TESIS DE DOCTORADO

THE APPLICATION OF THE NANOTECHNOLOGY IN THE ELIMINATION OF PHYCOTOXINS AND MYCOTOXINS AND IMPLEMENTATION OF MASS SPECTROMETRY FOR THEIR DETECTION

Jesús María González Jartín

ESCUELA DE DOCTORADO INTERNACIONAL CAMPUS TERRA
PROGRAMA DE DOCTORADO EN INVESTIGACIÓN BÁSICA Y APLICADA EN CIENCIAS VETERINARIAS

LUGO
2019

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

DECLARACIÓN DEL AUTOR DE LA TESIS

The application of the nanotechnology in the elimination of phycotoxins and mycotoxins and implementation of mass spectrometry for their detection

D. Jesús María González Jartín

Presento mi tesis, siguiendo el procedimiento adecuado al Reglamento, y declaro que:

1) *La tesis abarca los resultados de la elaboración de mi trabajo.*
2) *En su caso, en la tesis se hace referencia a las colaboraciones que tuvo este trabajo.*
3) *La tesis es la versión definitiva presentada para su defensa y coincide con la versión enviada en formato electrónico.*
4) *Confirmo que la tesis no incurre en ningún tipo de plagio de otros autores ni de trabajos presentados por mí para la obtención de otros títulos.*

En Lugo 5 de diciembre de 2019

Fdo. Jesús María González Jartín

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

AUTORIZACIÓN DEL DIRECTOR / TUTOR DE LA TESIS

The application of the nanotechnology in the elimination of phycotoxins and mycotoxins and implementation of mass spectrometry for their detection

D. Luis Miguel Botana López
Dña. María Amparo Alfonso Rancaño
D. José Manuel Cifuentes Martínez

INFORMAN:

Que la presente tesis, se corresponde con el trabajo realizado por D. Jesús María González Jartín, bajo mi dirección, y autorizo su presentación, considerando que reúne los requisitos exigidos en el Reglamento de Estudios de Doctorado de la USC, y que como director de esta no incurre en las causas de abstención establecidas en la Ley 40/2015.

De acuerdo con el artículo 41 del Reglamento de Estudios de Doctorado, declara también que la presente tesis doctoral es idónea para ser defendida en base a la modalidad de COMPENDIO DE PUBLICACIONES, en los que la participación del doctorando/a fue decisiva para su elaboración.

La utilización de estos artículos en esta memoria, está en conocimiento de los coautores, tanto doctores como no doctores. Además, estos últimos tienen conocimiento de que ninguno de los trabajos aquí reunidos podrá ser presentado en ninguna otra tesis doctoral.

En Lugo 5 de diciembre de 2019

Fdo Luis M. Botana López	Fdo Mª Amparo Alfonso Rancaño	Fdo José Manuel Cifuentes Martínez
Director	Director	Tutor

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Acknowledgements

En primer lugar, quiero agradecer a Luis y a Mercedes por darme la oportunidad de unirme a este grupo de investigación y hacer la tesis.

A Amparo, por todo lo que he aprendido contigo, por estar ahí cuando los experimentos no salían, por saber escuchar, por tu paciencia y por tu gran dedicación.

A Chus, por abrirme las puertas de su laboratorio, por su ánimo y apoyo.

A mis compañeros, en especial a aquellos con los que más horas he pasado, Rebeca, Paula, Eva, Sandra, Jon, Lara y, sobre todo, Inés. Gracias por aguantarme quejándome todos los días y por dejar que me coma vuestros pinchos en el café. Gracias por los momentos fuera del laboratorio, los congresos, las cañas y esas cenas en las que nadie quiere llamar para reservar.

A mi familia, en especial mis tíos, por apoyarme en todas las decisiones que he tomado.

A mis amigos de la Facultad, Sonia, Laura, Paula y J, por esos días y noches en la biblioteca, y por lo bien que me lo hacíais pasar con los trabajos de clase.

A mis amigas de siempre, Patri y Ali, y a todos los nuevos que he hecho en Lugo, en especial Diego, Laia, Anita, Antia, Parga, Patri y Jorge. Gracias por hacerme la vida más fácil.

A todos los que han contribuido a esta tesis, en mayor o menor medida, muchas gracias.

Al Ministerio de Educación, Cultura y Deporte por la concesión de la ayuda para la formación de profesorado universitario (FPU), con la que fue posible la realización de esta tesis.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Utilización de la nanotecnología en la eliminación de ficotoxinas y micotoxinas e implementación de la espectrometría de masas para su detección.

RESUMEN

La presencia de contaminantes en productos destinados al consumo humano y animal ha causado una gran preocupación en los últimos años debido al mayor conocimiento de sus efectos nocivos. Por ello, asegurar la inocuidad de alimentos y piensos ha sido uno de los principales objetivos de organismos nacionales e internacionales.

Dentro de los contaminantes de la cadena alimentaria, las toxinas naturales como micotoxinas, ficotoxinas y cianotoxinas tienen una especial relevancia ya que se encuentran frecuentemente en distintas matrices y, en ocasiones, en niveles que pueden suponer un riesgo para la salud pública. Las micotoxinas son compuestos producidos por hongos filamentosos, principalmente de los géneros *Aspergillus*, *Penicillium*, *Fusarium* y *Claviceps* que pueden crecer sobre los alimentos en determinadas condiciones de humedad y temperatura. De esta forma contaminan materias primas como cereales, alimentos y piensos. Las ficotoxinas o toxinas marinas, son compuestos producidos principalmente por organismos fotosintéticos y en especial dinoflagelados de los géneros *Azadinium*, *Gambierdiscus*, *Prorocentrum*, *Dinophysis* y *Alexandrium*. Los moluscos filtran grandes cantidades de agua acumulando ficotoxinas que, de esta forma, llegan a la cadena alimentaria humana. Finalmente, las cianotoxinas son compuestos producidos por cianobacterias que se pueden acumular en ríos y lagos causando intoxicaciones por la ingesta de agua contaminada.

Los efectos perjudiciales de las toxinas son muy diversos, las ficotoxinas causan intoxicaciones agudas, mientras que las micotoxinas se relacionan con efectos tóxicos a largo plazo, aunque también pueden causar intoxicaciones agudas. Por ello, los niveles de estos contaminantes deben mantenerse en valores aceptables desde el punto de vista toxicológico en productos destinados al consumo. Con este fin, la Unión Europea (EU) ha establecido el contenido máximo de micotoxinas y toxinas marinas en distintas matrices. Además, la legislación incluye métodos oficiales y de referencia para el análisis de toxinas marinas y, en el caso de las micotoxinas, fija los criterios de funcionamiento que deben tener los métodos empleados para su análisis. Además de las toxinas reguladas, los productos alimentarios pueden contener toxinas emergentes y modificadas las cuales no son detectadas con los métodos de análisis empleados de forma rutinaria, pero suponen un riesgo para la salud pública.

En la presente tesis doctoral se han optimizado distintos métodos de cromatografía líquida acoplada a espectrometría de masas (LC-MS) para la detección de toxinas reguladas, emergentes y modificadas empleando un equipo de triple cuadrupolo (QqQ), y un equipo

híbrido de alta resolución con trampa iónica (IT) y analizador de masas de tiempo de vuelo (TOF). Además, se ha desarrollado la aplicación de la nanotecnología a la detoxificación de distintas matrices alimentarias.

En la primera parte de la tesis doctoral, se han desarrollado métodos "untarget" para la detección de toxinas, en los que se realiza un barrido completo de todas las masas presentes en la muestra. Para ello, se empleó el equipo de cromatografía de ultra alto rendimiento acoplado a un espectrómetro de masas de trampa de iones-tiempo de vuelo (UHPLC-MS-IT-TOF). La principal ventaja de este instrumento es que permite la detección de la masa exacta de los compuestos y su fragmentación en la trampa de iones. De esta forma es posible estudiar el patrón de fragmentación y obtener la fórmula molecular de los compuestos presentes en las muestras empleando un software específico que utiliza la masa exacta de las moléculas y de sus fragmentos para determinar su composición elemental.

En primer lugar, se estudió el perfil micotoxigénico de una cepa comercial de *Aspergillus ochraceus* (CBS 108.08). Para ello, se incubó sobre maíz a 24 °C durante 3 meses. A continuación, el cultivo se extrajo usando un método previamente validado para el análisis de micotoxinas, y el extracto obtenido con este método se analizó por UHPLC-MS-IT-TOT en modo scan. De esta forma se encontraron varios picos que no estaban presentes en la muestra control. Los espectros de masas mostraban el ion molecular ($[M + H]^+$) y los aductos de sodio ($[M + Na]^+$) y potasio ($[M + K]^+$). La masa exacta de los compuestos coincidía con varios análogos de la familia de las circumdatinas. Con el fin de identificarlos de forma definitiva, se estudió su patrón de fragmentación empleando como modelo un estándar de circumdatina A. En este sentido, se determinó que la principal ruta de fragmentación de este compuesto consistía en la pérdida de un grupo nitrogenado seguida por pérdidas sucesivas de grupos carbonilo. Este mismo patrón de fragmentación se obtuvo para todos los compuestos detectados en las muestras. A continuación, se empleó el software de predicción de fórmula molecular y, de esta forma, se identificaron las circumdatinas A-E, G y I. Cabe destacar que se encontró por primera vez la producción de circumdatina I por un hongo terrestre. Aunque no existen datos toxicológicos *in vivo*, la toxicidad hallada en estudios *in vitro* indica que las circumdatinas son micotoxinas. Por lo tanto, es necesario disponer de métodos que permitan su detección de forma rutinaria. Con este fin, se optimizó un método de UHPLC con detección por espectrometría de masas en tándem (UHPLC-MS/MS). El método se desarrolló teniendo en cuenta los productos de fragmentación establecidos con el IT-TOF y, como resultado, se obtuvo un método con una alta sensibilidad, ya que el límite de detección (LOD) para la circumdatina A fue de 0.19 ng/mL.

A continuación, se estudió el perfil micotoxigénico de *Fusarium sporotrichioides*. Para ello se incubó en agar de patata y dextrosa (PDA) durante 7 días a 25 °C y a continuación se extrajo con una disolución de acetonitrilo/agua/ácido acético (79:20:1, v/v/v). El extracto se analizó mediante UHPLC-MS-IT-TOF. Al buscar la masa exacta de las toxinas T-2, HT-2 y neosolaniol (NEO), se obtuvieron 5 picos. La masa exacta y los tiempos de retención de 3 de ellos permitieron su identificarlos como las toxinas T-2, HT-2 y NEO, mientras que los otros 2 podían corresponder a compuestos relacionados. Para establecer su identidad se fragmentaron los iones más intensos de los compuestos desconocidos y de las toxinas ya identificadas. En ambos casos, estos iones correspondían a los aductos de amonio ($[M+NH_4]^+$) y $[M+Na]^+$, y

como resultado de la fragmentación, se obtuvieron perdidas de ácido isovalérico, ácido acético, formaldeido y agua. Empleando el software de predicción de fórmula molecular, los compuestos se identificaron como iso-NEO y NEO monoacetato. Además, en la ruta de fragmentación se encontraron fragmentos comunes a todas las toxinas, más concretamente los iones *m/z* 245.1172 ($C_{15}H_{17}O_3$), *m/z* 215.1067 ($C_{14}H_{15}O_2$), y *m/z* 197.0961 ($C_{14}H_{13}O$). Estas masas fueron buscadas en modo scan y se obtuvieron 11 picos, 5 de ellos pertenecían a las toxinas previamente identificadas mientras que el resto eran moléculas relacionadas. Para establecer su identidad se siguió el mismo proceso y, de esta forma, se identificó el NEO 8-propionato, NEO 8-isobutirato, 3′-hidroxi T-2 toxina, 3-acetil T-2 toxina, y el α,β T-2 análogo o β, γ T-2 análogo, el cual no había sido descrito previamente como un producto natural. A continuación, basándose en los fragmentos identificados, se desarrollaron 2 métodos para detectar estos tricotecenos con instrumentos QqQ. En este sentido, se optimizó un método de UHPLC-MS/MS, y un método de escaneo del ion precursor. Este último, permite detectar cualquier análogo de este grupo de toxinas. Por lo tanto, en este trabajo se desarrolló un método para cuantificar estas toxinas con LOD inferior a 3.25 ng/mL y 2 métodos "untarget" para detectar cualquier tricoteceno del tipo A.

Fusarium foetens ha sido recientemente descrito como una nueva especie fúngica. Con el fin de estudiar su perfil micotoxigénico, un aislado se incubó en PDA durante una semana a 25 ºC. Después de este periodo, el cultivo se extrajo y se analizó por UHPLC-MS-IT-TOF. En base a la masa exacta y al tiempo de retención se identificó la producción de beauvericina (BEA). Además, en la muestra se detectó el ion *m/z* 180.1013, que podía corresponder al ácido fusárico (FA), pero las condiciones cromatográficas no eran adecuadas para la detección de esta micotoxina ya que el tiempo de retención no era reproducible. Por lo tanto, se optimizaron las condiciones de cromatografía para su análisis. A continuación, se estudió el patrón de fragmentación empleando un estándar de FA, encontrando pérdidas de agua y carbonilos. La coincidencia de la masa exacta, el tiempo de retención y el patrón de fragmentación del estándar y del compuesto detectado en el extracto permiten asegurar que *F. foetens* produce FA. Para determinar si este hongo sintetiza algún análogo del FA se buscaron compuestos que, al igual que la toxina, mostraran pérdidas de una molécula de agua en modo scan. De esta forma se detectaron 4 nuevos picos, y estudiando su patrón de fragmentación se obtuvieron las mismas perdidas que para el FA. Empleando el software de predicción de fórmula molecular, fueron identificados como ácido 10,11-dihidroxifusárico, ácido hidroxifusárico, ácido dehidrofusárico, y un análogo insaturado hidroxilado del FA. Con el fin de establecer la capacidad de *F. foetens* de producir micotoxinas de una forma más sólida, el hongo se cultivó sobre distintos medios. En todos ellos, salvo en el medio pobre en nutrientes Spezieller Nährstoffarmer agar, se encontró la producción de toxina. Por lo tanto, se ha demostrado que *F. foetens* es un hongo micotoxigénico.

Por otra parte, se estudió la incidencia de hongos del género *Fusarium* en plantas de maíz procedentes de distintas localizaciones. Se aislaron especies fúngicas de prácticamente todos los granos, hojas y tallos. A continuación, se estudió el perfil micotoxigénico de las especias detectadas con mayor frecuencia cultivando los aislados en PDA y en trigo. Después de la incubación, los cultivos fueron extraídos y analizados por UHPLC-MS-IT-TOF. En el medio

PDA se detectó una mayor variedad de micotoxinas. En general, la BEA y la moniliformina (MON) fueron las toxinas más producidas por las especies aisladas.

En la segunda parte de la tesis doctoral se desarrollaron métodos para la extracción y cuantificación de micotoxinas en matrices sólidas y líquidas. Además, se desarrollaron métodos de detección de para ficotoxinas y cianotoxinas.

