	+	+	+	+	+	+	-	+	+
Ergogaline (= Unk 8)	$C_{33}H_{44}N_5O_5$	590.3331	590.3342	-1.9	+	-	-	+	-	-	+	-	+	+	+	-	-	-	+
Ergostinine (= Unk 9)	$C_{34}H_{38}N_5O_5$	596.2888	596.2873	2.5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Ergokryptine isomer (= Unk 10)	$C_{32}H_{42}N_5O_5$	576.3195	576.3186	1.6	+	-	-	-	-	-	+	+	+	+	+	+	-	+	+

[a] Assignment of compounds as Unk 1 to Unk 10 refers to Figure 6.9

[b] '+': detected: '-': not detected

6.4. Conclusions

A method based on HRMS mass spectrometry and IT-MS technology has been developed for identification of less studied or novel ergot alkaloid derivatives. The combination of the two systems allows obtaining reliable and reproducible analytical results for multi-component analysis. The fragmentation of nine ergot alkaloids (Et, Es, Em, Eco, Ekr, Ecr, MeEM, MeErgi and DHEt) was studied to develop a strategy for the identification of novel ergot alkaloids. The ions at *m/z* 223 and 251 were found to be common for all the ergopeptines, ergoamides and ergopeptams. Subsequently, the parent scan experiments using this ion were performed to screen wheat and rye samples for the presence of ergot alkaloid derivatives. Besides the 6 most common ergot alkaloids and their corresponding epimers (for which standards were available), ten other ergot alkaloid derivatives were identified following the proposed strategy.

This work was carried out during the predoctoral stay at the Faculty of Pharmaceutical Sciences, University of Ghent (Ghent, Belgium) and has been submitted for publication as:

"Holistic approach based on high resolution and multi-stage mass spectrometry to investigate ergot alkaloids in cereals". N. Arroyo-Manzanares, S. Malysheva, J. Vanden Bussche, L. Vanhaecke, J. D. Di Mavungu, S. De Saeger, Talanta (submitted for publication).

CHAPTER 7

Perfil metabolómico del *Aspergillus flavus*: Identificación y fragmentación de los metabolitos producidos por el *cluster* 27 mediante espectrometría de masas de alta resolución y de múltiples etapas

Metabolic profiling of *Aspergillus flavus*: Identification and fragmentation study of the cluster 27 polyketide synthase metabolites by high resolution and multi-stage mass spectrometry

Resumen

En este capítulo se han determinado lo metabolitos producidos por el conjunto de genes *27 policétido sintasa* (*pks*27) en *Aspergillus flavus*. La comparación metabolómica, usando cromatografía de líquidos de alta eficacia acoplada a espectrometría de masas de alta resolución, permitió detectar los metabolitos que se expresaban de manera diferente en el *A. flavus* silvestre y el mutante (*Δpks*27) de *A. flavus*. Los datos se analizaron estadísticamente usando el programa SIEVE. La espectrometría de masas de alta resolución (Orbitrap) se utilizó para determinar la composición elemental de los compuestos de interés y por lo tanto, llevar a cabo una asignación preliminar del pico, mientras que la trampa de iones se usó para obtener información estructural y confirmar los metabolitos. El metabolito más abundante producido por el *cluster* 27 de *A. flavus*, fue asparanona A, un pigmento de antraquinona. En este capítulo también se ha estudiado por primera vez el patrón de fragmentación de este compuesto usando ionización por electrospray en modo negativo. Además, tres nuevos compuestos concretamente los resultantes de las correspondientes reacciones de deshidratación y desacetilación así como el oxi-derivado de asparasona A, fueron identificados en *A. flavus*. Tanto la asparasona A como sus derivados, se detectaron simultáneamente con algunos metabolitos conocidos de *A. flavus*, tales como las aflatoxinas, aflatrem, ácido ciclopiazónico y ditriptofenalina, en otras *Aspergillus spp.*

Abstract

The present study was designed to determine metabolites produced by the *Aspergillus flavus* cluster 27 polyketide synthase gene (*pks27*). Comparative metabolomics, using ultra-high performance liquid chromatography coupled to high resolution mass spectrometry (Orbitrap), allowed to detect metabolites that were differentially expressed in *A. flav*us wild-type and *Δpks2*7 mutant strains. This was supported by a statistical differential analysis of mass spectrometry data using SIEVE software. Accurate mass data from the Orbitrap mass spectrometry instrument was used to determine the elemental composition of the compounds of interest and thereby to perform a preliminary peak assignment, while ion trap multi-stage mass spectrometry was utilized to obtain structural information and to confirm the tentatively identified metabolites. Asparasone A, an anthraquinone pigment, was the major metabolite produced by the *A. flav*us cluster 27. The fragmentation pattern of this compound in the negative electrospray ionization mode was elucidated for the first time. In addition, three novel compounds, namely the dehydration, deacetyl- and oxy-derivatives of asparasone A, were identified in *A. flavus*. Asparasone A and its derivatives were detected simultaneously with some known *A. flavus* metabolites, such as aflatoxins, aflatrem, cyclopiazonic acid and ditryptophenaline, in other *Aspergillus spp*.

7.1. Introduction

Secondary fungal metabolites have been long used as drugs and still serve as structural inspiration for new compounds. To date, the genomes of several filamentous fungi have been sequenced which led to extensive investigation of biosynthetic metabolic pathways and identification of a number of secondary metabolites.

The filamentous fungus *Aspergillus flavus* is one of the most important species in *Aspergillus* genus and is distributed world-wide infecting food and feed [1-3]. Such prevalent occurrence can possibly be attributed to the production of numerous airborne conidia, which are easily dispersed by wind and/or insects [4]. *A. flavus* produces a number of toxic secondary metabolites (mycotoxins), such as aflatoxins, sterigmatocystin, aflatrem, cyclopiazonic acid and gliotoxin [1,5-8].

Analysis of the *A. flavus* genome has allowed the identification of many other putative secondary metabolic gene clusters predicted to encode metabolites derived from polyketide synthases (*pks*), non-ribosomal peptide synthetases (*nrps*), hybrid *pks-nrps* and prenyltransferases (*ptr*). *In silico* analysis of the *A. flavus* genome identified 55 gene clusters predicted to be associated with secondary metabolism [9,10]. Although approximately 20 metabolites have been identified in *A. flavus* cultures, the gene

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clusters responsible for biosynthesis are known only for six of those [11]. To determine the function of these gene clusters, gene targeting (deletion for a specific gene of interest), followed by metabolite profiling using appropriate analytical tools, is a commonly used approach [12]. Although TLC and HPLC coupled to UV are still routinely utilized in metabolite screening of fungi (especially at the initial steps of analysis) [13,14], LC-HRMS is getting increased interest due to its ability of achieving sensitive simultaneous analysis of a wide range of compounds and has been suggested for fungal metabolite identification in mouldy food and feed samples [15].

Previously, inactivation of the cluster pks gene 27 (*Δpks27*), produced a modified gene resulted in production of greyish-yellow sclerotia compared to the dark pigment normally observed for *A. flavus* sclerotia (a dense mass of branched hyphae, as in certain fungi, that contain stored food and are capable of remaining dormant for long periods) [16]. Therefore, the specific aim of this study was to disclose the metabolites that are differentially expressed in wild-type *A. flavus* and *A. flavus Δpks27*. A comparative metabolomic approach, based on UHPLC coupled to Orbitrap MS, was applied to identify the metabolites associated with *A. flavus* cluster 27 pks gene (*pks27*). The sclerotial metabolites of the wild-type strain and *Δpks27* mutant were compared using differential statistical analysis to disclose metabolites that were only present in the wild-type strain. Identification of the metabolites was based on accurate mass and supported by multi-stage fragmentation experiments.

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7.2. Experimental

7.2.1. Chemicals and solvents

MeOH and MeCN both of LC-MS grade were obtained from Biosolve (Valkenswaard, The Netherlands), whereas HPLC-grade MeOH and MeCN were from VWR International (Zaventem, Belgium). Ethyl acetate (EtOAc), dichloromethane (DCM) and ACO were purchased from Acros Organics (Geel, Belgium). Sigma-Aldrich (Bornem, Belgium) was the supplier of ammonium formate, agar, uracil, corn steep solids, dextrose, peptone, sucrose, yeast extract, glucose, ammonium tartrate, dipotassium hydrogen phosphate trihydrate, magnesium sulphate heptahydrate and iron(II) sulphate heptahydrate. Ammonium sulphate, potassium chloride, sodium nitrate, sorbitol and formic acid were from Merck (Darmstadt, Germany). Ultrapure water was produced by a Milli-Q Gradient System (Millipore, Brussels, Belgium). Ultrafree®-MC centrifugal filter units (0.22 µm) from Millipore (Bedford, MA, USA) were used.

Standards of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and cyclopiazonic acid were purchased from Sigma-Aldrich. Ready-made solid portions of commercial standards were dissolved in MeOH to reach the concentration of 1 mg mL^{-1}. Stock solutions were stored at -20 °C. Standard of asparasone A was kindly supplied by Dr. Victor Sobolev (US Department of Agriculture, Agricultural Research Service, National Peanut Research Laboratory, Dawson, Georgia).

7.2.2. Strains and growth conditions

An *Aspergillus flavus* 70 *niaD*−, *pyrG*− parental strain (referred to as AF70) was used as host for transformation. Three Δ*pks27* (Af70 *niaD*−, Δ*pks27*) mutants were obtained following transformation of the parental strain with the *pks27* knockout vector pks27-pyrG. An AF70 pyrG-1 (*niaD*−, *pyrG*+) strain transformed with the pPG2.8 vector expressing the *A. parasiticus pyrG* gene [17] was used as a transformation control.

[17] C. D. Skory, J. S. Horng, J. J. Pestka, J. E. Linz, App. Environ. Microbiol. 56 (1990) 3315.

Vector construction and fungal transformation were carried out at the USDA Southern Regional Research Center (New Orleans, LA, USA). A polimerase chain reaction (PCR) based method was used to construct the *A. flavus* 70 Δ*pks27* in which a 770 bp region within the *pks27* coding region was replaced by the *A. parasiticus pyrG* selectable marker gene. Briefly, 5' and 3' regions of the *pks27* gene were amplified using oligonucleotide primers. Following PCR amplification of *A. flavus* genomic DNA with ExTaq HS polymerase (Takara, Pittsburg, PA, USA), PCR products of the expected size of 1218 bp for the 5' *pks27* amplification and 1042 bp for the 3' *pks27* amplification were obtained. PCR products were subcloned into TOPO pCR2.1 (Invitrogen, Carlsbad, CA, USA) and verified by DNA sequencing. The 5' and 3' *pks27* PCR products were released from their TOPO vectors using EcoRI-BamHI and SalI-HindIII digestion respectively and subcloned in a stepwise manner into the analogous restriction digested pPG2.8 vector harboring the *A. parasiticus pyrG* selectable marker gene. The resulting knockout vector was designated pks27-pyrG. Transformation was performed as described in Cary *et al.* [18] using *A. flavus* 70 (AF70) as the host.

For the investigation of cluster 27 metabolites, conidia were grown for 10 days at 30 °C in the dark onto GMM agar supplemented with 2 % sorbitol (GMMS; per liter: glucose, 10 g; ammonium tartrate, 2.0 g; sorbitol, 2.0 g; Cove's salts, 20 mL; pH 6.5).

The strains used in the investigation of asparasone A production by other *Aspergillus* spp. were obtained from the USDA Southern Regional Research Center (New Orleans, LA, USA). These strains were grown on Wickerham medium (WATM), containing 2.0 g yeast extract, 3.0 g peptone, 5.0 g corn steep solids, 2.0 g dextrose, 30.0 g sucrose, 2.0 g $NaNO_3$, 1.0 g $K_2HPO_4 \cdot 3H_2O$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.1 g $FeSO_4 \cdot 7H_2O$ and 15.0 g agar per liter (pH 5.5).

7.2.3. Sample preparation

Sclerotia were collected from the Petri dish by adding 10 mL of sterile deionized water to the agar surface and gently scraping colonies with a cell scraper. The collected sclerotia were washed two times by addition of 40 mL water to remove residual conidia

[18] J. W. Cary, K. C. Ehrlich, J. M. Bland, B. G. Montalbano, App. Environ. Microbiol. 72 (2006) 1096.

(asexually produced spores) and mycelia (the vegetative part of a fungus, consisting of a mass of branching, threadlike hyphae). After centrifugation and removal of water, metabolites were extracted with 30 mL MeOH:DCM:EtOAc (10:20:30, v/v/v) containing 1 % (v/v) formic acid. The samples were shaken during 60 min on an Agitelec overhead shaker (J. Toulemonde & Cie, Paris, France). Four mL of extract was transferred into a glass tube and evaporated till dryness under a stream of nitrogen. The residue was reconstituted with 200 µL MeOH:MeCN:H_2O (30:30:40, v/v/v), centrifuged in a Ultrafree®-MC centrifugal device for 5 min at 14000 g and injected into the MS.

7.2.4. UHPLC-HRMS analysis

An Accela™ High Speed LC (UHPLC) (Thermo Fisher Scientific, San José, USA) was used for separation. The column was a Zorbax RRHD Eclipse Plus C_{18} (2.1 x 100 mm, 1.8 µm) from Agilent Technologies (Diegem, Belgium). The mobile phase consisted of H_2O:MeOH (95:5, v/v) containing 0.1 % formic acid and 10 mM ammonium formate [solvent A] and MeOH:H_2O (95:5, v/v) containing 0.1 % formic acid and 10 mM ammonium formate [solvent B]. The gradient elution program for UHPLC-HRMS analyses was applied as follows: 0 min: 0 % B, 0.5 min: 0, 20 min: 99 % B, 21 min: 99 % B, 24 min: 0 % B, 28 min: 0 % B. The flow rate was 0.4 mL min^{-1}. The column temperature was set at 30 °C and temperature of the autosampler was 10 °C. Five µL of the sample were injected.

Accurate mass measurements of the precursor and fragment ions were carried out on an Orbitrap Exactive™ mass analyzer (Thermo Fisher Scientific) equipped with HESI and APCI interface. The final MS parameters in the HESI in negative mode were the following: spray voltage 4.5 kV, capillary temperature 250 °C, heater temperature 250 °C, sheath gas flow rate 45 arbitrary units (a.u.), auxiliary gas flow rate 10 a.u. The data were processed using the Xcalibur™ 2.1 and Exactive Tune software (Thermo Fisher Scientific). The instrument was operated in full scan mode with a resolution of 100000 FWHM. The maximum injection time was 200 ms and the number of microscans per scan was 1. Each full scan was followed by a same-polarity “all ion fragmentation” higher energy collisional dissociation scan.

7.2.5. HPLC-MS^n analysis

A Surveyor Plus HPLC System (Thermo Fisher Scientific) was used for separation. The column was an XBridge MS C_{18} column (150 x 2.1 mm, 3.5 µm) with an XBridge Sentry guard column (10 x 2.1 mm, 3.5 µm) both supplied by Waters (Milford, MA, USA). The mobile phase was as described in section 7.2.4. The gradient elution program was the following: 0 min: 0 % B, 1 min: 0% B, 24 min: 99 % B, 25 min: 99 % B, 28 min: 0 % B; 35 min: 0 % B. The flow rate was 0.25 mL min^{-1}. The column temperature was set at 40 °C and temperature of the autosampler was 10 °C. A 5 µL aliquot of the sample were injected.

An LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific) equipped with an HESI source was used for the investigation of fragmentation behavior. The set MS parameters were: source voltage 5 kV, capillary temperature 250 °C, heater temperature 175 °C, sheath gas flow rate 40 a.u., auxiliary gas flow rate 10 a.u. The maximum injection time was 200 ms and the number of microscans per scan was 3. When performing MS^n experiments, the precursor ion was isolated in the ion trap with an isolation width of 3 Da and activated at different CELs to find the optimal conditions for distinct fragmentation. Xcalibur™ 2.0.7 software (Thermo Fisher Scientific) was used for instrument control, data acquisition and processing.

7.2.6. Statistical data treatment

SIEVE 1.3 software (Thermo Fisher Scientific) was used for LC-MS data mining. SIEVE aligns the MS spectra over time for different experimental conditions and determines features in the dataset. Parameters for the SIEVE analysis were set as follows: frame time width 0.05 min, frame *m/z* width 0.020, peak intensity threshold 10000, maximum number of frames 5000, alignment bypass: False, *m/z* min: 100, *m/z* max: 1000, retention time start: 0.01 min, retention time stop: 28 min, PCA V1.0. Statistical significance of a frame was calculated within the SIEVE package using a t-test.

To identify the extracted frames, initial database searching was performed using the MZLookup function in SIEVE where a home-made database was uploaded. The

database was constructed based on literature data and consisted of secondary metabolites produced by *Aspergillus* spp.

7.3. Results and discussion

7.3.1. Metabolic profiling and preliminary metabolite assignment

Recombinational inactivation of the *pks*27 resulted in *A. flavus* transformants that no longer produced characteristic dark brown sclerotia but instead produced sclerotia that were greyish-yellow (Figure 7.1.).