La cerveza es una de las bebidas más consumidas a nivel mundial. Este producto suele estar contaminado con micotoxinas tales como el deoxinivalenol (DON) y las fumonisinas (FBs). Los métodos de detección actuales están limitados por el gran efecto matriz que presenta este producto. Por lo tanto, con el fin de desarrollar un método sensible para la cuantificación de micotoxinas en cerveza, en primer lugar, se estableció un método de UHPLC-MS/MS. Mediante el uso de estándares analíticos se optimizaron las condiciones para la detección de 23 micotoxinas. La cromatografía empleada permitió la separación de todos los compuestos con la excepción de las toxinas modificadas 3 acetil deoxinivalenol (3Ac-DON) y 15 acetil deoxinivalenol (15Ac-DON) para los cuales se obtuvo un único pico. Por lo tanto, estos análogos del DON se cuantificaron como la suma de ambos isómeros. Para establecer el disolvente de análisis se evaluaron distintas mezclas, y se determinó que el más adecuado era la mezcla de acetonitrilo/agua/ácido acético 49/50/1 (v/v/v). En estas condiciones se obtuvieron unos límites de cuantificación (LOQs) bajos, inferiores a 1 ng/mL y unas curvas de calibración con coeficientes superiores a 0.995. A continuación, se desarrolló un metodo de extracción y limpieza tipo QuEChERS para la determinación de micotoxinas en cerveza. En este caso se realiza una preconcentración de la muestra que luego es extraída con agua acidificada y acetonitrilo. Después de inducir la separación de las fases acuosa y orgánica con las sales dispersivas ($MgSO_4$ y NaCl), se realiza un proceso de limpieza adicional a la fase orgánica empleando una extracción en fase sólida (SPE) dispersiva con C18 como adsorbente. El protocolo optimizado da lugar a una muestra 1.25 veces concentrada que presenta un efecto matriz con un factor de supresión/aumento de señal que se sitúa entre el 10% del DON y el 109% de la ENNA, con valores superiores al 100% indicando aumento de señal y por debajo supresión. La recuperación del método está entre el 70% obtenido para la AFB_1 y el 115% para la toxina HT-2, cumpliendo con los criterios de análisis establecidos en la legislación europea para las toxinas reguladas. Además, para evaluar la aceptabilidad de los métodos de análisis la legislación también permite emplear criterios mínimos de funcionamiento basados en la incertidumbre de la medición. Según estos criterios, el método es apto para el análisis de todas las toxinas con la excepción de la MON.

En el campo de la alimentación animal se han desarrollado pocos protocolos que permitan el análisis de múltiples toxinas en las muestras. Por ello, hemos optimizado un método de extracción y análisis para la detección de micotoxinas reguladas, enmascaradas y emergentes en materias primas y piensos. Las condiciones de UHPLC-MS/MS habían sido optimizadas en el estudio anterior, por lo que el desarrollo del método se centró en el proceso de extracción y en la reducción del efecto matriz. En primer lugar, el método se puso a punto para maíz. La extracción se basó en la metodología QuEChERS y se realizó empleando 2.5 g de muestra, 10 mL de ácido acético (2%) y 10 mL de acetonitrilo. Además, se estudió el efecto de una limpieza adicional basada en una SPE dispersiva con C18 como adsorbente. Se determinó el efecto

matriz empleando distintos niveles de dilución de los extractos obtenidos con y sin la aplicación de SPE. En este sentido, a mayor dilución menor efecto matriz, sin embargo, diluir la muestra más de 8 veces no disminuyó el efecto matriz de forma significativa, y, en general, no hubo diferencias entre la muestra tratada y sin tratar con C18. Por lo tanto, para estudiar la recuperación del método, se empleó una muestra 8 veces diluida y sin usar la SPE dispersiva. En este sentido, la recuperación de las micotoxinas rondaba el 80%, cumpliendo con los criterios de análisis de micotoxinas marcados por la legislación. A continuación, se estudió el funcionamiento para otras matrices empleadas de forma habitual en la fabricación de piensos. El trigo, el salvado de trigo, la cebada y la soja mostraron un bajo efecto matriz, con un factor de supresión/aumento de señal que vario en torno al 70% y 120%, mientras que los granos secos de destilería con solubles (DDGS), la alfalfa y el girasol causaron la mayor supresión de señal, especialmente para DON y sus formas modificadas. La recuperación en todas estas matrices se situó en torno 80-110%, por lo que el método también cumple con los criterios de análisis de micotoxinas marcados en la legislación. Después de la validación, se analizaron 61 muestras de materias primas. Los productos más contaminados fueron los DDGS, el maíz y el trigo, aunque en todos los casos los niveles de toxina detectados fueron inferiores al máximo permitido o recomendado por la EU. Además, el método se validó para piensos de los principales animales de cría incluyendo vacas, terneros, conejos, gallinas, pollos de engorde y cerdos. En estos piensos, el efecto matriz fue ligeramente superior al de las materias primas pero la recuperación se mantuvo en el rango del 80-120% y, por lo tanto, es adecuado para su análisis. En este sentido, se estudió la presencia de micotoxinas en 14 productos encontrando una alta prevalencia de FBs, eniatinas (ENNs), DON y zearalenona (ZEN), aunque en bajas cantidades.

En la tercera parte de la tesis doctoral se estudiaron distintas estrategias para la detoxificación de alimentos y piensos, incluyendo la implementación de la nanotecnología para la eliminación de ficotoxinas, micotoxinas y cianotoxinas.

En general, existe una alta presencia de micotoxinas en los productos destinados a la alimentación animal. Con el fin de reducir su impacto, en primer lugar, se ha estudiado la capacidad de 5 materiales para eliminar micotoxinas de materias primas y piensos. Para ello, se puso en contacto material contaminado de forma natural, maíz y pienso, con esferas de distintos diámetros de vidrio y alúmina. Se mantuvieron en contacto durante 180 min empleando un agitador y al final se separaron empleando un tamiz. La cantidad de toxinas en el maíz y pienso se estudió antes y después del tratamiento. En este sentido, el empleo de esferas de vidrio de 2 mm de diámetro permite la reducción de hasta el 28% del contenido de micotoxinas de las matrices, incluyendo AFs, FBs, ZEN y tricotecenos.

Por otra parte, se determinó la capacidad de adsorción de micotoxinas de 25 partículas magnéticas nanoestructuradas de distintos tamaños, pequeño (inferior a 400 nm), mediano (15 μm) y grande (1-3 mm). Para ello, se emplearon soluciones acuosas contaminadas con una mezcla de las principales micotoxinas, DON, ZEN, fumonisin B_1 (FB_1), y AFs. Estas soluciones se incubaron con las partículas durante 180 min en agitación. En general, las partículas de tamaño pequeño, formadas de Fe_3O_4 con cubiertas de carbono, sílice o distintas proporciones de hidróxido de aluminio, fueron muy efectivas para la eliminación de FB_1, mientras que solo las partículas con cubiertas de carbono adsorbieron todas las toxinas, aunque en proporción

variable, desde un 15% de DON hasta un 94% de ZEN. Las partículas de tamaño medio están formadas por mezclas de bentonita, carbón activado, óxido de aluminio y gelatina. Las partículas sin gelatina adsorben hasta un 20% de DON y alrededor del 90% de las otras toxinas, mientras que cuando se incluye este biopolímero en su composición, la adsorción se ve muy disminuida. Las partículas de tamaño grande están formadas por alginato y combinaciones de biopolímeros, carbón activado o sales como el sulfito de sodio. Las partículas de alginato eliminaron hasta el 90% de ZEN y AFs. Cuando se añadió carbón activado a su composición, además de las toxinas anteriores, se eliminó un 54% de DON y un 71% de FB_1. En general, la sustitución de carbón activado por biopolímeros o sales disminuye la capacidad de adsorción. A continuación, la capacidad de eliminar toxina de las partículas se probó en cerveza. Para ello se escogieron las dos partículas de tamaño mediano y las dos de tamaño grande que habían mostrado una mayor adsorción de toxina de las disoluciones acuosas. En este caso, la cerveza se contaminó con una mezcla de micotoxinas DON, ZEN, FB_1, AFs (AFB_1, AFB_2, AFG_1 and AFG_2) y OTA por encima de los niveles de contaminación habituales. En esta matriz, las nanoestructuras de tamaño grande continúan teniendo una alta eficacia de detoxificación. En este sentido, las partículas formadas por mezclas de alginato y carbón activado redujeron el 20% de DON, el 50% de FB_1, el 57% de ZEN, el 80% de AFs y el 90% de OTA. Todas las partículas empleadas eran superparamagnéticas, lo que permitió separarlas de la matriz con un imán. Después del estudio de detoxificación, las toxinas fueron extraídas de las partículas empleando una mezcla de disolventes orgánicos sin ocasionarles daños estructurales. Por lo tanto, pueden ser utilizadas en sucesivos procesos de detoxificación.

Además, se evaluó la eficacia de dos partículas magnéticas para eliminar ficotoxinas y cianotoxinas de agua contaminada. Las partículas empleadas estaban formadas por Fe_3O_4, empleado como fase magnética, y carbono o sílice mesoporosa para facilitar la afinidad química con las toxinas. Inicialmente, se prepararon soluciones contaminadas con una mezcla de ficotoxinas lipofílicas o hidrofílicas, que contenían desde 10 a 200 µg/L de cada toxina. Estas soluciones se trataron con 125 mg/L de partículas. De esta forma, las cubiertas de carbono lograron reducciones cercanas al 45% de las toxinas hidrofílicas, y alrededor del 90% de las ficotoxinas lipofílicas, mientras que las partículas de sílice mostraron una menor capacidad de adsorción. A continuación, se estudió la adsorción de cada grupo de compuestos de forma independiente empleando las partículas con cubiertas de carbono. En este caso, se contaminó agua con 20 µg/L de toxinas paralizantes (PSPs), diarreicas (DSPs), azaspirácidos (AZAs), yesotoxinas (YTXs) o espirólidos (SPXs). Las disoluciones se trataron con 125 o 250 mg/L de partículas, tomando alícuotas a distintos tiempos para evaluar la adsorción a lo largo del tiempo. El uso de 250 mg/L eliminó hasta el 72% de compuestos del grupo de la saxitoxina (STXs), sin embargo, la eliminación de otras PSPs, como las gonyautoxinas (GTXs), fue inferior al 15%. La disminución de la cantidad de partículas a 125 mg/L ocasiono una reducción proporcional de la toxina eliminada de las soluciones. Por ello, la adsorción de toxinas lipofílicas se estudió empleando 125 mg/L. Después de 60 min de incubación se obtuvieron reducciones de hasta el 8.5% de las YTXs, 16% de las DSPs, 68% de los AZAs y del 71.1% de los SPXs. Por lo tanto, la capacidad de detoxificación está condicionada por la estructura de la toxina. Los compuestos que presentan grupos sulfato como las GTXs y YTXs son poco adsorbidas por las cubiertas de

carbono. A continuación, se empleó la misma cantidad de partículas con cubiertas de carbono para tratar agua contaminada con cianotoxinas. De esta forma, se obtuvieron reducciones de hasta el 81.90% de microcistinas (MCs), 29.57% de nodularina (NOD), e inferiores al 10% de anatoxina A (ATX-a) y cilindrospermopsina (CYN). De nuevo, la adsorción de estos compuestos depende de su estructura, ya que los péptidos cíclicos, MC y NOD se eliminaron en un alto porcentaje, mientras que la concentración de los alcaloides ATX-a y CYN en la disolución solo se redujo en pequeñas cantidades. Por otra parte, las toxinas marinas se adsorben en los primeros 5 minutos, mientras que las toxinas de agua dulce necesitan estar en contacto con las partículas durante 90 min para alcanzar el máximo de eliminación. Además, las toxinas se recuperan de las partículas mediante la extracción con distintos disolventes y, por ello, los materiales nanoestructurados pueden ser usadas en sucesivos ciclos de detoxificación.

En resumen, en esta tesis doctoral se han desarrollado varios métodos de extracción y análisis de toxinas de distintas matrices, y se ha estudiado la aplicación de la nanotecnología para la detoxificación de aguas y alimentos contaminados con micotoxinas, ficotoxinas y cianotoxinas.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Abbreviations and acronyms

$[M + H]^+$	Molecular ion
$[M + K]^+$	Potassium adduct
$[M + Na]^+$	Sodium adduct
$[M + NH_4]^+$	Ammonium adduct
µg	Microgramos
µM	Micromolar
15Ac-DON	15-acetyl-deoxynivalenol
1a-homoYTX	1a-homo yessotoxin
3Ac-DON	3-acetyl- deoxynivalenol
45-hydroxy-1a-homoYTX	45-hydroxy-1a-homo yessotoxin
45-hydroxyYTX	45-hydroxy yessotoxin
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFs	Aflatoxins
AOAC	Association of Official Analytical Chemists
ARfD	Acute reference dose
ASP	Amnesic shellfish poisoning
ATA	Alimentary toxic aleukia
aW	Water activity
AZA 1	Azaspiracid 1
AZA 2	Azaspiracid 2
AZA 3	Azaspiracid 3
AZA 4	Azaspiracid 4
AZA 5	Azaspiracid 5
AZAs	Azaspiracids
AZP	Azaspiracid poisoning
b. w.	Body weight
BEA	Beauvericin
C1	N-sulfocarbamoyl Gonyautoxin-2
C1	N-sulfocarbamoyl Gonyautoxin-2
C2	N-sulfocarbamoyl Gonyautoxin-3
C2	N-sulfocarbamoyl Gonyautoxin-3
C4	N-sulfocarbamoyl Gonyautoxin-4

CFP	Ciguateric fish poisoning
CIs	Cyclic imines
CTN	Citrinin
CTXs	Ciguatoxins
DA	Domoic acid
DC	Direct current voltage
dcGTX1	Decarbamoylgonyautoxin 1
dcGTX2	Decarbamoylgonyautoxin 2
dcGTX3	Decarbamoylgonyautoxin 3
dcGTX4	Decarbamoylgonyautoxin 4
dcNEO	Decarbamoylneosaxitoxin
dcSTX	Decarbamoylsaxitoxin
DDGS	Distillers dried grains with solubles
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
DON-3-Gluc	Deoxynivalenol -3-glucoside
DSP	Diarrhoeic shellfish poisoning
DTX1	Dinophysistoxin 1
DTX2	Dinophysistoxin 2
DTX3	Dinophysistoxin 3
DTXs	Dinophysistoxins
EAs	Ergot alkaloids
EC	European Commission
EFSA	European Food Safety Authority
ENNA	Enniatins A
$ENNA_1$	Enniatins A_1
ENNB	Enniatins B
$ENNB_1$	Enniatins B_1
ENNs	Enniatins
epi-DA	C5´- Domoic acid
ESI	Electrospray ionisation
EU	European Union
EU-RL-MB	European Union Reference Laboratory for marine biotoxins
FA	Fusaric acid
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FB_3	Fumonisin B_3
FBs	Fumonisins