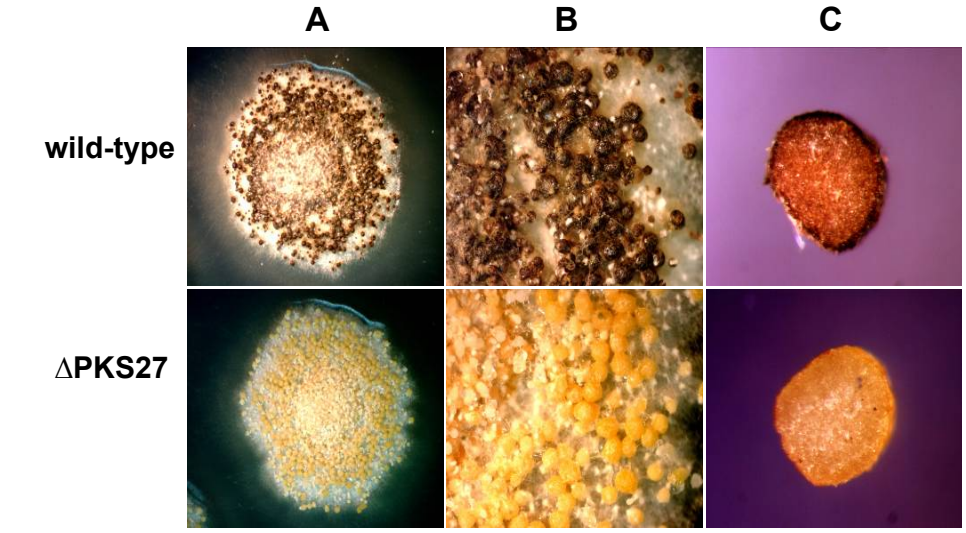


Figure 7.1. Microscopic examination of sclerotia. Sclerotium production in wild-type Af70, *Δpks27* mutant strain was observed using an Olympus SZH10 stereomicroscope and Nikon DS-Qi1 camera. Strains were grown on GMMS agar for 10 days in the dark at 30°C. Panel: colony surface, X10 magnification (a); colony surface, X25 magnification (b); sclerotium cross-section, X140 magnification (c)

Metabolite screening of wild-type *A. flavus* AF70 and mutant *A. flavus* AF70 was performed on an UHPLC-Orbitrap MS in ESI and APCI, both in positive and negative mode. The total ion chromatograms (TICs) for the wild-type and mutant are presented in Figure 7.2.

Visual inspection of the TIC of the wild-type revealed presence of four clear peaks, which were seen only in the TIC of the parental strain. As low-abundance compounds might not be apparent, a statistical differential analysis of the LC-MS data was performed using SIEVE software. SIEVE included principal component analysis (PCA) to highlight differences between the two groups. To compare chromatograms of the groups on the basis of peak intensities, peak alignment was done using wild-type as reference sample. Significant differences in the metabolite profile were observed between the sample groups. On PCA score plot, the wild-type group was clearly separated from the other samples, which included the blank media and replicates of the mutant (Figure 7.3a). On PCA loading plot (Figure 7.3b), variables relevant for the clustering pattern are shown.

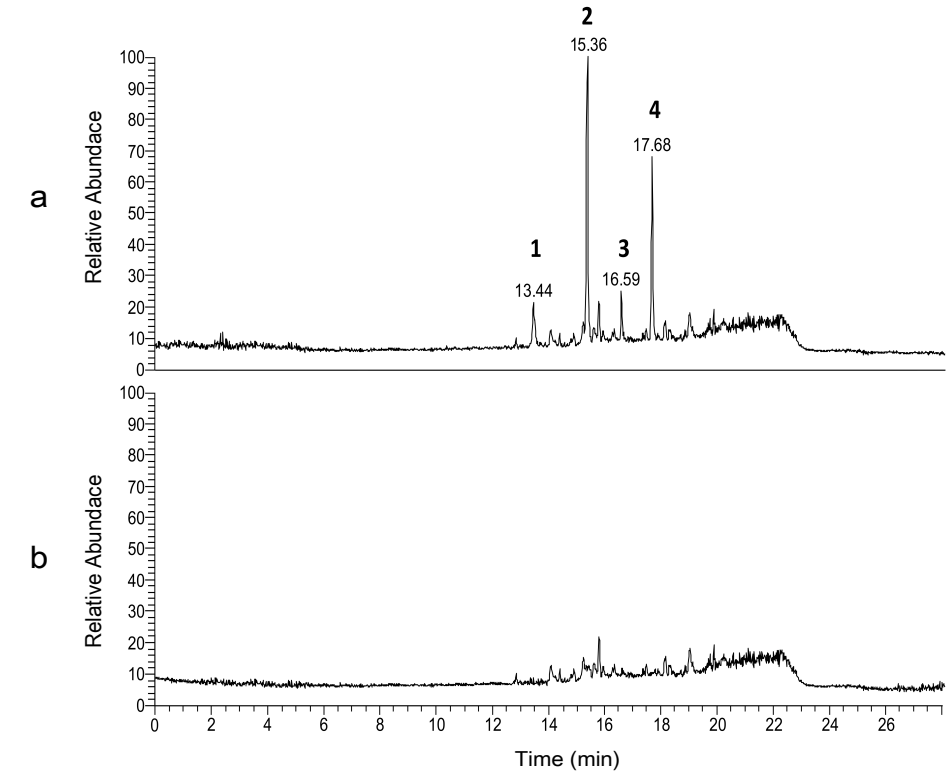


Figure 7.2. Total ion chromatogram of wild-type *A. flavus* AF70 (a) and mutant *A. flavus* AF70 (b) obtained in ESI (−). 1: m/z 373; 2: m/z 357; 3: m/z 315; 4: m/z 339

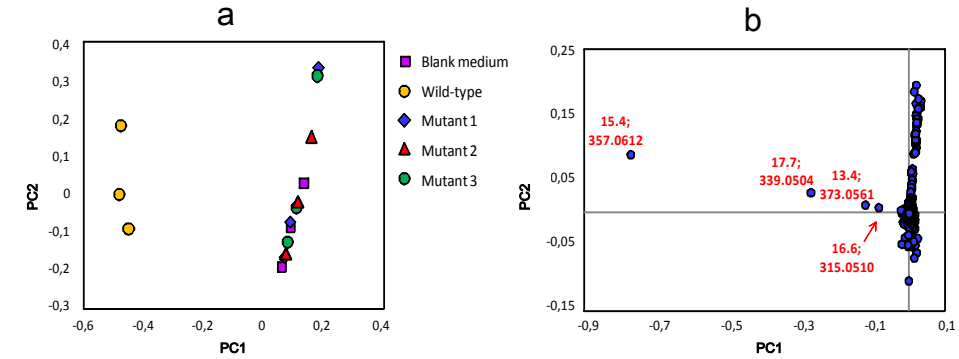


Figure 7.3. PCA score plot (a) and loading plot (b)

A set of four markers, corresponding to 373.0561 (13.4 min) (**1**), *m/z* 357.0612 (15.4 min) (**2**), *m/z* 315.0510 (16.6 min) (**3**), and *m/z* 339.0504 (17.7 min) (**4**) could be distinguished for the wild-type. The reconstructed ion chromatograms confirmed absence of these ions in the mutant.

7.3.2. Fragmentation pathway and metabolite identification

To identify the detected markers, firstly, accurate mass was used to generate elemental compositions leading to tentative metabolite identification by searching in a home-made and public databases. At this stage, a match was found only for *m/z* 357.0612 corresponding to asparasone A (1,3,6,8-tetrahydroxy-2-(1′-hydroxy-3′-oxobutyl)-anthraquinone) with molecular formula $C_{18}H_{14}O_8$; an anthraquinone pigment previously detected in *A. parasiticus* cultures [19]. Identification of *m/z* 357.0612 as asparasone A (**2**) was confirmed by comparison with an authentic standard. A complete correspondence of RT, MS and MS^n spectra for the identified metabolite and the standard was observed. MS^2 spectra (Figure 7.4) were characterized by abundant product ions at *m/z* 339, and *m/z* 299 corresponding to loss of H_2O (-18 Da) and C_3H_6O (-58 Da) from the precursor ion at *m/z* 357, respectively. Subsequent fragmentation of the ion at *m/z* 339 yielded a product ion at *m/z* 297 corresponding to a loss of 42 Da

[19] V. S. Sobolev, R. J. Cole, J. W. Dorner, B. W. Horn, J. Nat. Prod. 60 (1997) 847.

(C_2H_2O), whereas a fragment at *m/z* 271, corresponding to a loss of CO (-28 Da), was formed through fragmentation of the ion at *m/z* 299.

MS^n data of the *m/z* 297 and *m/z* 271 (Figure 7.5) indicated consecutive losses of 28 Da and 44 Da corresponding to CO and CO_2, respectively. The fragmentation mechanism for these losses of CO and CO_2 moieties from the anthraquinone structure was similar to that described by Kang *et al.* [20] for isoflavone derivatives and by Proctor *et al.* [21] for quinones. This involved a rearrangement of the molecule leading to the loss of the carbonyl and hydroxyl moieties, and closing of ring; however, the order in which CO or CO_2 were eliminated from the structure could not be established.

[20] J. Kang, L. A. Hick, W. E. Price, Rapid Commun. Mass Spectrom. 21 (2007) 857.

[21] C. J. Proctor, B. Kralj, E. A. Larka, C. J. Porter, A. Maquestiaul, J. H. Beynon, Org. Mass Spectrom. 16 (1981) 312.

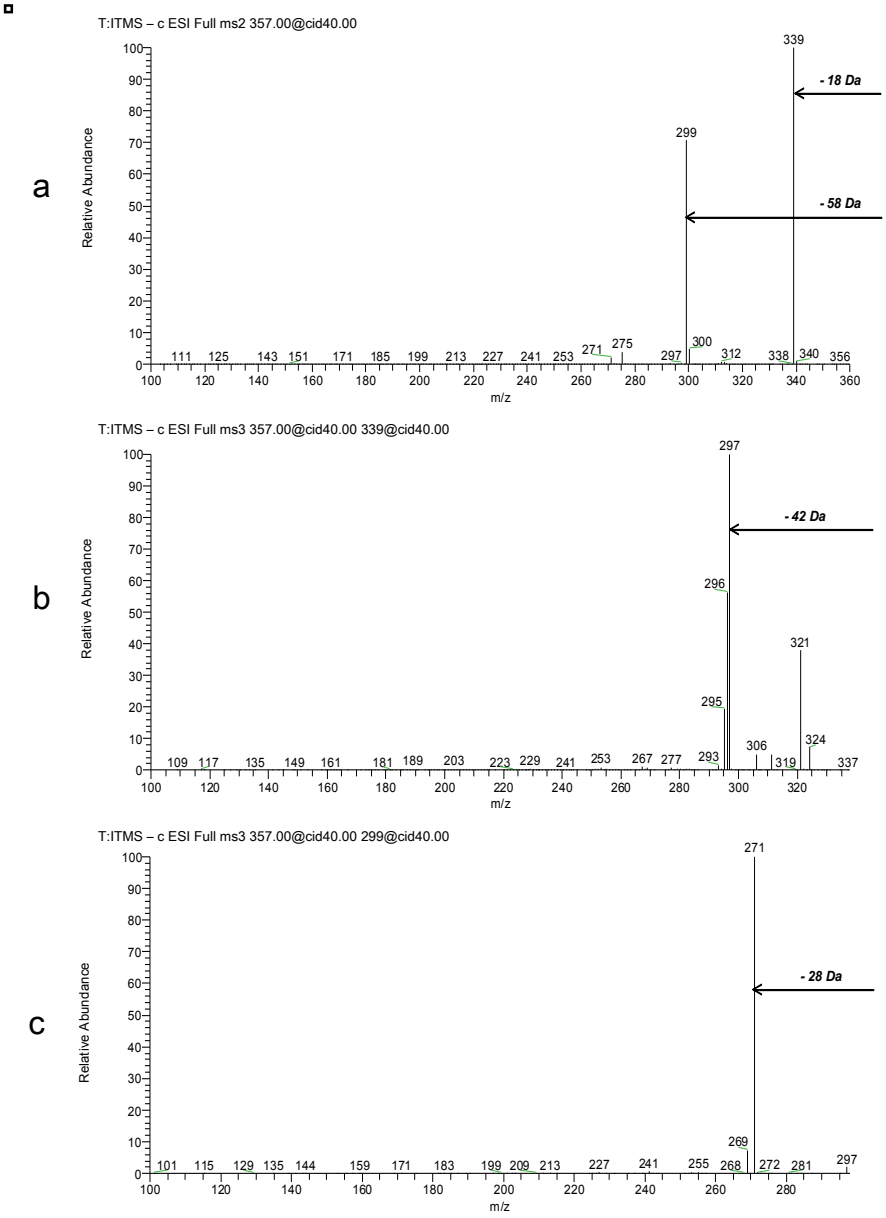


Figure 7.4. CID spectrum of the precursor ion at *m/z* 357 (a) and second-generation spectra (b, c) acquired for compound **2**. The product ions at *m/z* 339 (b) and *m/z* 299 (c) were isolated and collisionally activated at 40 % CEL

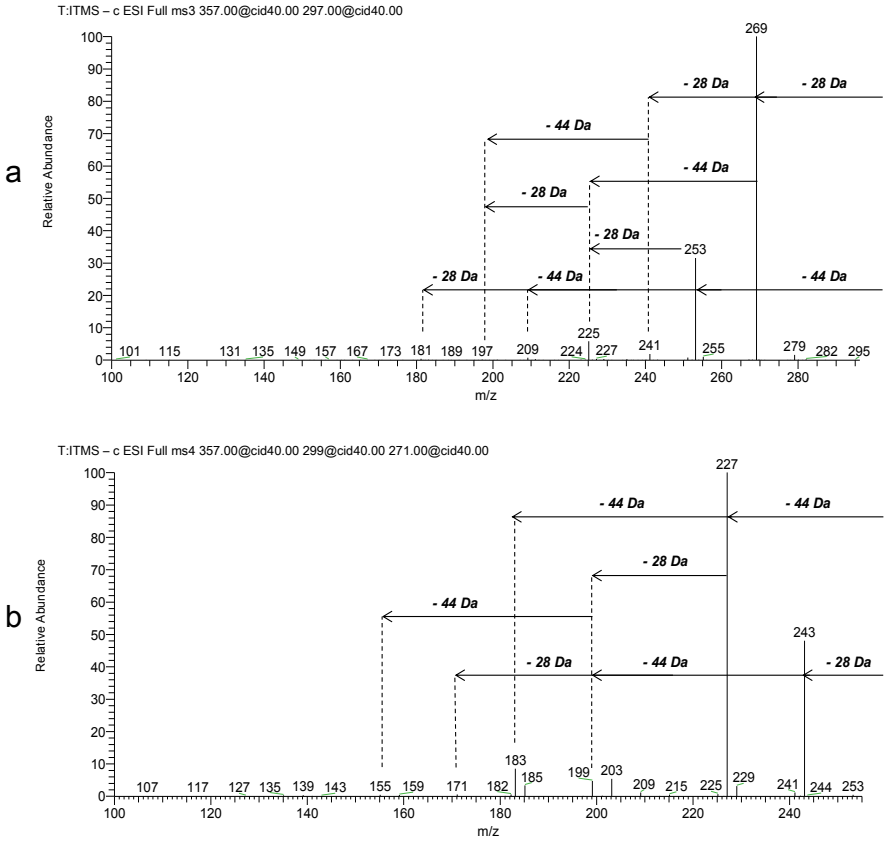


Figure 7.5. Second- (a) and third- (b) generation spectra acquired for compound **2**. The product ions at *m/z* 297 (a) and *m/z* 271 (b) were isolated and collisionally activated at 40 % CEL

Combining these fragmentation data and accurate mass measurements, a fragmentation pathway of asparasone A in ESI (−) was proposed (Figure 7.6). The above described consecutive losses of CO and CO_2 led to the fragments at *m/z* 141 and 157, and *m/z* 155 from the ions at *m/z* 297 and *m/z* 271, respectively.