GTXs	Gonyautoxins
GTX1	Gonyautoxin 1
GTX2	Gonyautoxin 2
GTX3	Gonyautoxin 3
GTX3	Gonyautoxin 3
GTX4	Gonyautoxin 4
GTX5	Gonyautoxin 5
GTX6	Gonyautoxin 6
GYMs	Gymnodimines
HABs	Harmful algal blooms
HPLC	High-performance liquid chromatography
IAC	Immunoaffinity columns
IARC	International Agency for Research on Cancer
IT	Quadrupole ion trap
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography–mass spectrometry
LD50	Lethal Dose 50%
LEM	Equine Leukoencephalomalacia
LOAEL	Lowest observed adverse effect level
LOD	Limit of detection
LOQ	limit of quantification
MBA	Mouse Bioassay
$MgSO_4$	Anhydrous magnesium sulphate
MON	Moniliformin
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NaCl	Sodium chloride
NEO	Neosaxitoxin
NOAEL	No observable adverse effect level
NPs	Magnetic nanoparticles
NSP	Neurotoxic shellfish poisoning
OA	Okadaic acid
OTA	Ochratoxin A
OTB	Ochratoxin B
OTB	Ochratoxin B
OTC	Ochratoxin C
PAT	Patulin
PbTXs	Brevetoxins

PCR	Polymerase chain reaction
ppm	Parts per million
PSA	Primary Secondary Amine
PSP	Paralytic shellfish poisoning
PTX1	Pectenotoxin 1
PTX11	Pectenotoxin 11
PTX2	Pectenotoxin 2
PTX3	Pectenotoxin 3
PTX4	Pectenotoxin 4
PTX6	Pectenotoxin 6
PTXs	Pectenotoxins
Q	Quadrupole
QqQ	Triple quadrupole
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
RF	Radio frequency voltage
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPs	Relative potencies
SIM	Selected-ion monitoring
SPE	Solid phase extraction
SPXs	Spirolides
SRM	Selected reaction monitoring
STX	Saxitoxin
STXs	Saxitoxins
TDI	Tolerable daily intake
TEFs	Toxic equivalency factors
TOF	Time of flight
TTX	Tetrodotoxin
TTXs	Tetrodotoxins
UHPLC	Ultra high perfomance liquid chromatography
UHPLC-MS-IT-TOF	Ultra high perfomance liquid chromatography coupled to mass spectrometry-ion trap-time-of-flight
UHPLC-MS/MS	Ultra high perfomance liquid chromatography coupled to tandem mass spectrometry
WHO	World Health Organization
YTXs	Yessotoxins
ZEN	Zearalenone
α-ZEN	α-zearalenol
β-ZEN	β-zearalenol

Index

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1 INTRODUCTION

The outbreaks of foodborne illnesses occurred in the 1990s made consumers aware of the importance of food safety. In that decade, the Single European Market was being implemented and the huge impact of food scandals put this topic at the top of the political agenda (1). After the bovine spongiform encephalopathy crisis, in 1997 the European Commission (EC) published the Green Paper on the general principles of food law in the European Union (EU). In this text, the EC established the basic objectives for Community food law including to ensure a high level of protection of public health, safety and the consumer. To achieve the goals of the new food law, it was necessary for the regulatory approach to cover the whole food chain and legislation based on scientific evidence (2). Next, the EC White Paper on Food Safety, published in 2000, proposed a series of measures to coordinate and integrate the food safety policy. The new regulation was based on covering all aspects of food products "from farm to table", harmonising control systems and implementing dialogue with consumers. In addition, the establishment of an independent European Food Authority with responsibility for the identification of food and feed hazards was envisaged (3). The following step was the approval of Regulation (EC) Nº 178/2002, known as the General Food Law, which provides a general framework to protect consumers. This regulation laid down general principles, requirements and procedures that support decisions on food and feed safety. In order to achieve a high level of health protection, food law was based on risk assessment. In 2004, the EC published the hygiene package, a series of rules on hygiene of foodstuffs (Regulation (EC) Nº 852/2004, 853/2004, and 854/2004) to merge, harmonise and simplify the previous hygiene requirements. In addition, these regulations included tools to manage food security and any food crisis (4-7).

The European Food Safety Authority (EFSA) is established as an independent authority to provide scientific advice in all fields related to food and feed safety (8). EFSA provides risk assessment procedures based on scientific studies with quantitative estimations of the probability of adverse health effects associated with exposure to toxic agents (9, 10). For the assessment elaboration, threshold doses must be calculated including the no observable adverse effect level (NOAEL) and the lowest observed adverse effect level (LOAEL). From these data, health-based guidance values are derived, including the acute reference dose (ARfD) for acute toxicity, and the tolerable daily intake (TDI) as well as the tolerable weekly intake (TWI) to characterise chronic health risks (10).

Foodborne illness can be caused by microbiological, chemical or physical hazards. Among these major issues in food safety, chemical contaminants are substances that have not been intentionally added to food or feed and can affect animal and human health after single or long-term exposure. Within this classification are included environmental contaminants, naturally occurring chemicals in plants and natural toxicants such as mycotoxins and phycotoxins. In the framework of the annual collection of analytical results on chemical contaminants in food and

feed, EFSA uses a hierarchical classification system with different levels of aggregation. In this classification, mycotoxins and phycotoxins are grouped together within the cluster of toxins as chemical contaminants. In order to protect public health, the EC has established maximum tolerances for these specific contaminants, including limits in different foods and analytical detection limits (11-13).

Toxic effects of mycotoxins and phycotoxins have been known for hundreds of years. In fact, the Bible relates several incidents that can be attributed to these compounds (14, 15). In order to avoid intoxications, many strategies have been developed. In this sense, different methods to detect the presence of mycotoxins and phycotoxins have been implemented. In this way, the consumption of contaminated food and feed should be avoided if toxins are present in unsafe amounts. On the other hand, some techniques have been developed to treat foodstuffs in order to eliminate or reduce the quantity of toxins. Due to the growing awareness of the etiological role of mycotoxins and phycotoxins in human and animal diseases, there is an increased interest in improving both detection and detoxification methods (16-18).

1.1 PHYCOTOXINS

Phycotoxins, also called marine toxins, are a large and diverse group of chemical compounds produced by phytoplankton, mainly dinoflagellates and diatoms, which rise a food-safety hazard for humans. Filtering bivalves such as mussels, oysters, clams, scallops and cockles feed on phytoplankton. When toxin-producing phytoplankton blooms occur, shellfish accumulate toxins and the subsequent consumption of contaminated products by humans leads to intoxication. Human exposure can also occur through the intake of contaminated fish, breathing aerosols, or direct skin contact (19).

Phycotoxins have been traditionally classified based on toxicological effects in paralytic shellfish poisoning (PSP), amnesic shellfish poisoning (ASP), neurotoxic shellfish poisoning, (NSP), ciguateric fish poisoning (CFP), azaspiracid poisoning (AZP) and diarrhoeic shellfish poisoning (DSP). Although this classification has been used for many years, it has fallen into disuse due to better knowledge of the chemistry and mechanism of action of phycotoxins (20). Nowadays, it is common to classify these compounds based on their chemical properties into hydrophilic, lipophilic and amphiphilic compounds. Hydrophilic toxins include domoic acid (DA), the saxitoxin group (STXs) and the tetrodotoxin group (TTXs). Lipophilic toxins include okadaic acid (OA), dinophysistoxins (DTXs), azaspiracids (AZAs), yessotoxins (YTXs), pectenotoxins (PTXs), cyclic imines (CIs), brevetoxins (PbTXs) and ciguatoxins (CTXs). Finally, the amphiphilic group include maitotoxins and palytoxins (21). Table 1 summarises the main toxins with their related syndromes.

Marine toxins have a worldwide distribution, although NSP is mainly limited to USA and New Zealand coasts, while DSP and AZP occur most frequently in Europe. Hence, regulatory limits have been established around the world (22). In the EU, maximum levels for marine biotoxins were established in Regulations (EC) Nº 853/2004 and Nº 786/2013 (6, 23, 24). In this sense, bivalve molluscs placed on the market for human consumption should not exceed 800 µg STX equivalents/kg for PSPs, 20 mg/kg for DA, 160 µg of OA equivalents/kg for the sum of OA, DTXs and PTXs, 3.75 mg of YTX equivalents/kg for YTXs, and 160 µg of AZA

equivalents/kg for AZAs. EU regulations also include reference methods for the detection of these compounds, and gives EU Member States the mandate to monitor the presence of biotoxin-forming phytoplankton (7, 25, 26).

Toxin group		Syndrome
Hydrophilic	DA	ASP
	STXs	PSP
Lipophilic	OA	DSP
	DTXs	DSP
	PbTXs	NSP
	CTXs	CFP
	AZAs	AZP

Table 1. Classification of the main phycotoxins according to their chemical properties and characteristic syndrome

1.1.1 Phytoplankton

Phytoplankton is a community of microalgae that includes cyanobacteria, diatoms, and dinoflagellates, among other microorganisms. Phytoplankton plays major roles in coastal primary production, nutrient cycling, and food web dynamics (27).

Figure 1. Red tide in Galicia

Under certain ecological conditions, algae populations may grow out of control and form dense concentrations of cells that form visible blooms which can discolour the water (Figure 1). These events are known as 'harmful algal blooms' (HABs) since negative environmental impacts occur both as a consequence of the accumulation of algal biomass, which affects marine life, or as a consequence of the production of phycotoxins. Approximately 4000 phytoplankton species cause HABs, although only 60-80 can produce toxins, of which up to 75% are dinoflagellates (28).

In the last few decades, there was an increase in locations where HABs occurs, and also in their frequency and the duration of the event. Eutrophication and climate change may be the primary causes of the increasing trend for the occurrence of HABs over the world. Climate

change was also related to the appearance of toxins in new locations, some examples of these emerging compounds include the detection of TTXs in the South of Europe, CTXs in the South of Spain, palytoxin in the Mediterranean Sea, and CIs all over Europe (29-31).

1.1.2 EU regulated phycotoxins

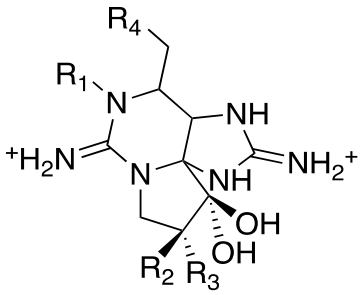
1.1.2.1 Saxitoxin group

STXs are a group of related hydrophilic compounds causing PSP in humans. They are mainly produced by dinoflagellates belonging to the genus *Alexandrium* including *A. tamarensis*, *A. minutum*, and *A. catenella*. Other STXs producing dinoflagellates are *Pyrodinium bahamense* and *Gymnodinium catenatum.* These species occur worldwide, both in tropical and moderate climate zones (32). In addition, some freshwater cyanobacteria are also able to produce STXs (33). Saxitoxin (STX), the main representative compound, was first isolated in 1957 from clams as the compound responsible for PSP, although in 1937 a bloom of *A. catenella* had been suggested as responsible for a PSP event which resulted in 102 people falling ill and six dying (34-36).

STXs are a family of tetrahydropurines containing guanidino groups, which are responsible for the high polarity of these toxins (37). To date, 57 analogues have been identified which mainly differ in the position of the hydroxyl and sulfate groups in the molecule (Figure 2). The most commonly occurring and toxic STXs can be classified based on the substituent on the R4 side chain as N-sulfocarbamoyl, decarbamoyl, or carbamoyl compounds (38). In addition, a new subclass of hydrophobic analogues (GC toxins) was described in 2003, that contains a hydroxybenzoate moiety in place of the carbamoyl group (39). STXs are stable to heat treatment up to 100 °C; however, different acid and base treatments will lead to various transformations. For instance, in acidic conditions, some toxins (C1, C2 and GTX5) can be partially hydrolysed into more toxic analogues (GTX2, GTX3, STX) (40).

These compounds are adsorbed through the buccal mucous membranes and local symptoms may appear within minutes after exposure (41). In the digestive tract, STXs are transported across the epithelium via the paracellular route. Next, these toxins are widely distributed in the body fluids, including the cerebrospinal fluid, and tissues (42, 43). In humans, STXs are removed within 24 hours, mainly by urine (44). These compounds have a high affinity for binding on site 1 of the voltage-gated sodium channels where block the ion conductance. As a consequence, STXs prevent depolarisation of the membrane leading to paralysis in peripheral nerves and skeletal muscles (45, 46).

STXs usually produce a tingling sensation around the lips, gums, and tongue. In addition, headache, dizziness, nausea, vomiting and diarrhoea are frequent. In more severe cases, weakness of the lower limbs, which progresses to the arms, legs and neck can be observed. In some cases, paralysis of respiratory muscles can occur, which results in respiratory arrest and death (37, 47). Hence, hospitalisation with mechanical ventilation is advisable in severe cases. STXs are eliminated from the blood within 24 h, leaving no organ damage or long-term effects (48).



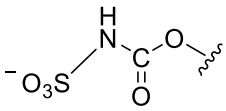
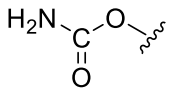
	Compound	R_1	R_2	R_3	R_4	Molecular formula
N-sulfo-carbamoyl	N-sulfocarbamoyl Gonyautoxin-2 (C1)	H	H	OSO_3^-		$C_{10}H_{17}N_7O_{11}S_2$
	N-sulfocarbamoyl Gonyautoxin-3 (C2)	H	OSO_3^-	H		$C_{10}H_{17}N_7O_{11}S_2$
	N-sulfocarbamoyl Gonyautoxin-4 (C4)	OH	OSO_3^-	H		$C_{10}H_{17}N_7O_{12}S_2$
	Gonyautoxin 5 (GTX5)	H	H	H		$C_{10}H_{17}N_7O_7S$
	Gonyautoxin 6 (GTX6)	OH	H	H		$C_{10}H_{17}N_7O_8S$
Decarbamoyl	Decarbamoylgonyautoxin 2 (dcGTX2)	H	H	OSO_3^-	OH	$C_9H_{16}N_6O_7S$
	Decarbamoylgonyautoxin 3 (dcGTX3)	H	OSO_3^-	H		$C_9H_{16}N_6O_7S$
	Decarbamoylgonyautoxin 1 (dcGTX1)	OH	H	OSO_3^-		$C_9H_{16}N_6O_8S$
	Decarbamoylgonyautoxin 4 (dcGTX4)	OH	OSO_3^-	H		$C_9H_{16}N_6O_8S$
	Decarbamoylsaxitoxin (dcSTX)	H	H	H		$C_9H_{16}N_6O_3$
	Decarbamoylneosaxitoxin (dcNEO)	OH	H	H		$C_9H_{16}N_6O_4$
Carbamoyl	Gonyautoxin 2 (GTX2)	H	H	OSO_3^-		$C_{10}H_{17}N_7O_8S$
	Gonyautoxin 3 (GTX3)	H	OSO_3^-	H		$C_{10}H_{17}N_7O_8S$
	Gonyautoxin 1 (GTX1)	OH	H	OSO_3^-		$C_{10}H_{17}N_7O_9S$
	Gonyautoxin 4 (GTX4)	OH	OSO_3^-	H		$C_{10}H_{17}N_7O_9S$
	Saxitoxin (STX)	H	H	H		$C_{10}H_{17}N_7O_4$
	Neosaxitoxin (NEO)	OH	H	H		$C_{10}H_{17}N_7O_5$

Figure 2. Structure of STXs

The toxicity of STXs is related to their structure. In this sense, toxicities, measured by the mouse bioassay and by electrophysiologic probes, increase in the following order N-sulfocarbamoyl < decarbamoyl < carbamoyl (24, 46, 49). Hence, the total toxicity of one sample can be calculated after converting the analytical results for individual toxins into STX equivalents by applying toxicity equivalency factors (TEFs). Based on data obtained in cerebellar neurons and mouse bioassay the EFSA has proposed the following TEFs: STX = 1, NEO = 1, dcSTX = 1, GTX1 = 1, GTX4 = 0.7, GTX3 = 0.6, GTX2 = 0.4, dcNEO = 0.4, GTX3 = 0.4, 11-hydroxy-STX = 0.3, dc GTX2 = 0.2, GTX5 = 0.1, GTX6 = 0.1, C2 = 0.1, and C4 = 0.1. In view of the acute toxicity, an ARfD of 0.5 µg STX equivalents/kg b.w. was established based on a LOAEL for mild symptoms in humans of 1.5 µg STX equivalents/kg b.w. (24, 46, 49).