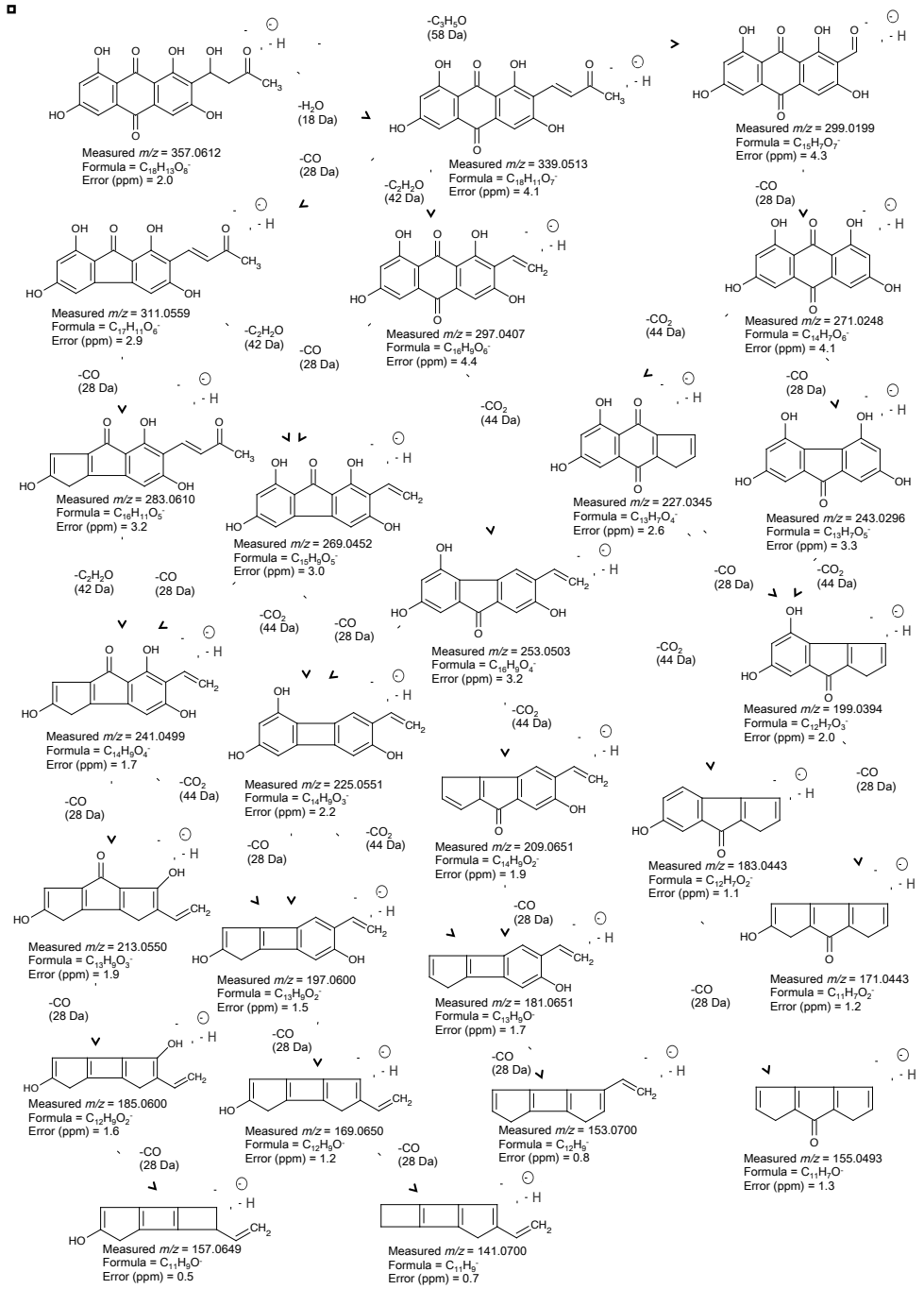


Figure 7.6. Proposed fragmentation pathway of asparasone A (2). The order of these losses could not be established; exemplary structures of fragments are shown

It was hypothesized that the four compounds detected in the wild-type *A. flavus* AF70 might be a part of the asparasone A biosynthetic pathway and therefore can be structurally related. Owing to mass differences of the abundant ions observed in the MS spectra, it was assumed that *m/z* 339 (**4**) and *m/z* 315 (**3**) could represent a molecule of asparasone A in which H_2O and C_2H_2O, respectively, were eliminated from the side chain. A difference of 16 Da for *m/z* 373 (**1**) could possibly account for an additional oxygen atom in the structure of asparasone A.

MS^2 spectra of compound **3** (*m/z* 315) showed initially a loss of 18 Da (corresponding to the elimination of a water molecule) yielding *m/z* 297 (Figure 7.7a), whose fragmentation was identical to that observed for asparasone A. Compound **4** (*m/z* 339) also gave the same fragment at *m/z* 297 (Figure 7.7b).

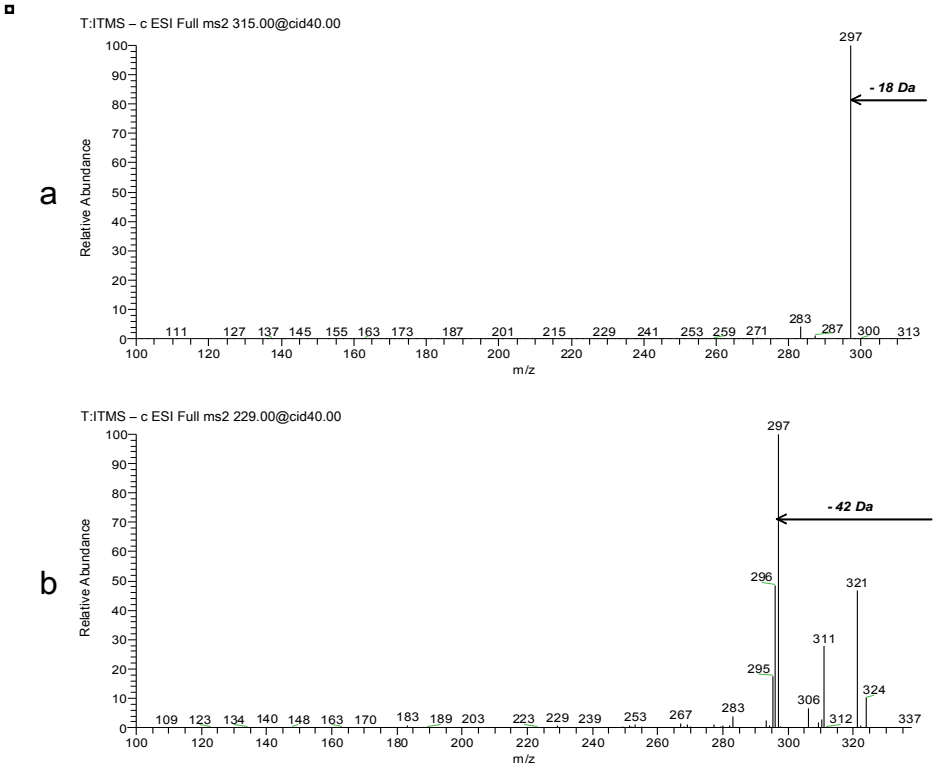


Figure 7.7. CID spectra of precursor ions at m/z 315 (compound 3) (a) and m/z 339 (compound 4) (b)

MS^2 of compound **1** (*m/z* 373) indicated losses of 18 and 58 Da (Figure 7.8a), as was observed with asparasone A (Figure 7.4a).

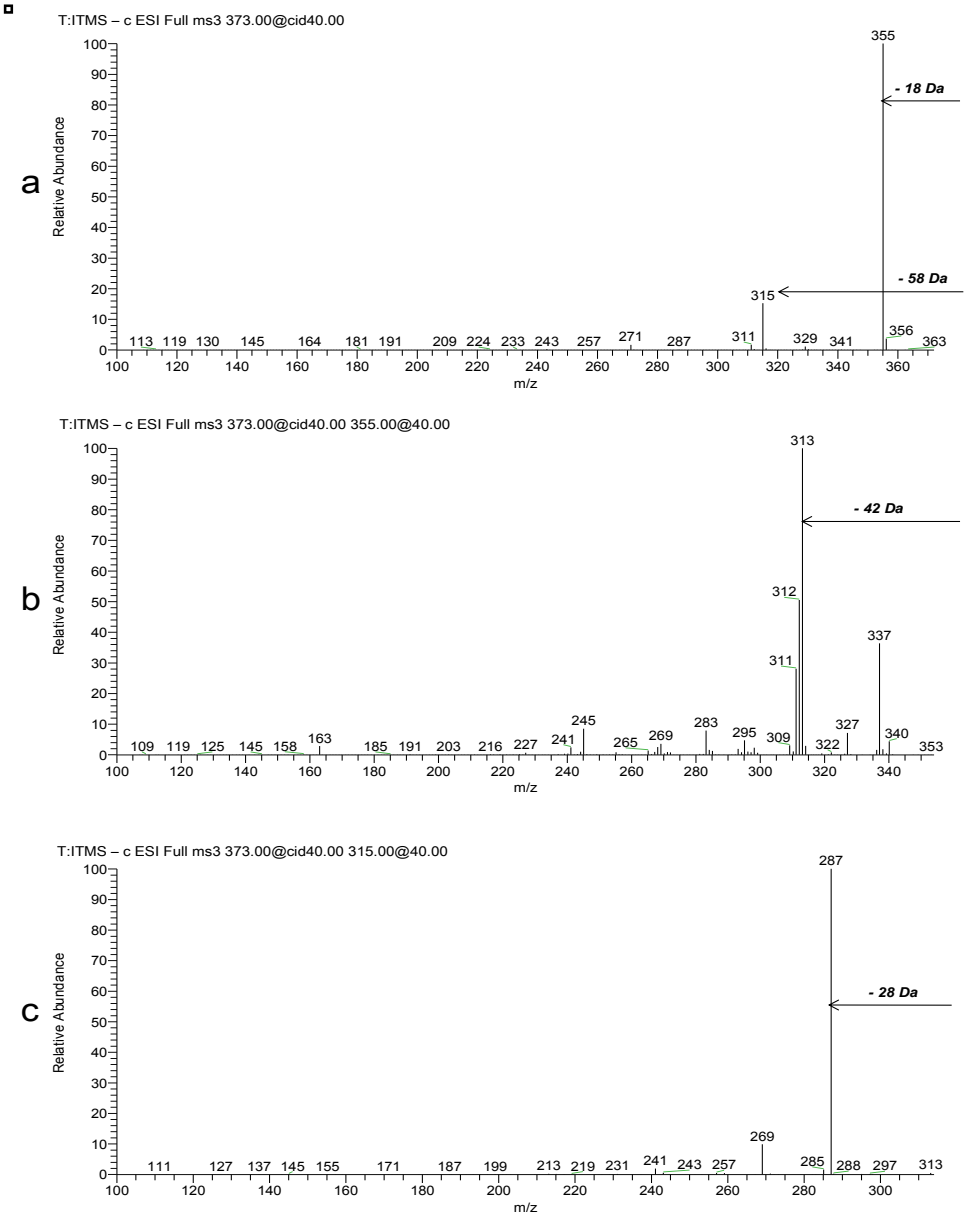


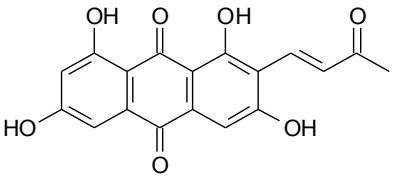
Figure 7.8. CID spectrum of a precursor ion at *m/z* 373 (a) and second-generation spectra (b, c) acquired for compound **1**. The product ions at *m/z* 355 (b) and *m/z* 315 (c) were isolated and collisionally activated at 40 % CEL