1.1.2.2 Domoic acid

DA and its isomers are hydrophilic phycotoxins which cause ASP in humans. DA was first isolated from the macro red algae *Chondria armata* in 1958 (50). Next, DA was identified as the compound responsible for the first reported ASP outbreak in Canada in 1987 (51). Diatoms in the genus *Pseudo-nitzschia* including *P. multiseries, P. pseudodelicatissima* and *P. australis*, as well as red algae of the genus *Chondria* are the main producers of DA and its isomers (52). *Pseudo-nitzschia spp*. are distributed in sea waters of both warm and cold climates world-wide. In general, warmer sea temperatures are associated to increased DA production, although some strains like *P. seriata* produce this toxin in cooler waters (53).

DA is a cyclic amino acid with three carboxyl groups which are responsible for its water solubility and relatively high polarity (Figure 3). Several isomers of DA have been identified; however, only its major isomer C5′-*epi*-DA (epi-DA) is typically present at significant amounts in seafood (53, 54)

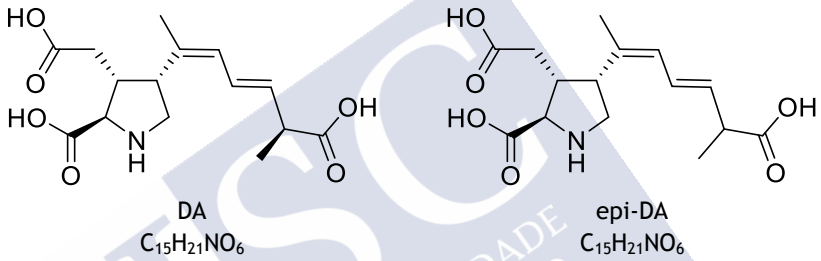


Figure 3. Structure of DA and epi-DA

Scallops, mussels, and razor clams are some of the species of most concern. However, mussels have relatively rapid depuration rates for these phycotoxins. The oral absorption of DA is low, around 7%. This compound is largely distributed in the blood compartment. After oral administration, the half-life of DA in monkeys is 11 h, and this compound is almost entirely excreted unchanged in the urine (55, 56). The primary mechanism of action of DA is via the activation of kainate receptors, which leads to an influx of extracellular ions into the cell, causing cell damage and neurotoxicity (57).

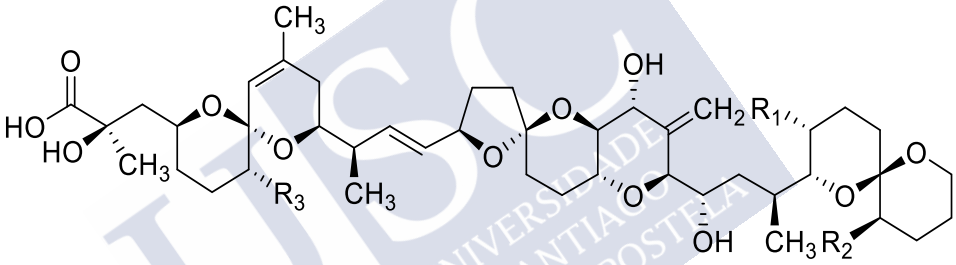
Acute intoxication, ASP, is characterised by the presence of gastrointestinal symptoms within 24 hours and neurological alterations within 48 hours. Dose-related symptoms include nausea, vomiting, abdominal cramps, diarrhoea, unstable blood pressure, headache, memory loss, convulsions, and, in some cases, death. In this last case, brain damage is characterised by neuronal necrosis and astrocytosis in the hippocampus and the amygdaloid nucleus (51, 58).

The isomers of DA show lower toxicity than the original compound, and occur at much lower concentrations; hence, the EFSA concluded that the setting of TEFs for DA analogues was not required. However, since DA can be converted to epi-DA during storage, the ARfD applies to the sum of DA and epi-DA. In this sense, an ARfD of 30 μg/kg b.w. was established based on a LOAEL of 0.9 mg/kg b.w. for mild symptoms (59).

1.1.2.3 Okadaic acid group

OA and its analogues, DTXs, are lipophilic phycotoxins that accumulate in the fatty tissue of shellfish. These compounds, responsible for DSP in humans, are produced by dinoflagellates of the genus *Prorocentrum* and *Dynophysis*, mainly *P. lima*, *P. concavum*, *D. acuta*, *D. acuminate*, and *D. fortii*. OA and DTXs are widely distributed worldwide and are especially abundant in Europe, Japan, and South America (60). OA was initially isolated in 1981 from the sponges *Halichondria okadaii* and *Halichondria melanodocia* (61). Next, the OA was identified as the causative agent of DSP after a series of events (62, 63).

Chemically, OA and its analogues dinophysistoxins 1 and 2 (DTX1 and DTX2) are polyether toxins characterised by a carboxylic acid group and three spiro-keto ring assemblies, one of which connects a five with a six-membered ring. These compounds can be esterified at the 7-hydroxy position with a range of fatty acids to form corresponding "acylated" derivatives known as dinophysistoxin 3 (DTX3) (Figure 4). It has been suggested that DTX3 is a metabolic product since it has only been found in shellfish (64). After consumption, DTX3 is hydrolysed in the gastrointestinal tract to OA, DTX1 or DTX2 (19).



Compound	R_1	R_2	R_3	Molecular formula
OA	CH_3	H	H	$C_{44}H_{68}O_{13}$
DTX1	CH_3	CH_3	H	$C_{45}H_{70}O_{13}$
DTX2	H	CH_3	H	$C_{44}H_{68}O_{13}$
DTX3	As parent	As parent	Acyl	-

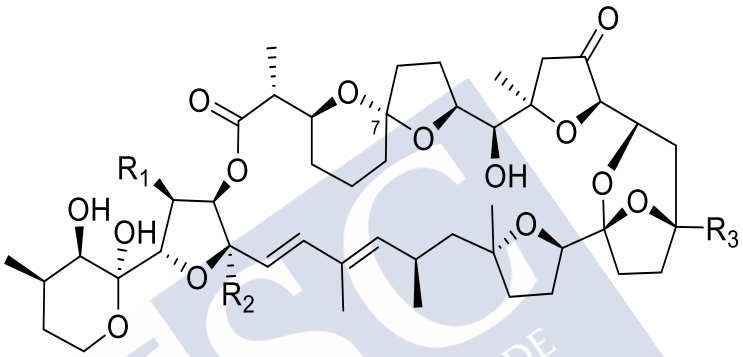
Figure 4. Structure of OA and DTXs

OA is well absorbed in the gastrointestinal tract and distributed to all internal organs. The elimination is slow, as this compound is submitted to biliary excretion, and enterohepatic circulation occurs (65, 66). OA and its analogues are potent inhibitors of the serine/threonine protein phosphatases 1 and 2A (67). These compounds cause acute diarrhoea, nausea, vomiting and abdominal pain after oral ingestion, and produce lesions in the liver, small intestine and forestomach (68-71). Also, OA was described as a tumour promoter in rodents (72). The EFSA has established TEFs for the OA group based on Lethal Dose 50% (LD50) experiments following intraperitoneal injection in mice as follows: OA = 1, DTX1 = 1 and DTX2 = 0.6. An ARfD of 0.3 μg OA equivalents/kg b.w. was calculated based on a LOAEL for human illness in the region of 0.8 μg OA equivalents/kg b.w. (73).

1.1.2.4 Pectenotoxins

PTXs are a group of lipophilic compounds produced by *Dinophysis spp*. These toxins were first isolated in 1985, and since then PTXs have been found in Australia, Japan, New Zealand and Europe. In shellfish, PTXs are always detected at the same time than OA and DTXs, being PTX2 the more frequently found analogue. To date, these compounds have not been related to intoxications in humans (47, 74, 75).

PTXs are a group of more than 15 macrocyclic polyethers whose common structural features include a spiroketal group, three oxolanes, a bicyclic ketal, and a six-membered cyclic hemiketal. The main differences between these compounds involve the stereochemistry of the spiroketal group and the level of oxidation at different radicals (Figure 5) (76, 77).



Compound	C_7	R_1	R_2	R_3	Molecular formula
PTX1	R	H	H	CH_2OH	$C_{47}H_{70}O_{15}$
PTX2	R	H	H	CH_3	$C_{47}H_{68}O_{14}$
PTX3	R	H	H	CHO	$C_{47}H_{68}O_{15}$
PTX4	S	H	H	CH_2OH	$C_{47}H_{70}O_{15}$
PTX6	R	H	H	CO_2H	$C_{47}H_{68}O_{16}$
PTX11	R	OH	H	CH_3	$C_{47}H_{70}O_{15}$

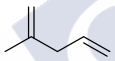
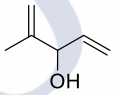
Figure 5. Structure of PTXs

Toxicokinetics and toxicity data are limited. It seems that the gastrointestinal absorption of PTXs is low, and these compounds are distributed to the liver, kidney, and heart. The toxicity is mediated by the inhibition of actin polymerisation, which disrupts the cells cytoskeleton (76, 78, 79). The oral toxicity of PTXs is low; these compounds do not induce diarrhoea, however they cause oedema at the lamina propria of villi in the jejunum-ileum, which leads to erosion (80). PTXs are acutely toxic in mice following intraperitoneal administration, causing injury to the liver and kidneys (81). Due to the limited toxicological data, EFSA has proposed a provisional TEF value of 1 for PTX1, PTX2, PTX3, PTX4, PTX6 and PTX11. An ARfD of 0.8 µg PTX2 equivalents/kg b.w was established based on a LOAEL of 250 µg/kg b.w. for intestinal toxicity of PTX2 observed in mice (77).

1.1.2.5 Yessotoxin group

YTXs are a group of lipophilic phycotoxins primarily produced by *Protoceratium reticulatum*. These compounds occur in various parts of the world including Australia, Canada, Japan, New Zealand, and Europe (47). YTX was isolated in 1987 from the digestive glands of scallops (82). Initially, YTXs were classified as DSPs but later it was demonstrated that these compounds lack diarrhoeagenic effects and therefore were considered a separate group of algal toxins. Also, there are no reports of human intoxications caused by YTXs (83).

YTXs are a group of ladder-shaped polycyclic and polyether compounds consisting of 11 contiguously transfused ether rings, an unsaturated side chain, and two sulphate esters (Figure 6). The presence of sulphate groups in the molecule made these toxins the most polar within the group of lipophilic phycotoxins. More than 90 analogues have been described to date, some of which, like YTX and 1a-homoYTX, are produced by dinoflagellates, while others such as 45-hydroxyYTX have only been isolated from shellfish (84, 85).

Compound	n	R_1	R_2	Molecular formula
YTX	1	OSO_3Na		$C_{55}H_{80}O_{21}S_2Na_2$
1a-homoYTX	2			$C_{56}H_{82}O_{21}S_2Na_2$
45-hydroxyYTX	1	OSO_3Na		$C_{55}H_{82}O_{22}S_2Na_2$
45-hydroxy-1a-homoYTX	2			$C_{56}H_{84}O_{22}S_2Na_2$

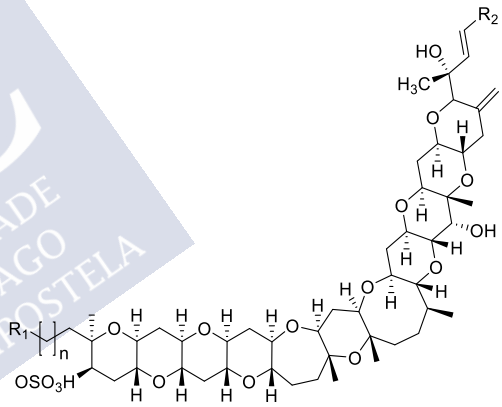


Figure 6. Structure of YTXs.
n=number of methyl groups in the molecule

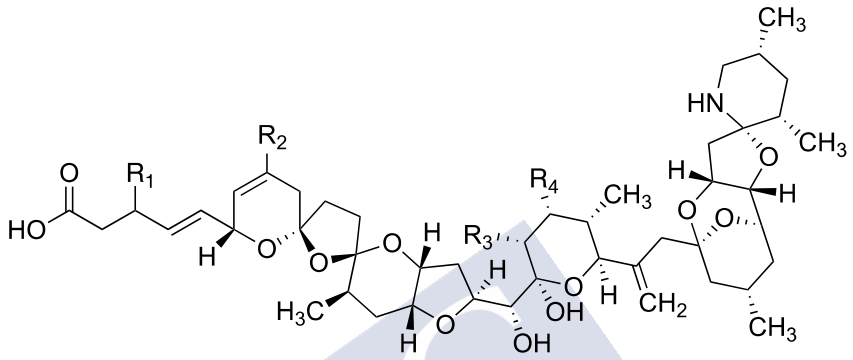
YTXs are poorly absorbed after oral intake, and most of the toxin is recovered from the lower intestine and faeces. These compounds modulate phosphodiesterases activity and calcium movements, inducing different types of cell death in several culture lines (85-88). YTXs are lethal after intraperitoneal injection to mice but not after single or repeated oral administration. The EFSA has established the following TEFs: YTX (TEF = 1), 1a-homoYTX (TEF = 1), 45-hydroxyYTX (TEF = 1), 45-hydroxy-1a-homoYTX (TEF = 0.5). Although the oral toxicity is not well defined, an ARfD of 25 µg YTX equivalents/kg b.w was established based on a NOAEL of 5 mg/kg b.w. for acute toxicity (89).