The resulting fragments (*m/z* 355 and *m/z* 315) had 16 mass units higher than the corresponding fragments in asparasone A, *i.e. m/z* 339 and *m/z* 299, respectively. This 16 Da difference pointed to an extra oxygen atom in the molecule, exact position of which in the structure could not be deduced from further MS^n fragmentation of the product ions at *m/z* 355 and *m/z* 315. Nevertheless, these fragmentation data indicated that the substitution with a hydroxyl-group in the side chain was not likely, as the difference of 16 Da between asparasone A (**2**) and compound **1** was still noted after elimination of the side chain from the molecule. Therefore, the hydroxyl-group could be at positions 4, 5 or 7, and more likely at positions 4 or 5. However, to designate the exact position of the hydroxyl-substituent, isolation of the compound and nuclear magnetic resonance (NMR) experiments are needed.

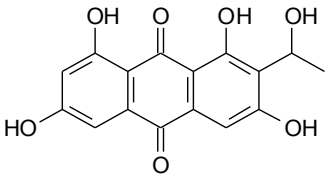
Based on data described above, compounds **1**, **3** and **4** were assigned respectively as (Figure 7.9):

- **Compound 1**: 1,3,4,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone or 1,3,5,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone
- **Compound 3**: 1,3,6,8-tetrahydroxy-2-(1'-hydroxyethyl)-anthraquinone
- **Compound 4**: 1,3,6,8-tetrahydroxy-2-(3'-oxobut-1'-en-1'-yl)-anthraquinone

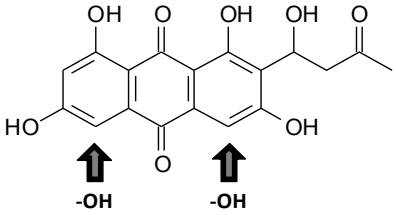
The elution order of these compounds under the applied chromatographic conditions (reversed-phase chromatography) was consistent with the proposed structures. Compounds **4** and **3** eluted later than asparasone A (**2**), being more hydrophobic due to absence of polar hydroxyl- and acetyl-groups, respectively. Compound **3** having a shorter side chain was eluted earlier than compound **4**. Compound **1**, bearing an additional hydroxyl-group is more polar and was eluted before asparasone A (**2**).



1,3,6,8-tetrahydroxy-2-(3'-oxobut-1'-en-1'-yl)-anthraquinone (compound **4**)



1,3,6,8-tetrahydroxy-2-(1'-hydroxyethyl)-anthraquinone (compound **3**)



1,3,4,6,8-pentahydroxy-2-(1'-hydroxy-3'oxobutyl)-anthraquinone or
1,3,5,6,8-pentahydroxy-2-(1'-hydroxy-3'oxobutyl)-anthraquinone
(compound **1**)

Figure 7.9. Structures of newly identified metabolites of *A. flavus* AF70. Probable positions of the additional hydroxyl-group in the structure of compound **1** are shown with the arrows

7.2.3. Production of asparasone A derivatives in selected fungal cultures

Asparasone A was first identified in *A. parasiticus* extracts as a pigment that was structurally related to *versicolorin* intermediates of the aflatoxin biosynthetic pathway [22]. In their study asparasone A was obtained from total extract of *A. parasiticus* culture.

In the investigation of the cluster 27 metabolites described in this work, an AF70 strain was used. Production of asparasone A and its derivatives (compounds 1, 3 and 4) was further investigated in extracts of other *Aspergillus* strains (Table 7.1). Asparasone A was detected in all fungal cultures studied and occurred together with its dehydrated product. In *A. terreus* culture, compounds 3 and 1, respectively, were not detected

[22] V. S. Sobolev, R. J. Cole, J. W. Dorner, J. Nat. Prod. 60 (1997) 847.

simultaneously with asparasone A. Investigation of other known *Aspergillus* metabolites revealed co-occurrence of asparasone A, AFB_1, AFB_2, cyclopiazonic acid and ditryptophenaline. However, a clear correlation between these metabolites could not be established. AFB_1, AFB_2, AFG_1 and AFG_2, and cyclopiazonic acid were confirmed with authentic standards, while aflatrem and ditryptophenaline were identified based on their retention time profile, which was in good agreement with the results of Rank *et al*. [23], and fragmentation pattern. Table 7.2 summarizes data on the detected metabolites. It is worth to mention that the dimeric character of ditryptophenaline was revealed in the MS spectra; an ion at *m/z* 346.1550 ($C_{21}H_{20}N_3O_2$; -2.0 ppm), corresponding to a monomeric fragment, accompanied the protonated ion at *m/z* 693.

[23] C. Rank, M. L. Klejnstrup, L. M. Petersen, S. Kildgaard, J. C. Frisvad, C. H. Gotfredsen, Metabolites 2 (2012) 39.

Table 7.1. Production of asparasone A and its derivatives, and a number of other metabolites by *Aspergillus* spp

Metabolite	Fungi							
	A. nidulans	*A. nomius*	*A. terreus*	*A. parasiticus BN8*	*A. parasiticus BN9*	*A. flavus AF36*	*A. flavus AF70*	*A. flavus CA14*
Asparasone A	++	++	+	++	+++	+++	++	+
1,3,6,8-tetrahydroxy-2-(3'-oxobut-1'-en-1'-yl)-anthraquinone	++	++	+	++	++	++	++	+
1,3,6,8-tetrahydroxy-2-(1'-hydroxyethyl)-anthraquinone	++	++	-	++	++	++	++	+
1,3,4,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone or 1,3,5,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone	++	++	-	++	++	++	++	+
Aflatoxin B_1	+	+++	++	+++	+++	++	+++	+
Aflatoxin B_2	+	+++	+	+++	+++	+	+++	+
Aflatoxin G_1	-	+++	++	+++	+++	++	++	-
Aflatoxin G_2	-	+++	+	+++	+++	-	++	-
Cyclopiazonic acid	++	++	++	+++	++	+++	+++	+++
Aflatrem	+	-	-	++	++	+++	+++	++
Ditryptophenaline	++	++	++	+++	+	+++	++	+++

-	No detected
+	Peak areal ≤ 10^6
++	10^6 < Peak área ≤ 10^8
+++	Peak área > 10^8

Table 7.2. Secondary metabolites identified in cultures of *Aspergillus*

Metabolite	Ionization mode	Formula	RT (min)	Calcul. *m/z*	Measur. *m/z*	Mass error (ppm)
Aflatoxin B_1	ESI (+)	$C_{17}H_{12}O_6$	11.6	313.0707	313.0703	-1.2
Aflatoxin B_2	ESI (+)	$C_{17}H_{14}O_6$	11.1	315.0863	315.086	-0.9
Aflatoxin G_1	ESI (+)	$C_{17}H_{12}O_7$	10.1	329.0656	329.0652	-1.2
Aflatoxin G_2	ESI (+)	$C_{17}H_{14}O_7$	9.9	331.0812	331.0808	-1.3
Cyclopiazonic acid	ESI (+)	$C_{20}H_{20}N_2O_3$	16.3	337.1547	337.1542	-1.4
Aflatrem	ESI (+)	$C_{32}H_{39}NO_4$	20.4	502.2952	502.2944	-1.5
Ditryptophenaline	ESI (+)	$C_{42}H_{40}N_6O_4$	15.1	693.3184	693.3178	-0.9
1,3,4,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone or 1,3,5,6,8-pentahydroxy-2-(1'-hydroxy-3'-oxobutyl)-anthraquinone	ESI (−)	$C_{18}H_{14}O_9$	13.4	373.0559	373.0561	1.9
Asparasone A	ESI (−)	$C_{18}H_{14}O_8$	15.4	357.0616	357.0612	2
1,3,6,8-tetrahydroxy-2-(1'-hydroxyethyl)-anthraquinone	ESI (−)	$C_{16}H_{12}O_7$	16.6	315.051	315.051	3.4

7.4. Conclusions

In this chapter the metabolites produced by the *Aspergillus flavus* cluster 27 *pks* gene (asparasone A, and dehydration, deacetyl- and oxy-derivatives of asparasone A) have been identified using UHPLC coupled to high resolution mass spectrometry (Orbitrap). Accurate mass data from the Orbitrap MS instrument was used to determine the elemental composition of the compounds of interest and thereby to perform a preliminary peak assignment, while ion trap multi-stage MS was utilized to obtain structural information and to confirm the tentatively identified metabolites. Finally, asparasone A and its derivatives were detected simultaneously with some known *A. flavus* metabolites, such as aflatoxins, aflatrem, cyclopiazonic acid and ditryptophenaline, in other *Aspergillus spp*.