1.1.2.6 Azaspiracids

AZAs are a group of approximately 50 analogues causing AZP. Among them, AZA1, AZA2, and AZA3 are the most relevant in terms of occurrence and toxicity. These compounds are produced by dinoflagellates, mainly *Azadinium spinosum*, although other species including *A. poporum*, *A. dexteroporum* and *Amphidoma languida* can produce these toxins (90, 91). In

recent years, the presence of AZAs in shellfish has been reported worldwide, and especially in regions of Western Europe, Northern Africa, China and North America (92). The AZA1 was first isolated in 1998 from mussels cultivated in Ireland. This toxin was related to an outbreak of human illness that occurred in the Netherlands in 1995 (93).

Chemically, these phycotoxins are nitrogen-containing polyether compounds comprising a unique tri-spiro ring assembly having a heterocyclic amine and an aliphatic carboxylic acid moiety (Figure 7). These toxins were called AZAs because of their spiro ring assemblies (94).



Compound	R1	R2	R3	R4	Molecular formula
AZA1	H	H	CH_3	H	$C_{47}H_{71}NO_{12}$
AZA2	H	CH_3	CH_3	H	$C_{48}H_{73}NO_{12}$
AZA3	H	H	H	H	$C_{46}H_{69}NO_{12}$

Figure 7. Structure of AZAs

Mussels and oysters are the shellfish that accumulate higher amounts of AZAs (95). These compounds are absorbed from the gastrointestinal tract in a dose-dependent manner and are widely distributed. The highest concentrations are detected in the intestine, liver, kidneys, spleen and lungs, while only trace amounts are reported in the brain (96, 97). The biological target of these compounds is still unknown, although several signal transduction pathways are affected (98, 99). The gastrointestinal tract is the primarily affected organ, the intoxication causes diverse symptoms including nausea, vomiting, diarrhoea and stomach cramps. Cardiotoxicity, neurotoxicity, liver alterations and necrotic lymphocytes in the lymphoid organs were also observed after exposure to AZAs (97, 100).

The EFSA has established the following TEFs based on the limited toxicological information for these groups of compounds AZA1 = 1, AZA2 = 1.8, AZA3 = 1.4, AZA4 = 0.4, and AZA5 = 0.2. Besides, an ARfD of 0.2 µg AZA1 equivalents/kg b.w was calculated based on a LOAEL of 1.9 µg AZA1 equivalents/kg b.w. for gastrointestinal symptoms in humans (101).

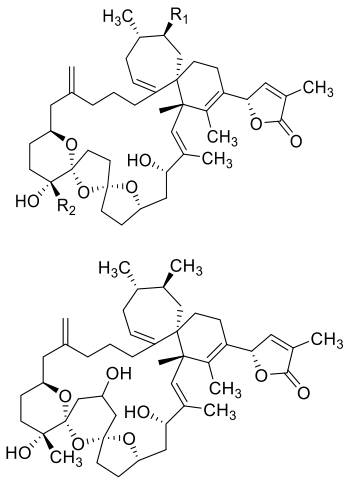
1.1.3 Emerging phycotoxins

Geographic ranges of phytoplankton are controlled by sea-surface temperatures. Climate change is increasing global ocean temperatures and, as consequence, the distribution of phytoplankton is also shifting (102). For instance, *Gambierdiscus spp.* endemic of tropical and subtropical marine areas, have been recently detected in Cabo Verde and Canary Islands (Macaronesia, Eastern Atlantic Ocean waters), Cameroon, Greece (Mediterranean Sea) (103). *Karenia brevis*, known to produce PbTXs, is characteristic from the Gulf of Mexico, the Southeast US coast and New Zealand, while *Karenia mikimotoi* is common in European waters (104). Therefore, toxins from tropical waters can emerge in northern areas where they have not been previously detected contaminating vectors that may reach the human food chain. In this way, TTXs, CTXs and palytoxin have been detected in marine products collected in the EU coasts, while PbTXs were not still reported (31, 104). Although there are no regulatory limits for these emerging toxins, legislation requires that no fish products containing CTXs should be placed on the market. Similarly, in the case of TTXs, fishery products derived from poisonous fish of the families *Tetraodontidae*, *Molidae*, *Diodontidae* and *Canthigasteridae* should not be on the market (6). However, no legislation about palytoxin presence or detection has been published so far, although this is the most toxic natural compound (105).

TTX is the causative agent responsible for pufferfish/fugu poisoning, frequently reported in Japan. TTX and its analogues are sodium channel blockers that affects both action potential generation and impulse conduction. In severe cases, death may occur due to respiratory failure and/or cardiac arrhythmias. In 2008, an intoxication with TTX occurred in Spain, since then low levels of this toxin have been detected in gastropods and bivalves from European waters (106-108). CTXs cause CFP, which is characterised by gastrointestinal, neurological and cardiovascular effects. These toxins are found in fish mainly in Pacific, Caribbean and Indian Ocean regions. Although there are very few occurrence data in Europe, a CFP case was confirmed after consumption of fish caught in the Canary Islands. In addition, CTXs were identified in fish from Madeira (109-111).

The group of emerging toxins also includes new toxins recently described. In this sense, the group of CIs were described in the 90's during a routine monitoring of lipophilic toxins in Canada and New Zealand (112). These toxins are now frequently detected across Europe, although they are not regulated (113). The largest group of CIs are the SPXs (Figure 8). These compounds occur in bivalve molluscs from several parts of the world and show high acute toxicity in mice upon intraperitoneal injection. However, no adverse effects in humans have been reported (114).

CIs have a common structural feature, a hexa- or heptacyclic imine ring, which is believed to contribute substantially to the bioactivity of these compounds. The toxicity of CIs is mediated by their ability to binds and block acetylcholine receptors in the central and peripheral nervous systems, leading to systemic neurotoxicity, including respiratory paralysis (19, 115).



Compound	Molecular formula	R_1	R_2
13-desmethyl SPX C	$C_{42}H_{62}NO_7$	CH_3	CH_3
13,19-didesmethyl SPX C	$C_{41}H_{60}NO_7$	CH_3	H

Compound	Molecular formula
20-methyl SPX G	$C_{43}H_{64}NO_7$

Figure 8. Structure of representative SPXs

PbTXs are polyether ladder-shaped compounds that cause significant mortalities of fish and other aquatic animals. These toxins are grouped in types A and B based on their backbones. PbTXs bind to voltage-gated sodium channels causing the persistent activation of neuronal, muscle and cardiac cells. Symptoms usually include nausea, vomiting, diarrhoea, paraesthesia, cramps, bronchoconstriction, paralysis, seizures, coma, and death (104).

CIs are extracted with the same procedure as regulated lipophilic toxins, so it is usual to include them in monitoring programs, although they are not regulated. On the other hand, it is not common to study the presence of PbTXs, TTXs or CTXs and, as a result, there is little data about their incidence in the EU. Therefore, in order to avoid public health impacts, there is a need for adequate monitoring programs, and optimizing effective methods of analysis for all of these emerging toxins (116)

1.1.4 Other aquatic toxins

Cyanobacteria are a diverse group of prokaryotes that inhabits freshwater reservoirs throughout the world. Several cyanobacteria species produce toxic metabolites called cyanotoxins or freshwater toxins (117). Depending on the mechanism of toxicity, cyanotoxins are classified as hepatotoxins (microcystins (MCs), nodularins and cylindrospermopsins), neurotoxins (anatoxin-a and analogues, STXs and ß-N-methylamino-L-alanine) and dermatotoxins (aplysiatoxins and lyngbyatoxins) (Figure 9). MCs are the largest group of cyanotoxins which include 100 analogues, some of the most relevant are microcystin LR (MC-LR) and microcystin (MC-RR) (118, 119).

Humans are mainly exposed to cyanotoxins though the consumption of contaminated drinking water or food such as fish, crops, food supplements based on algae, or items of animal origins, following the use of contaminated water for irrigation or in farming activities (119-121). MC-LR is the most toxic cyanotoxin. To protect consumers, the WHO proposed a limit of 1 µg/L of MC-LR in drinking water and a TDI of 0.04 µg/kg bw (122).

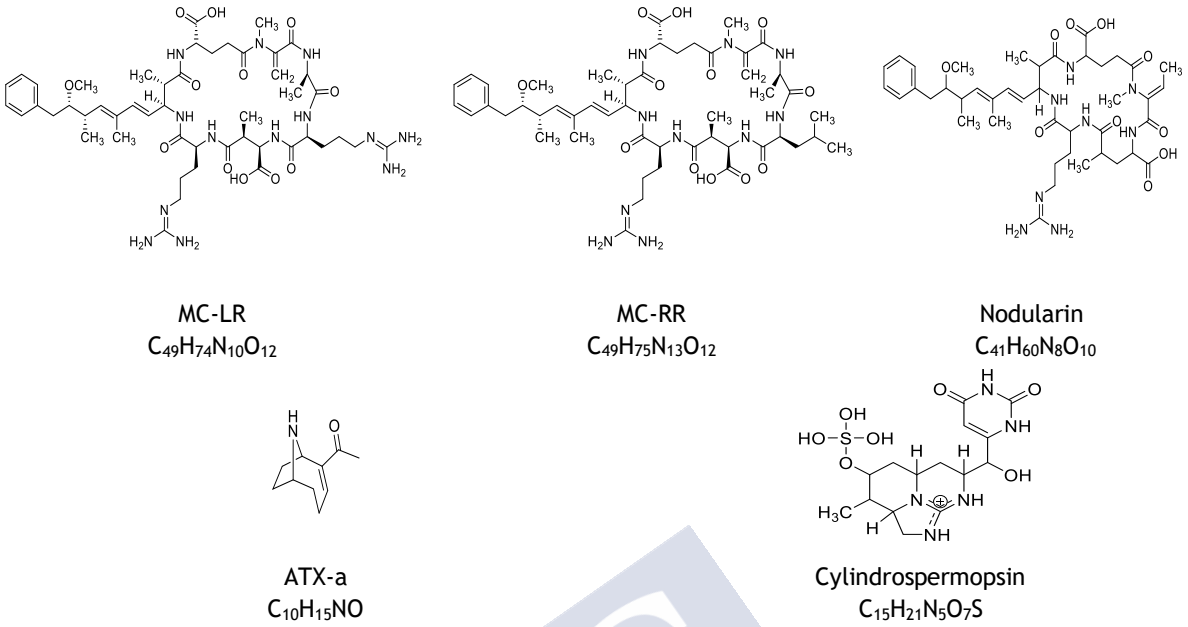


Figure 9. Structure of representative cyanotoxins

The presence of cyanotoxins should be controlled, although no universal threshold levels for all compounds have been established. However, legal regulations have been laid down in several countries (123-125).

1.2 MYCOTOXINS

Mycotoxins are low-molecular-weight secondary metabolites produced by filamentous fungi, which are toxic to humans and other vertebrates. Also, many mycotoxins are toxic to invertebrates, plants, and microorganisms. The term mycotoxin was proposed in the 1960s following the death of 100,000 turkey pouts in England due to the consumption of peanut contaminated with aflatoxins (AFs). After this veterinary crisis, the occurrence of other toxic fungal metabolites started to be studied. Nowadays, more than 400 compounds are recognised as mycotoxins, of which about 20 can be naturally present in food and feeds at significant levels. These toxins are mainly produced by species of *Fusarium*, *Aspergillus*, and *Penicillium* (126).

The presence of mycotoxins in food and feed causes a health risk for animals and humans. Hence, maximum levels in food have been legislated in many countries (127). In the EU, the Regulation (EC) Nº 1881/2006 establish maximum levels for AFs, ochratoxin A (OTA), patulin (PAT), deoxynivalenol (DON), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), and citrinin (CTN). In addition, there are maximum levels of ergot sclerotia (*Claviceps purpurea*) for unprocessed cereals, and indicative levels for T-2 and HT-2 toxins (128-131). Animal feed legislation include maximum levels for aflatoxin B_1 (AFB_1) and ergot sclerotia, as well as guidance values for DON, ZEN, OTA, fumonisins (FBs), T-2 and HT-2 toxin (132-134). Table 2 summarises maximum levels for mycotoxins in the EU.

Mycotoxin	Food commodity	Limits for food (µg/Kg)	Guidance values for feed (µg/Kg)
AFs	Cereal grains, peanut, pistachio, figs, almond, ground nuts, tree nuts, spices	0.1-12 (AFB_1) 4-15 (Sum of AFs)	5-20 (AFB_1)*
AFM_1	Milk, and milk products	0.025-0.05	-
CTN	Food supplements based on rice fermented	2,000	-
DON	Cereal grains	200-1,750	900-12,000
FB_1, FB_2	Maize, sorghum, asparagus	200-4,000	5,000-60,000
OTA	Barley, oats, grapes, wine, cocoa, coffee beans, cheese, rice	0.5-80	10-250
PAT	Apples, products derived from apples, pears, cherries	10-50	-
T-2 and HT-2	All cereal grains	50-1,000	500-2,000
ZEN	Cereal grains	20-400	100-3,000

Table 2. Major mycotoxins, affected commodities and range of maximum levels in EU. *In the case of feed, there are legal limits for AFB_1, values for the rest of mycotoxins are recommendations. Aflatoxin M_1 (AFM_1).

Exposure to fungal toxins is not restricted to regulated mycotoxins; these compounds can be modified by fungi, plant or animal metabolism, which leads to products with potential toxicity that are not considered in the legislation. These products have frequently been called masked, bound, conjugated or hidden mycotoxins, but these terms have been used inconsistently. The term "modified mycotoxins" was introduced to describe all types of mycotoxin modifications and was recently adopted by the EFSA in a scientific opinion about the modified forms of certain mycotoxins (135). In this sense, a systematic mycotoxin classification in four levels has been proposed to avoid misidentifications (Figure 10) (136). In the first level, three groups have been included: free, matrix-associated and modified mycotoxins. Free mycotoxins are the unmodified basic compounds formed as secondary metabolites in fungus. Matrix-associated mycotoxins are compounds that are trapped, dissolved or covalently bound to the matrix. While modified mycotoxins include biologically and chemically modified compounds. Biologically modified compounds are obtained after metabolism reactions of natural compounds, either in animals, plants or fungi, while chemically modified compounds are obtained under thermal, chemical or light conditions. Although this is a clear classification, some compounds can be defined in several categories. In any case, "modified mycotoxins" is a wide term where masked or conjugated compounds are included, while the term "masked mycotoxins" is reserved for metabolites produced in plants. Many modified mycotoxins have been recently discovered but others remain unknown. The most relevant are acetylated derivatives of DON, 3-acetyl-DON (3Ac-DON) and 15-acetyl-DON (15Ac-DON), phase I metabolites of ZEN α-zearalenol (α-ZEN) and β-zearalenol (β-ZEN), and the glucose conjugates DON-3-glucoside (DON-3-Gluc) and ZEN-14-glucoside. The lack of toxicological studies for these compounds, together with the hydrolysis that these toxins can suffer during the digestion process, presents a real hazard for public health (137, 138).

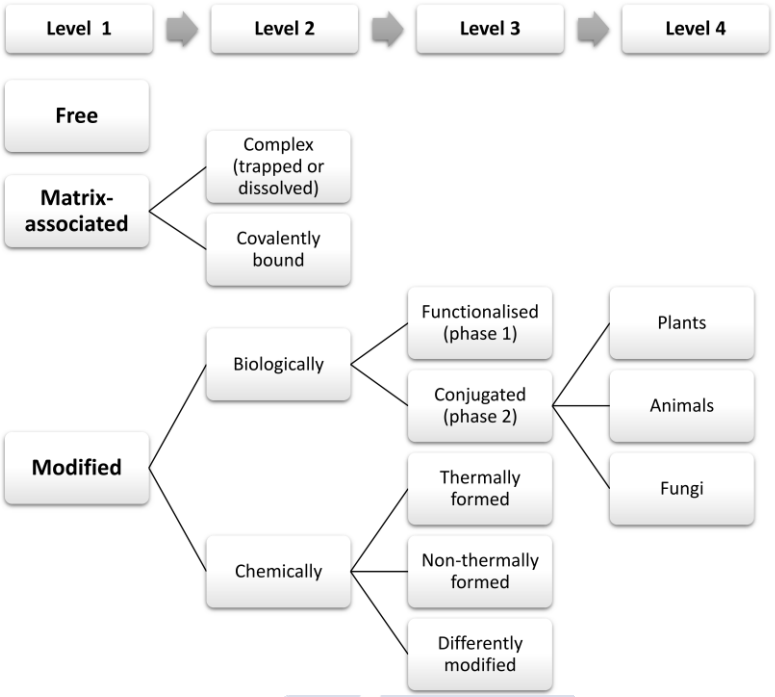


Figure 10. Systematic definition of mycotoxins. Adapted from Rychlik et al. (136)

The term "emerging mycotoxins" began to be widely used in 2008 to deal with toxic secondary metabolites that are not regulated or routinely determined, and for which the evidence of their incidence was rapidly increasing. Initially, this term was used for fusaproliferin, beauvericin (BEA), enniatins (ENNs), and moniliformin (MON). However, other metabolites such as sterigmatocystin, mycophenolic acid, alternariol or tenuazonic acid would fall into this category (139, 140).

1.2.1 Fungi

The kingdom of fungi comprises a group of organisms that are divided into yeasts and filamentous fungi or moulds. They are heterotrophic organisms, since they feed on organic matter, and can be classified as biotrophs, necrotrophs and saprotrophs based on their lifestyle and infection strategy. Biotrophic fungi live as obligate parasites that obtain nutrients from living host tissues, while saprotrophs and necrotrophs feed on dead tissues. In this sense, necrotrophs infect living plants and kill host cells to get the nutrients (141). Fungi are ubiquitous organisms, with activity and colonisation levels that are determined by environmental conditions such as temperature and the availability of nutrients (142).

Ascomycota is the largest phylum of Fungi, with more than sixty thousand species (143). Within this phylum are the main mycotoxigenic species, which belong to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps*. Fungal contamination can occur from pre-harvest to post-harvest stages, and fungi can be classified according to their predominance at each stage. *Alternaria* and *Fusarium* are field fungi, since they colonise crops in the field before harvesting. However, they can still produce mycotoxins before or just after harvesting. Storage fungi include species of *Aspergillus* and *Penicillium*, which contaminate

crops during or after harvesting, and grow throughout storage under favourable conditions (144). Mycotoxigenic fungi live in a wide range of conditions, generally between 10 and 40ºC, in a pH range of 4-8, and with water activity (a_w) greater than 0.70. Hence, fungi can be found worldwide in solid and liquid matrices. In this sense, *Aspergillus* and *Penicillium* grow at low a_w and high temperatures, while *Fusarium* species require high a_w and temperate temperatures (145).

Mycotoxins are considered secondary metabolites because they are not essential for fungal growth and are metabolic intermediates. These compounds are found as a differentiation product in restricted taxonomic groups. However, their functions have not been clearly established. Mycotoxins may be involved in the elimination of competing microorganisms, and in the invasion of host tissues by parasitic fungi (146, 147). These compounds, like many other secondary metabolites, are mainly produced during the sporulation, and it was proposed that this process is regulated by the G-protein signalling pathway (148).

Morphological characteristics of fungi have been traditionally used for their recognition. However, molecular techniques have become essential for the identification of fungal species. To perform molecular analysis, fungi must be first isolated from contaminated commodities. Different culture media can be employed for this purpose, the most common of which is potato dextrose agar (PDA), a general medium which allows the growth of a wide range of fungi. On the other hand, selective media such as Komada or Czapek yeast agar (CYA) can be used for the isolation of certain genera, particularly *Fusarium* or *Aspergillus* and *Penicillium*, respectively. In addition, antibiotics can be added to the media to inhibit bacterial growth. Several fungal colonies can be found in one sample; therefore, it is necessary to subculture at several levels until monosporic cultures are obtained (Figure 11) (149).

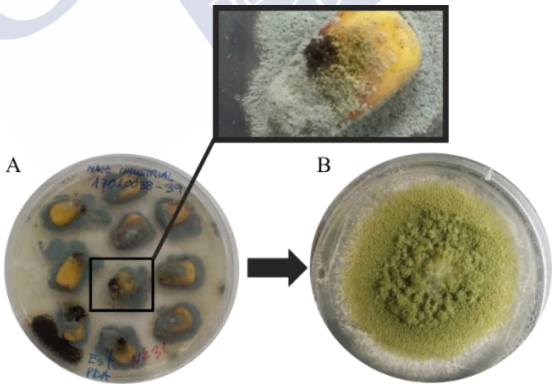


Figure 11. Isolation of mycotoxigenic fungi. Different species growing from maize kernels (A). Monosporic isolate obtained after several subculture (B)

The morphological identification is based on characters observed in different culture media such as colony morphology (shape and size), pigmentation, growth rates, and microscopic characteristics (Figure 12) (150). These phenotypic characteristics were employed until the 1990s for the identification of *Aspergillus, Fusarium* and *Penicillium* species. However, traditional phenotypic identification has low specificity for species-level discrimination.

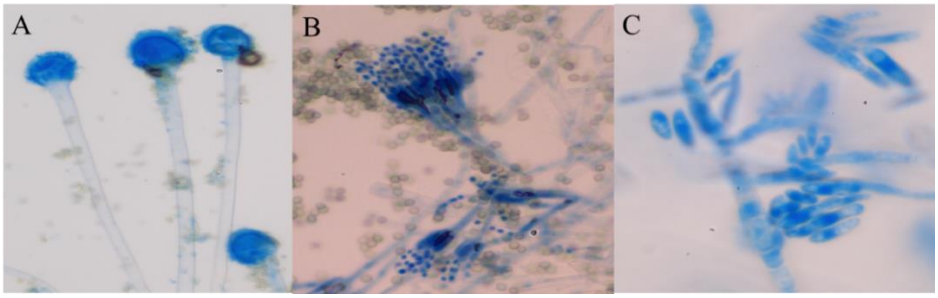


Figure 12. Microscopic observation of fungi stained with cotton blue. *A. flavus* (A), *P. purpurogenum* (B) and *F. verticillioides* (C).

Nowadays, molecular techniques are employed to complement morphological methods. To perform molecular and phylogenetic analysis, fungal deoxyribonucleic acid (DNA) is first extracted and then two or more loci are amplified by polymerase chain reaction (PCR). Next, PCR products are separated by electrophoresis and sequenced to construct gene genealogies. The obtained sequences are subsequently compared with databases. Finally, the phylogenetic approach identifies fungal species based on the concordance of multiple nucleotide sequences which are employed to construct and study phylogenetic trees (151). In addition to species identification, molecular techniques can be employed to study mycotoxin pathway gene sequences that allow the toxigenic potential of fungal species to be identified. For instance, these studies can be used to identify DON or nivalenol chemotypes of *Fusarium* species (152).

Chemotaxonomy is other approach to fungi identification; this technique is based on establishing the profiles of secondary metabolites, including mycotoxins, produced by fungal species. Some of these compounds are produced by a single species and, in general, all species produce a unique combination of secondary metabolites. Therefore, chemotaxonomy can be used together with other information such as phenotypic and physiological data to identify and classify fungi (153, 154).

1.2.2 EU regulated mycotoxins

1.2.2.1 Aflatoxins

AFs are a group of about 18 related compounds produced by *Aspergillus* species, predominantly *A. flavus* and *A. parasiticus*. These compounds were first isolated in the 1960s after the outbreak of an unknown disease which caused the death of 100,000 turkeys in England. This condition was called Turkey "X" disease and was eventually attributed to the consumption of contaminated peanut meal (155).

A. flavus is a ubiquitous contaminant present in aerial parts of plants, while *A. parasiticus* is more frequently detected in soils. These species are abundant in warm and humid areas, and hence are mainly found in tropical and subtropical regions of the world. However, the four major naturally occurring AFs (AFB_1, AFB_2, AFG_1 and AFG_2) have a worldwide occurrence in foodstuffs such as nuts, spices, cereals, oils, fruits, vegetables or meat (156). In fact, AFs are the group of mycotoxins that cause a higher number of border rejections in the EU. AFM_1 is

the hydroxylated metabolite of AFB_1 that can be found in the milk of animals that have ingested feed contaminated with AFB_1 (157).

AFs are difuranocoumarins composed of two furan rings joined to a coumarin moiety (Figure 13). These compounds are classified into two groups based on their chemical structure. The difurocoumarocyclopentenone group comprises, AFB_1, AFB_2 and AFM_1, among others, while the difurocoumarolactone group includes AFG_1 and AFG_2. These toxins are related, since AFB_2 and AFG_2 are dihydroxy derivatives of AFB_1 and AFG_1, while aflatoxin M_1 is the 4-hydroxy AFB_1 (158).

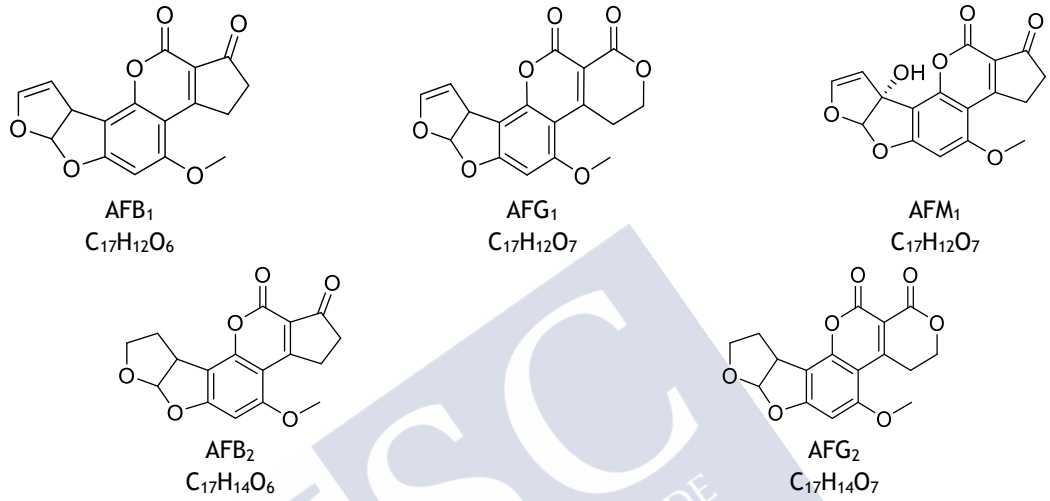


Figure 13. Structures of AFs

Humans and animals are mainly exposed to AFs through the ingestion of contaminated food and feed. These lipophilic compounds are readily absorbed from the gastrointestinal tract into the blood stream. Then, AFs are distributed to various body organs, but primarily the liver, where they are biotransformed through hydroxylation, demethylation and epoxidation (159). These reactions are catalysed by cytochrome P450, leading to the formation of a series of metabolites. One of these metabolites, AF-8,9-epoxide, binds to proteins, causing acute toxicity, aflatoxicosis, or to DNA, and induces cancer. This metabolite occurs in endo and exo forms with different activities, being the exo form (AF-exo-8,9-epoxide) the most reactive. Their formation is determined by the presence of a double bond at the 8,9-position in the precursor AFs, as in AFB_1, AFG_1 and AFM_1. Nonetheless, AFB_1 presents a greater degree of epoxidation, and is therefore the most toxic compound. Metabolites obtained by biotransformation are excreted in bile, urine, milk and eggs (160, 161).

There have been sporadic reports of human aflatoxicosis. The latest outbreak happened in Kenya in 2004 and caused the death of 125 people (162). Acute aflatoxicosis is caused by the intake of large doses of AFs, which result in damage to the liver, usually through liver cirrhosis. In this sense, bile duct proliferation, hepatocyte necrosis, and early fibrosis of the liver occur. In addition, hepatic encephalopathy can happen because of liver damage. Other lesions include haemorrhage into the gastrointestinal tract, and renal lesions in the proximal tubules and glomerular spaces (163). Chronic exposure to sublethal doses of AFs leads to decreased protein synthesis, nutritional alterations, thymic aplasia and the suppression of cell-mediated immune

responses (164). Long-term exposure causes cancer in many animal species. These compounds are considered by the International Agency for Research on Cancer (IARC) as carcinogenic in humans. AFB_1, AFG_1 and AFM_1 cause hepatocellular carcinoma. In addition, these mycotoxins have been related to tumours in other organs such as the kidney or colon. The carcinogenicity is mediated by a genotoxic mechanism; the AF-exo-8,9-epoxide metabolite binds to DNA, which lead to mutations in p53 tumour suppressor gene, resulting in carcinogenesis (165, 166). A TDI cannot be defined for AFs since, due to the carcinogenicity of these compounds, exposure at any level is considered unsafe. However, the World Health Organisation (WHO) has proposed a provisional maximum TDI of 1 ng/kg b.w. for AFB_1 (167).

1.2.2.2 Trichothecenes

Trichothecenes are a group of related compounds which include more than 180 analogues produced by diverse fungi. These mycotoxins were discovered in the 1940s during a screening program for antifungal agents. However, it was not until the 1980s that trichothecenes were related to diseases including the alimentary toxic aleukia (ATA) in the Soviet Union and red-mould disease in Japan (168-170).

Fusarium species are the main producers of trichothecenes; this plant pathogenic fungus is found worldwide in cereals such as wheat and maize, especially in northern temperate regions. Other genera of fungi such as *Stachybotrys*, *Trichoderma*, *Trichothecium*, *Cephalosporium*, and *Cylindrocarpon* can also produce some of these compounds (147, 171).