This methodology could be applied to study other clusters, allowing elucidating which of them is responsible of the production of different fungi metabolites, including mycotoxins.

This work is in the process of drafting:

“Metabolic profiling of Aspergillus flavus: Identification and fragmentation study of the cluster 27 polyketide synthase metabolites by high resolution and multi-stage mass spectrometry”. N. Arroyo-Manzanares, S. V. Malysheva, J. W. Cary, K. C. Ehrlich, J. Vanden Bussche, L. Vanhaecke, D. Bhatnagar, J. D. Di Mavungu, S. De Saeger.

CONCLUSIONES FINALES

Conclusiones finales

La problemática principal abordada en esta Tesis ha sido el desarrollo de diferentes métodos para la determinación de un conjunto de contaminantes naturales de gran interés por su toxicidad en alimentos, las micotoxinas. Para ello se han evaluado diferentes técnicas instrumentales separativas, tanto miniaturizadas (CE y HPLC capilar) como de ultra resolución (UHPLC) acopladas a diferentes sistemas de detección de gran sensibilidad y selectividad como LIF y MS/MS. Con ello se ha contribuido al aumento y la mejora de los métodos de control de calidad de alimentos. Además se han estudiado y propuesto metodologías de tratamiento de muestra alternativas a las ya existentes que permiten aumentar la eficacia y el rendimiento de los análisis, además de ser más respetuosas con el medioambiente, permitiendo la determinación simultánea de micotoxinas de diversas familias.

Durante el estudio de las diferentes técnicas utilizadas, LC y CE acopladas a los métodos de detección indicados, se han encontrado importantes diferencias que se comentan a continuación:

- El método basado en CE implicó un menor consumo de reactivos y disolventes, especialmente disolventes orgánicos, que los métodos cromatográficos. Además los consumibles empleados supusieron un coste inferior a los de LC. Este trabajo demostró el potencial de la CE (técnica poco usual en este campo) para la determinación de micotoxinas, en concreto de aflatoxinas.

- UHPLC ha permitido determinar un mayor número de analitos y en menor tiempo que las otras técnicas. Así, mientras que con CE se determinaban 4 micotoxinas en aproximadamente 20 min, la metodología UHPLC conseguía determinar 15 micotoxinas en menos de 4 min. Además, acoplada a MS/MS ha demostrado ser una técnica muy eficaz para los análisis multimicotoxinas.

- En cuanto a los métodos de detección, hay que señalar que CE-LIF permitió alcanzar LOQs por debajo de los contenidos máximos establecido en

legislación para aflatoxinas, sin necesidad de derivatización previa. Además, para la determinación de OTA, la adición de SDS a la fase móvil en el acoplamiento HPLC-LIF, mejoró considerablemente el LOQ.

- No obstante, a pesar de la alta selectividad y sensibilidad de la detección LIF, esta técnica está restringida sólo a aquellas micotoxinas que presentan fluorescencia nativa a la longitud de onda seleccionada, no permitiendo detectar un alto número de analitos simultáneamente, como es el caso de la MS/MS, sistema de detección universal que además permite la identificación inequívoca de los mismos.

Otro de los objetivos planteados fue el estudio de tratamientos de muestra alternativos para evaluar la eficacia, simplicidad, versatilidad y precisión de los mismos, además de su impacto en la generación de residuos al medioambiente. Para ello se han evaluado métodos relativamente recientes como DLLME, IL-DLLME y QuEChERS, poco explorados en el análisis de micotoxinas:

- En general, todos los métodos estudiados presentaban las ventajas generales de menor coste y menor tiempo requerido, en comparación con el método convencional de IACs.

- Con los métodos DLLME y IL-DLLME se consiguieron LOQs más bajos que los obtenidos con la metodología QuEChERS cuando estos tres tratamientos fueron aplicados para la el análisis de ocratoxina A en muestras de vino. Esto fue debido a la preconcentración asociada a DLLME y IL-DLLME que no se llevaba a cabo con la metodología QuEChERS, que implicó la dilución de la muestra. Sin embargo, la precisión fue ligeramente mejor con DLLME y QuEChERS que con IL-DLLME.

- Los tratamientos basados en QuEChERS resultaron muy adecuados cuando se utilizan métodos de detección muy selectivos como LIF y MS/MS, siendo especialmente efectivos cuando se quiere estudiar un gran número de compuestos de naturaleza muy diferente, como es el caso del análisis multirresiduo.

- Sin embargo, la metodología QuEChERS no resultó suficiente en muestras complejas, como en el caso del complemento alimentico estudiado en esta Tesis y los frutos secos. En estos casos, fue necesaria una etapa de limpieza que disminuyera el efecto matriz en MS/MS, con objeto de conseguir los LOQs adecuados para algunas micotoxinas, proponiéndose un paso adicional de limpieza basada en DLLME.

En la Tabla C1, y para acompañar las conclusiones anteriores, se pueden observar las características más notables de cada uno de los métodos desarrollados en esta Tesis.

Por último, en los trabajos llevados a cabo en la Facultad de Ciencias Farmacéuticas de la Universidad de Gante (Bélgica) durante la estancia predoctoral, se ha aplicado por primera vez la combinación de UHPLC-Orbitap-MS y HPLC-MS^n, para el estudio del metaboloma de algunos de los hongos productores de micotoxinas. Las conclusiones más importantes derivadas de estos estudios fueron las siguientes:

- Se ha desarrollado una estrategia para el estudio de alcaloides ergóticos nuevos o poco explorados, planteando un método para su rápida identificación y elucidación estructural.

- Se han identificado los metabolitos producidos por el cluster 27 *pks* (*pks*27) en el *Aspergillus flavus*: asparanona A (un pigmento derivado de la antraquinona) y tres nuevos compuestos, concretamente los resultantes de las correspondientes reacciones de deshidratación y desacetilación así como el oxi-derivado de asparasona A. Tanto la asparasona A como sus derivados, se detectaron simultáneamente con algunos metabolitos conocidos de *A. flavus*, tales como las aflatoxinas, aflatrem, ácido ciclopiazónico y ditriptofenalina, en otras especies de *Aspergillus.* Sin embargo, no se pudo establecer una correlación clara para la presencia de estos metabolitos.

Analitos	Matriz	Tratamiento de muestras	Técnica instrumental	Tiempo de análisis (min)	LOQs
AFB_1, AFB_2, AFG_1, AFG_2	Arroz	IACs	MEKC-LIF BGE: 20 mM tampón borato y 30 mM SDS a pH 8.5, con 7% de MeCN (v/v)	20	0.13-1.74 µg L^{-1}
OTA	Vino	DLLME	HPLC capilar-LIF Fase móvil: A- H_2O con 2% ácido acético y 0.2 M SDS B- MeOH	4	18.4 ng L^{-1}
OTA	Vino	IL-DLLME			17.5 ng L^{-1}
OTA	Vino	QuEChsERS			286 ng L^{-1}
AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, NIV, F-X, ZEN	Cardo mariano	QuEChERS+ DLLME	UHPLC – MS/MS Fase móvil: A- H_2O con 0.3% ácido fórmico y 5 mM fomiato amónico B- MeOH con 0.3% ácido fórmico y 5 mM fomiato amónico	4	1.50-1530 µg Kg^{-1}
AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, NIV, F-X, ZEN	Cereales y pseudocereales	QuEChERS			0.23-233 µg Kg^{-1}
OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, F-X, ZEN	Melazas de cereal	QuEChERS			0.45-75.2 µg Kg^{-1}
AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, F-X, ZEN	Frutos secos	QuEChERS+ DLLME			0.57- 150 µg Kg^{-1}

FINAL CONCLUSIONS

Final conclusions

The main objective of this Thesis was the development of analytical methods for the determination of an important kind of natural contaminants in foods, the mycotoxins. Thus, different techniques have been evaluated, both miniaturized, as CE and capillary HPLC, and of ultra-resolution as UHPLC. They have been coupled to different detection systems of great sensitivity and selectivity, such as LIF and MS/MS. This fact has contributed to increase the number and to improve the analytical methods for food quality control. In addition, in order to increase efficiency and sample throughput, different sample treatments have been assessed as an alternative to those previously reported.

During the assessment of these analytical techniques (LC and CE) coupled to the above-mentioned detection systems, the advantages and drawbacks of each methodology have been emphasized, finding significant differences among them:

- The CE method implied lower solvent and reagent consumption, especially organic solvents, compared with chromatographic methods and also lower in consumables than for LC. This work showed the applicability of CE for the determination of mycotoxins, specifically for aflatoxins.