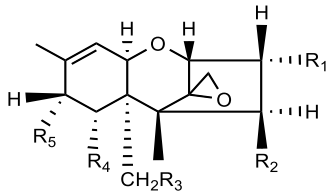
Trichothecenes are sesquiterpenes that share a tricyclic nucleus, known as trichothecene, with a spiro-epoxide group at C-12 and C-13 and an olefinic double bond between C-9 and C-10, which are essential for their toxicity. These compounds are divided into four groups (types A–D) according to their structure (Figure 14). Type A trichothecenes have an esterified or free hydroxyl group at C-8 (R_5 in Figure 14), or an unsubstituted C-8; the most relevant compounds within this group are T-2 toxin, HT-2 toxin and neosolaniol (NEO). Type B trichothecenes have a keto group at C-8 and include nivalenol, DON and its modified forms 3-AcDON, 15-AcDON and DON-3-Gluc (172). Type C trichothecenes have a second epoxide, while type D trichothecenes incorporate a macrocyclic ring. These last two groups of mycotoxins are less common than the others and are not produced by *Fusarium* species (173).

Trichothecenes have related toxicokinetic properties, although there are some variations depending on the structure and the animal species. The absorption, distribution and excretion of type-A trichothecenes is rapid (174). These mycotoxins are metabolised in the liver and other tissues through hydroxylation, de-epoxidation, acetylation and conjugation. The obtained products are excreted in urine and faeces, including the HT-2 toxin, which is the main product obtained from T-2 metabolism (175). The oral absorption of type-B trichothecenes is high in monogastric animals such as swine and rodents. For instance, up to 82% of DON reaches the systemic circulation in pigs. Then, this toxin is extensively biotransformed to glucuronide conjugates in the liver, which are subsequently excreted in urine and faeces (176, 177).

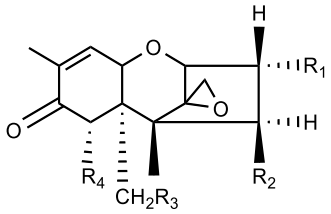
In general, trichothecenes bind to the 60_S subunit of ribosomes, this binding explains most of their toxicity. At the cellular level, trichothecenes show multiple effects such as the inhibition

of protein, ribonucleic acid (RNA) and DNA synthesis, the impairment of mitochondria function, the alteration of membrane structure, the activation of cytokines and cell death (172).

Type A trichothecenes



Type B trichothecenes



Type A trichothecenes

Toxin	R_1	R_2	R_3	R_4	R_5	Molecular formula
T-2 toxin	OH	$OCOCH_3$	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$	$C_{24}H_{34}O_9$
HT-2 toxin	OH	OH	$OCOCH_3$	H	$OCOCH_2CH(CH_3)_2$	$C_{22}H_{32}O_8$
NEO	OH	$OCOCH_3$	$OCOCH_3$	H	OH	$C_{19}H_{26}O_8$

Type B trichothecenes

Toxin	R_1	R_2	R_3	R_4	Molecular formula
DON	OH	H	OH	OH	$C_{15}H_{20}O_6$
3-AcDON	$OCOCH_3$	H	OH	OH	$C_{17}H_{22}O_7$
15-AcDON	OH	H	$OCOCH_3$	OH	$C_{17}H_{22}O_7$
DON-3Gluc	$C_6H_{11}O_6$	H	OH	OH	$C_{21}H_{30}O_{11}$
Nivalenol	OH	$OCOCH_3$	$OCOCH_3$	H	$C_{15}H_{20}O_7$

Figure 14. Structures of trichothecenes type A and B

Type-A trichothecenes inhibit haematopoiesis by apoptosis and disrupt the differentiation of monocytes into macrophages and dendritic cells (178). Acute intoxication with type-A trichothecenes, mainly T-2 toxin, leads to the development of ATA. This mycotoxicosis is characterised by leukopenia, agranulocytosis, exhaustion of the bone marrow, necrotic angina and death (179). An outbreak of ATA caused the death of thousands of people in Russia during World War II in the 1940s (180). Chronic exposure to type-A trichothecenes is associated with anorexia, reduced body weight gain and lesions in the upper digestive tract (181).

Type-B trichothecenes produce gastrointestinal tract toxicity. Acute intoxication with DON leads to anorexia and emesis; because of that, this compound is also known as vomitoxin. Chronic exposure to low concentrations of DON produces feed refusal and weight-gain suppression. Swine and other susceptible animals exposed to high doses of type-B trichothecenes during prolonged periods have developed lesions in the gastrointestinal tract and lymphoid tissues (171, 176). The EFSA established a TDI of 1 μg/kg b.w. for DON based on a NOAEL of 100 μg/kg b.w. in mice (182). Besides, a TDI of 100 ng/kg b.w. was calculated for the sum of T-2 and HT-2 toxins (183).

1.2.2.3 Zearalenone

ZEN, previously known as F-2 toxin, is a non-steroidal estrogenic mycotoxin, mainly produced by *F. graminearum* and *F. culmorum*, which are common soil fungi in temperate and warm regions. This mycotoxin can be found in cereal crops such as maize, wheat, barley, oats or rice (184). ZEN was isolated in 1962 from a *F. graminearum* strain obtained from the feed of pigs with vulvar and mammary enlargement (185, 186).

Chemically, ZEN consists of a resorcinol moiety fused with a 14-membered macrocyclic lactone, which includes a trans double bond, a ketone and a methyl branch (Figure 15). The reduction of the keto group to an alcoholic hydroxyl group leads to the stereoisomers α-ZEN and β-ZEN (187).

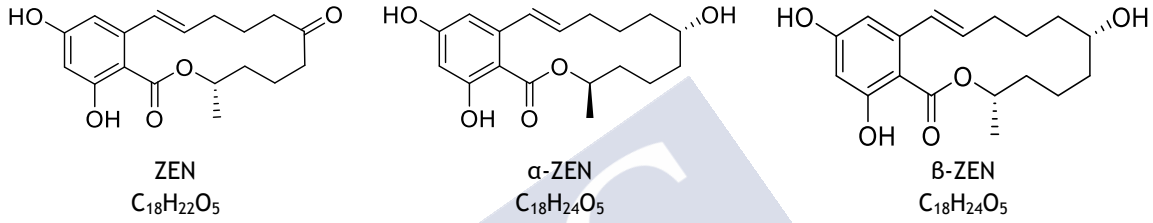


Figure 15. Structure of ZEN and its modified forms.

ZEN is poorly absorbed into the systemic circulation, with an absolute oral bioavailability of 2.7% due to an extensive pre-systemic metabolism (188). This compound is biotransformed in the liver and intestines to α-ZEN and β-ZEN, which are subsequently transformed in α-zearalanol and β-zearalanol (189). Finally, these metabolites are conjugated with glucuronic acid and excreted in the urine and bile (190).

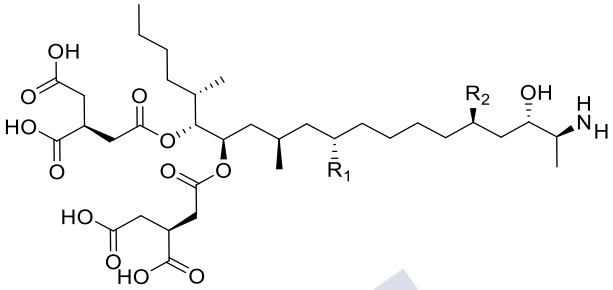
The toxicity of ZEN and its metabolites is mediated by their binding to cytosolic oestrogen receptors with the subsequent activation of gene expression and the synthesis of new proteins. ZEN and its analogues have different estrogenic potencies (α-ZEN > ZEN > β-ZEN) according to their affinity for oestrogen receptors (191). In this sense, swine shows a preferential conversion of ZEN into α-ZEN, which makes this species the most sensitive to toxin exposure (189).

ZEN possesses low acute toxicity after oral ingestion. Chronic exposure causes estrogenic effects including decreased body weight gain, changes in serum levels of progesterone and oestradiol, decreased fertility rates and abortion. Moreover, alterations in the mammary gland and fibrosis of the uterus were reported (192, 193). A TDI of 0.25 μg/kg b.w. was established by the EFSA for ZEN based on its oestrogenic effects (194).

1.2.2.4 Fumonisins

FBs are a group of more than 25 analogues produced by *Fusarium* species, mainly *F. verticillioides* and *F. proliferatum*, which frequently infect maize and other crops (195, 196). These compounds were first isolated in 1988 from cultures of *F. verticillioides* (formerly *F. moniliforme*). These strains were obtained from feed whose consumption had been previously related to the development of leukoencephalomalacia (LEM) in horses (197).

This group of mycotoxins is classified as A-, B-, C- and P- series based on their different substituent groups. However, only fumonisins type B (FBs) are relevant in terms of toxicity and occurrence. In this sense, the sum of FB_1, FB_2 and FB_3 supposes more than 95% of the total content of fumonisins present in contaminated samples. Among these, FB_1 is usually found at the highest levels in food and feed (195). Chemically, FBs are long-chain aminopolyols with two tricarballylic acid side chains (198). FB_1, FB_2 and FB_3 differ in the position of hydroxyl groups (Figure 16).



Toxin	R_1	R_2	Molecular formula
FB_1	OH	OH	$C_{34}H_{59}NO_{15}$
FB_2	H	OH	$C_{34}H_{59}NO_{14}$
FB_3	OH	H	$C_{34}H_{59}NO_{14}$

Figure 16. Structure of FBs

FBs are poorly absorbed from the gastrointestinal tract as less than 5% of the ingested compounds reach the bloodstream (199). Absorbed fractions are rapidly distributed, mainly to the liver and kidneys. The biotransformation of these compounds is limited and they are eliminated in both faeces and urine (200).

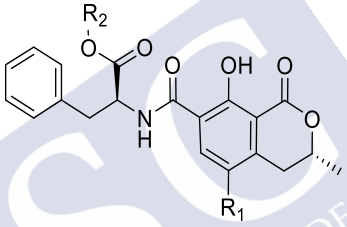
These compounds are structural analogues of sphingosine, a component of sphingolipids. Consequently, FBs act as competitive inhibitors of sphinganine and ceramide synthase (sphingosine N-acyltransferase). In this way, these mycotoxins inhibit the biosynthesis of ceramide and disrupt the sphingolipid metabolism. As a result, FBs cause an increase in the amount of sphingainine in serum, tissues, and urine. In addition, the depletion of ceramide, sphingomyelin, and glycosphingolipids may occur (201-203).

Following FBs ingestion, different symptoms have been described for various animal species. In horses, liquefactive necrosis of the white matter happens. In swine, pulmonary oedema, hepatocellular injury, and increased serum cholesterol levels are observed. Other toxic effects such as progressive renal tubular necrosis have been observed in laboratory animals (204-206). In humans, epidemiological studies relate FBs with neural tube defects and oesophageal cancer (203, 207). A provisional maximum TDI of 2 μg/kg b.w. for the sum of FB_1, FB_2 and FB_3 was established by the WHO based on the incidence of megalocytic hepatocytes observed in a chronic study with mice (208).

1.2.2.5 Ochratoxins

Ochratoxins are produced by several *Aspergillus* and *Penicillium* species, mostly *P. verrucosum*, *A. ochraceus* and *A. carbonarius*. The incidence of these fungi varies according to geographic regions. *P. verrucosum* is the most important source of the contamination of cereals in countries with cold and temperate climates, while *A. ochraceus* infects cereals, coffee, cocoa and edible nuts in tropical climates. In addition, *Aspergillus* section *Nigri*, mainly *A. carbonarius*, is the main source of grape and wine OTA contamination in Europe (209, 210). OTA was isolated in 1965 from cultures of *A. ochraceus* obtained from cereals that had caused the death of animals (211).

OTA comprises a dihydrocoumarin moiety linked to a molecule of L-β-phenylalanine by an amide bond (Figure 17). Other ochratoxins are usually found in contaminated products such as ochratoxin B (OTB), a dechlorinated derivative of OTA, or ochratoxin C (OTC), an ethyl ester of OTA. However, OTA is the most relevant analogue in terms of occurrence and toxicity (212).



Compound	R_1	R_2	Molecular formula
OTA	Cl	H	$C_{20}H_{18}ClNO_6$
OTB	H	H	$C_{20}H_{19}NO_6$
OTC	Cl	C_2H_5	$C_{22}H_{22}ClNO_6$

Figure 17. Structure of ochratoxins

OTA is rapidly absorbed through the gastrointestinal tract. In the bloodstream, this mycotoxin is bound to albumin and other plasma proteins, having a long serum half-life, 35 days in humans (213, 214). OTA is metabolised in the liver, mainly by hydroxylation, and it is kidney excreted (215). OTA and OTB have similar in vitro activity. However, OTA is more harmful to humans and animals. The difference in toxicity could be due to the poor accumulation of OTB in plasma and tissues (216).

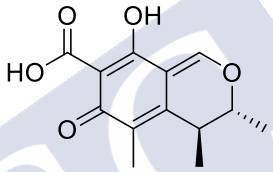
The toxic effects of OTA are related to its capacity to inhibit protein synthesis and with the induction of lipid peroxidation (217, 218). In addition, this compound increases the production of reactive oxygen species (ROS), inhibits the mitochondrial respiration, and disrupts the calcium homeostasis (219).

OTA acute intoxications are related to haemorrhages, intravascular coagulation and necrosis of the liver, kidney and lymphoid organs (220). Chronic exposure causes lesions in epithelial cells of the proximal tubules which evolve towards a cellular hypertrophy of the basement tubular membranes and, in later stages, interstitial fibrosis. In addition, this compound cause immunotoxicity and hepatotoxicity (221-223). OTA can cross the placenta, resulting in reduced birth weight and craniofacial abnormalities (224). This mycotoxin is classified as

possibly carcinogenic to humans (Group 2B IARC) since 1993 (225). However, new data regarding to the production of reactive oxygen species (ROS) and the formation of DNA adducts makes necessary a re-evaluation of OTA to increase its level of carcinogenicity (226). A TWI of 120 ng/kg b.w. was derived by the EFSA for OTA based on renal toxicity in pigs (227).

1.2.2.6 Citrinin

CTN is produced by several fungal species, including *Monascus*, *Penicillium* and *Aspergillus*. This mycotoxin is mainly synthesised under storage conditions and it occurs in grains, beans, fruits, and spices. In addition, CTN is usually found in red mould rice, a rice product that is fermented by *Monascus spp.* (228). This mycotoxin was isolated in the 1930s from a *P. citrinum* strain (229). This compound was recognised as a promising antibiotic, but the initial interest decreased when its toxicity was demonstrated (230, 231). Structurally, CIT is a quinone with two intramolecular hydrogen bonds (Figure 18) (232).