- A larger number of analytes in a shorter time was determined with UHPLC. Thus, only 4 mycotoxins were analysed in 20 min using CE, in comparison to UHPLC, where 15 mycotoxins were separated in less than 4 min. So, UHPLC methodology has proven to be a very efficient technique for multiclass analysis.

- In terms of sensitivity, LIF showed LOQs below the maximum levels established for aflatoxins by EU regulation in food, avoiding a previous derivatization step. Moreover, the addition of SDS micellar solution in the mobile phase allowed increasing the native fluorescence of OTA in capillary HPLC-LIF, decreasing LOQ.

- However, although LIF detection is highly selective and sensitive, this technique is only restricted to those mycotoxins showing native fluorescence at the specific wavelength of the laser source, not being appropriate to determine simultaneously a large number of analytes. In this sense, MS/MS has shown its potential for multimycotoxin determinations, allowing also the unequivocal identification of the compounds.

Another aim of this Thesis has been the study of alternative sample treatments for mycotoxins, in order to evaluate their efficacy, simplicity, versatility and precision, as well as their impact on the enviroment. So, different sample treatments, as DLLME, IL-DLLME and QuEChERS have been assessed for the analysis of mycotoxins.

- The proposed methods showed as general advantages their shorter time and the relatively low-cost, compared with conventional IAC.

- The LOQs obtained with DLLME and IL-DLLME were lower than those obtained with QuEChERS procedure. This difference was due to the preconcentration obtained with DLLMEs, instead of the dilution produced by QuEChERS. However, the precision, evaluated in terms of repeatability and intermediate precision, was slightly better with QuEChERS and DLLME than that obtained with IL-DLLME.

- QuEChERS-based methodologies have shown to be ideal when very selective detection techniques, such as LIF and MS/MS, were used, especially when it was necessary to determine a high number of analytes, as in multiclass methods.

- However QuEChERS methodology was insufficient in complex samples, such as milk thistle or edible nuts and seeds studied in this Thesis. In these cases, an additional clean-up step was necessary to decrease the matrix effect in MS/MS techniques, and reach the appropriate LOQs for some compounds. Thus, an additional cleaning-up DLLME step has been proposed.

As a summary, the most significant analytical characteristics of the developed methods are shown in Table C1.

Finally, during the work developed in the Faculty of Pharmaceutical Sciences, University of Ghent (Belgium) during the predoctoral stay, the combination of UHPLC-Orbitap-MS and HPLC-MS^n for the study of metabolomics of mycotoxin-producing fungi has been used for the first time, being the main conclusions:

- A strategy for studying less known or novel ergot alkaloid derivatives was developed, establishing a method for their identification and structural elucidation.

- Asparasone A, and their dehydration, deacetyl- and oxy-derivatives were identified as metabolites produced by the *Aspergillus flavus* cluster 27 *pks* gene (*pks27*). The fragmentation pattern of these compounds in the negative electrospray ionization mode was elucidated for the first time. In addition, Asparasone A and its derivatives were detected simultaneously with some known *A. flavus* metabolites, such as aflatoxins, aflatrem, cyclopiazonic acid and ditryptophenaline, in other *Aspergillus spp*. However, a clear correlation between these metabolites could not be established.

Analytes	Matrix	Sample treatment	Instrumental technique	Analysis time (min)	LOQs
AFB_1, AFB_2, AFG_1, AFG_2	Rice	IACs	MEKC-LIF BGE: 20 mM borate buffer and 30 mM SDS at pH 8.5, with 7% MeCN (v/v)	20	0.13-1.74 μg L^{-1}
OTA	Wine	DLLME	HPLC capilar-LIF Mobile phase: A- H_2O with 2% acetic acid and 0.2 M SDS B- MeOH	4	18.4 ng L^{-1}
OTA	Wine	IL-DLLME			17.5 ng L^{-1}
OTA	Wine	QuEChERS			286 ng L^{-1}
AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, NIV, F-X, ZEN	Milk thistle	QuEChERS + DLLME	UHPLC – MS/MS Mobile phase: A- H_2O with 0.3% formic acid and 5 mM ammonium formate B- MeOH with 0.3% formic acid and 5 mM ammonium formate	4	1.50-1530 μg Kg^{-1}
AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, NIV, F-X, ZEN	Cereals and pseudocereals	QuEChERS			0.23-233 μg Kg^{-1}
OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, F-X, ZEN	Cereal syrups	QuEChERS			0.45-75.2 μg Kg^{-1}
AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2, STE, CIT, DON, F-X, ZEN	Edible nuts and seeds	QuEChERS + DLLME			0.57- 150 μg Kg^{-1}

ABREVIATURAS Y ACRÓNIMOS

ABREVIATURAS Y ACRÓNIMOS

En la mayoría de los casos se ha empleado la abreviatura o acrónimo anglosajón debido a su empleo más generalizado.

ACO:	Acetone
AFB_1:	Aflatoxin B_1
AFB_2:	Aflatoxin B_2
AFG_1:	Aflatoxin G_1
AFG_2:	Aflatoxin G_2
BGE:	Background electrolyte
CD:	Cyclodextrin
CE:	Capillary electrophoresis
CEn:	Collision Energy
CEL:	collision energy levels
CEP:	Collision Cell Entrance Potential
CID:	collision-induced dissociation
CIT:	Citrinin
CMC:	critical micellar concentration
CXP:	Collision Cell Exit Potential
CZE:	Capillary zone electrophoresis
DAD:	diode array detection
DHEt:	Dihydroergotamine
DLLME:	Dispersive liquid-liquid microextraction
DON:	Deoxynivalenol
DP:	Declustering potential
dSPE:	Dispersive SPE
Eco:	Ergocornine
Ecom:	Ergocornam
Econ:	Ergocorninine
Ecr:	Ergocristine
Ecrism:	Ergocristam
Ecrn:	Ergocristinine

Ecrypm:	Ergocryptam
EFSA:	European Food Safety Authority
Ek:	Ergokryptine
Ekn:	Ergokryptinine
ELISA:	Enzyme-linked immuno sorbent assay
Em:	Ergometrine
Emn:	Ergometrinine
EP:	Entrance potential
Es:	Ergosine
Esn:	Ergosinine
ESI:	Electrospray ionization
ESI (+):	Electrospray ionization positive mode
Et:	Ergotamine
Etn:	Ergotaminine
EtOAc:	Ethyl acetate (EtOAc)
EtOH:	Ethanol
EU:	European Union
FB_1:	Fumonisin B_1
FB_2:	Fumonisin B_2
FL:	Fluorescent
FWHM:	Full width at half maximum
F-X:	Fusarenon-X
GC:	Gas chromatography
GCB:	graphitized carbon
HT-2:	HT-2 toxin
HPLC:	High performance liquid chromatography
HESI:	Heated Electrospray Ionization
HESI(+):	Heated Electrospray Ionization in positive mode
HRMS:	High resolution mass spectrometry
IAC:	Immunoaffinity column
IARC:	Agency for Research on Cancer
I.D.:	Internal diameter
ILs:	Ionic liquids

IL-DLLME:	Dispersive liquid-liquid microextraction with ionic liquid
LC:	Liquid chromatography
LIF:	Laser induced fluorescence
LLE:	Liquid-liquid extraction
LOD:	Limit of detection
LOQ:	Limit of quantification
ME:	matrix effect
MeCN:	Acetonitrile
MeEm:	Methylergometrine
MeErgi:	Methysergide
MEKC:	Micellar electrokinetic chromatography
MeOH:	Methanol
MISPE:	Molecularly imprinted solid phase extraction
MRM:	Multiple reaction monitoring
MS:	Mass spectrometry
MS^n:	Multi-stage mass spectrometry
MS/MS:	Tandem mass spectrometry
NIV:	Nivalenol
OTA:	Ochratoxin A
PLE:	Pressurized liquid extraction
PSA:	Primary secondary amine
R:	Recovery
rpm:	Revolution per minute
RSD:	Relative standard deviation
S/N:	Signal to noise ratio
SDS:	Sodium dodecyl sulphate
SLE:	Solid-liquid extraction
SPE:	Solid phase extraction
STE:	Sterigmatocystin
THF:	Tetrahydrofuran
TLC:	Thin layer chromatography
Tris:	Tris(hydroxymethyl)aminomethan
T-2:	T-2 toxin

UHPLC:	Ultra-high performance liquid chromatography
XLC:	Extreme pressure liquid chromatography
ZEN:	Zearalenone
α-CD:	α-cyclodextrin
β-CD:	β-cyclodextrin
γ-CD:	γ-cyclodextrin
2-hpβ-CD:	2-hydroxi-propil-β-cyclodextrin
$[C_4MIM][PF_6]$:	1-butyl-3-methylimidazolium hexafluorophosphate
$[C_6MIM][PF_6]$:	1-Hexyl-3-methylimidazolium hexafluorophosphate