$C_{13}H_{14}O_5$

Figure 18. Structure of CTN

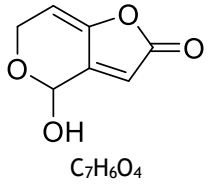
Intravenous administration indicates that CTN is extensively distributed in the body and accumulated in the liver and kidneys. The elimination is renal; however, small amounts of CTN are excreted in faeces (233). The mechanism of CTN toxicity is not completely understood. It may involve multiple pathways like DNA and RNA synthesis inhibition, an increase in ROS production, and activation of the caspase-cascade system that results in apoptotic cell death (234).

The kidney is the principal target organ of CTN. Acute toxicity involves kidney necrosis and hepatic impairment (235, 236). Chronic exposure causes progressive histopathological changes in the kidneys, which leads to the development of interstitial nephritis and cell adenomas (237). CTN has shown embryotoxic effects by promoting cell apoptosis and blastocysts damage (238). Moreover, genotoxicity and reproductive toxicity were reported (239). A level of no concern for nephrotoxicity in humans of 0.2 μg/kg b.w. per day was established by the EFSA. However, genotoxicity or carcinogenicity cannot be excluded at that level (234).

1.2.2.7 Patulin

PAT is produced by various species of *Penicillium*, *Aspergillus* and *Byssochlamys*. However, *P. expansum*, a spoilage fungus commonly found in fruits, is considered the primary producer of this mycotoxin. Apples and apple juice are the main products affected by PAT contamination. In addition, it can be found in other mouldy fruits such as pears, grapes, cherries

or peaches (240). PAT was first isolated in 1943 as an antibiotic effective against gram-positive and gram-negative organisms (241). Chemically, this compound is an unsaturated heterocyclic lactone (Figure 19) (242).



$C_7H_6O_4$

Figure 19. Structure of PAT

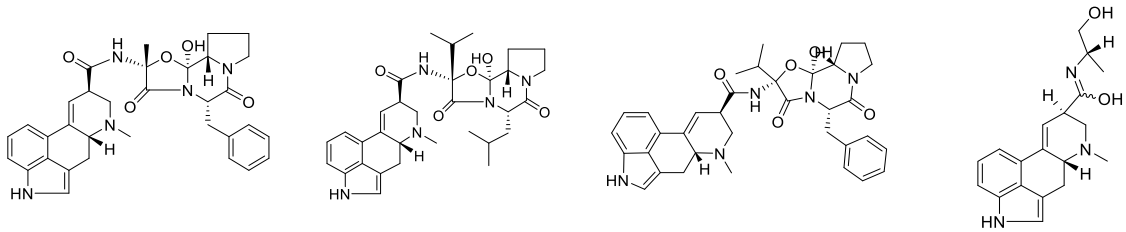
There are almost no data about the absorption and metabolism of PAT. This toxin is mainly accumulated in red blood cells and excreted within 24 h after exposure. However, low amounts of PAT can be detected in tissues after 7 days (243). PAT inhibits many enzymes including ATPase, lysosomal enzymes, RNA polymerase, due to its affinity for sulfphydryl-containing molecules such as cysteine or glutathione (244, 245). The suppression of catalase activity leads to an increase of ROS and oxidative stress (246).

Acute toxic effects of PAT are associated with inflammatory alterations of the gastrointestinal tract, as well as ulceration and inflammation of the mucosa of the stomach (247). These symptoms may be caused by the destruction of the tight junctions in the epithelial cell layer (248). Chronic exposure to PAT modifies the intestinal flora composition and may involve neurotoxic and immunosuppressive effects (249, 250). In addition, this mycotoxin is considered as a genotoxic compound (251). A provisional maximum TDI of 0.4 μg/kg b.w. per day has been set for PAT by the WHO (252).

1.2.2.8 Ergot alkaloids

Ergot Alkaloids (EAs) are produced by *Claviceps* species, mainly *C. purpurea.* This fungus infects grasses and cereals (rye, wheat, barley, millets and oats) replacing host seeds by their sclerotia, known as ergot. EAs are subsequently produced and accumulated inside of the sclerotia (253). In the Middle Ages, the consumption of food contaminated with EAs caused many outbreaks of a human poisoning known as St. Anthony's fire. Nowadays, agricultural practices and food processing techniques avoid severe outbreaks of this mycotoxicosis, currently known as ergotism. However, the removal of EAs from cereals is not complete and there is still a food safety hazard (254). More than 50 different EAs have been identified; these compounds are characterised by the presence of a tetracyclic ergoline ring system. Some of the more relevant analogues are the peptide EAs ergotamine, ergocryptine or ergocristine as well as the lysergic acid amide ergometrine (Figure 20) (255).

There are toxicokinetic data in humans and animals for ergometrine and ergotamine since they are used as pharmaceuticals. However, the majority of naturally occurring EAs have not been studied (256). EAs have a wide range of activities since these molecules have different affinities for noradrenaline, dopamine and serotonin receptors (254).



Ergotamine
$C_{33}H_{35}N_5O_5$

Ergocryptine
$C_{32}H_{41}N_5O_5$

Ergocristine
$C_{35}H_{39}N_5O_5$

Ergometrine
$C_{19}H_{23}N_3O_2$

Figure 20. Structure of some EAs

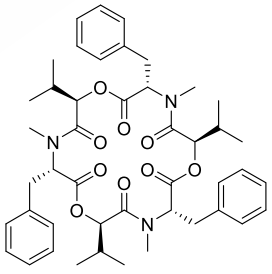
Acute exposure to EAs induces signs of neurotoxicity like muscular weakness, tremor or rigidity. Repeated exposure causes ischaemia in some parts of the body due to vasoconstriction. In addition, EAs induce adverse effects on the reproductive system, causing abortion and the inhibition of lactation (257). The EFSA has considered an equal relative potency for all EAs since relative potencies cannot be calculated with the available data, and a TDI of 0.6 µg/kg b.w. was established for the sum of EAs (255).

1.2.3 Emerging mycotoxins

As it was mentioned, the term emerging mycotoxins includes secondary metabolites that are not regulated or routinely determined. Although in this category many different compounds can be included, BEA, ENNs and MON are the more frequently detected.

1.2.3.1 Beauvericin

BEA is produced by some phytopathogenic *Fusarium* species including *F. proliferatum*, *F. semitectum*, and *F. subglutinans*. This toxin is produced under moist and cool conditions and is mainly found in maize and maize-based products (258). BEA is a cyclic hexadepsipeptide that contains three D-hidroxyisovaleryl and three N-methylphenylalanil residues in an alternating sequence (Figure 21) (259).



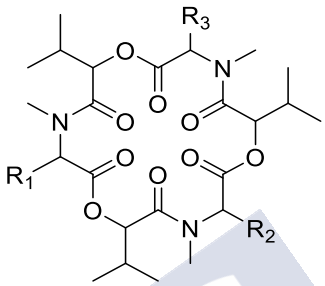
$C_{45}H_{57}N_3O_9$

Figure 21. Structure of BEA

The mechanism of action is related to its ionophoric activity. BEA is incorporated in mammalian cells forming a cation-selective channel, which increases ion permeability in biological membranes. This mycotoxin causes cytotoxicity in several cell lines, produces oxidative stress and it is an effective cholesterol acyltransferase inhibitor in rat liver microsomes (260). There are no data on subchronic or chronic toxicity. Hence, a TDI for BEA cannot be established (261).

1.2.3.2 Enniatins

ENNs are mainly produced by *Fusarium* species. These compounds are commonly found in cereals including maize, barley and wheat. ENNs are cyclohexadepsipeptides composed of alternating residues of three N-methyl amino acids, usually valine, leucine, and isoleucine, and three hydroxy acids, mainly hydroxyisovaleric acid (Figure 22) (262). To date, 29 naturally occurring analogues have been identified. However, only ENNA, $ENNA_1$, ENNB, $ENNB_1$ are frequently detected in food commodities (263).



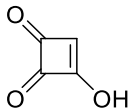
Compound	R_1	R_2	R_3	Molecular formula
ENNA	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$C_{36}H_{63}N_3O_9$
$ENNA_1$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$	$C_{35}H_{61}N_3O_9$
ENNB	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$	$C_{33}H_{57}N_3O_9$
$ENNB_1$	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$	$C_{34}H_{59}N_3O_9$

Figure 22. Structure of the main naturally occurring ENNs

The toxicity of ENNs, similarly to the BEA, is mediated by their ionophoric activity. These compounds are incorporated into cell membranes where form cation-selective pores. As a result, ENNS increase the alkali ion flux through biological membranes (264). These compounds are cytotoxic for several cell types; however, sub-acute exposure to ENNA showed no adverse effects in rats (265). There is no enough data to establish TDI (261).

1.2.3.3 Moniliformin

MON is mainly produced by *Fusarium* species including *F. verticillioides*, *F. avenaceum*, *F. proliferatum*, and *F. subglutinans*, which are found in maize and other cereals. MON, 3-hydroxy-3-cyclobutene-1, 2-dione (Figure 23), is a low molecular weight mycotoxin that occurs in nature as sodium or potassium salts (266).



$C_4H_2O_3$

Figure 23. Structure of MON

MON causes respiratory diseases, and myocardial degeneration probably as a consequence of the inhibition of the oxidation of intermediates in the tricarboxylic acid cycle. It is also proposed that MON disrupts the synthesis of enzymes (267). Based on cardiotoxicity, a

NOAEL of 6.0 mg/kg b.w. was observed in rats. However, a TDI could not be established due to limitations in the available data on chronic effects (268) .

1.3 ANALYTICAL TECHNIQUES FOR TOXIN DETECTION

Different physicochemical techniques have been developed for the detection of phycotoxins and mycotoxins. In general, the separation of sample components is done by using a technique such as liquid chromatography (LC) followed by detection with techniques such as fluorescence (FD), ultra-violet (UV), diode-array, electrochemical, and mass spectrometry (MS) (269). The application of a technique or a combination of techniques to analyse a specific compound is known as method. In general, the methods used for the analysis of food contaminants should be developed according to the Commission Decision 2002/657/EC, which established criteria and procedures to validate analytical methods, ensuring the quality and comparability of the results (270). Traditionally, EU legislation uses specific methods of analysis to determine the presence of different compounds in food and feed. For instance, there are official methods for the detection of all regulated phycotoxins, namely DA, PSPs, and lipophilic toxins (25, 26, 271, 272). This approach disincentive the development of new procedures since it is difficult to change the method for another one (273, 274). On the other hand, EU regulations do not establish official methods to detect mycotoxins, but only validated methods can be employed for the analysis of these compounds. It is recommended to use methods validated by collaborative trials. However, if they are not available, in-house validated methods for the commodity of interest can be used when these methods fulfil the performance criteria set out in the regulation EC Nº 401/2006 (275).

1.3.1 Liquid chromatography separation

The high-performance liquid chromatography (HPLC) is an analytical technique widely used to separate different components in a mixture (276). In this kind of chromatography, the separation occurs in a column where the sample is carried by a mobile phase, a solvent moving through the column. The column is formed by a stationary phase, a granular material made of small solid particles, tightly packed. Hence, a pump is necessary to force the elution of mobile phase through the column at high pressures of up to 400 bars. The ultra-high performance liquid chromatography (UHPLC) employs columns packed with particles lower than 2 μm, which increases the separation performance and allows a faster analysis compared with HPLC columns with higher particle sizes. This technology started to be used in 2004 when the first chromatographic system compatible with a pressure of 1000 bar was commercialised (277).

Methanol, water, and acetonitrile are usually employed as mobile phase. Samples containing ionizable compounds are strongly influenced by pH of the mobile phase. In order to choose the buffer of mobile phases, in addition to the chemical nature of the analytes, the stability of the column must be considered since it depends on the pH. A pumping systems force flow through the column. In this sense, binary and quaternary pumps are commonly employed. Binary pumps have two channels connected through a low-volume mixer chamber, while quaternary pumps are based in a system of one channel with two plungers and a valve to select the solvent line from four. If the composition of the mobile phase is constant during the

separation, it is referred to as an isocratic elution. While, gradient elution occurs when the composition of the solvent is changed among the elution. Between the pumping system and the column is located the injection system, which must introduce the sample in the mobile phase stream without modifying pressures or flow.

Different columns can be chosen for the separation, normal-phase columns are packed with polar resins like silica, amino or cyano, while reverse-phase columns usually employ C6, C8, C18 and phenyl resins. Other stationary phases can be selected depending on the analyte including ion-exchange, ligand exchange, or size exclusion resins (269, 278, 279). Normal-phase chromatography consists in the mentioned polar stationary phases and a nonpolar mobile phase. In this mode of chromatography, nonpolar compounds will not be fixed by the column packing, eluting faster than polar compounds, which are highly retained on the stationary phase. These retained compounds can be eluted by increasing the polarity of the mobile phase. Hydrophilic interaction liquid chromatography (HILIC) is a variant of normal phase liquid chromatography that can be used to separate small polar compounds by hydrophilic interactions using mobile phases like those employed in the reverse-phase chromatography (methanol, water, and acetonitrile) and traditional polar stationary phases. On the other hand, reverse-phase chromatography uses a polar mobile phase and the nonpolar stationary phase. In this way, the more polar compounds will be eluted first while nonpolar components will be retained on the stationary phase. In this case, the elution of adsorbed compounds can be made by decreasing the polarities of the mobile phase (278, 280, 281).

Over the last years the frequency of using UHPLC for pharmaceutical, forensic, environmental and food analysis has increased significantly. The UHPLC have several advantages such as short analysis time, reduce the use of organic solvents, and increases sensitivity and resolution of determination (282).

1.3.2 Mass spectrometry detection

After separation, the molecules can be identified using a mass spectrometer. MS is a powerful tool that provides structural information of the analyte by measuring the mass-to-charge ratio (*m/z*) values of ions that are positively or negatively charged (283). In this instrument, the first step is the production of gas-phase ions of the compound. In this sense, several ionisation techniques have been developed including electrospray ionisation (ESI), which achieved the nebulisation of the mobile phase eluting from the column through the application of a strong electrical field. In this way, ions are produced from the evaporating droplets which are subsequently transferred to a low-pressure region and finally are guided to the mass analyser through an ion-optical system (284). The mass analyser separates ions based on their *m/z*, and it is mainly electrically driven. Different mass devices were developed; some of the most widely employed are quadrupole (Q), time-of-flight (TOF), quadrupole ion trap (IT) (285).

Q instruments use the stability of *m/*z trajectories in oscillating electric fields to separate compounds. The ions transmitted through the quadrupole are determined by the Radio Frequency (RF) and Direct Current (DC) voltages applied to four parallel rods of circular or hyperbolic sections. These voltages produce an oscillating electric field that functions as a mass

filter; in this way, only certain *m/z* values will reach the detector for a given ratio of RF and DC voltages, while other ions will collide with the rods (286). In TOF instruments, the ions formed in the ion source are accelerated through a fixed potential into a drift tube, where they travel a controlled distance before reaching the detector. Ions are separated based on the velocity reached in the drift tube, which is inversely related to the square root of *m/z*. Hence, the *m/z* of the ion can be determined by measuring the time it takes to reach the detector. In IT analysers, ions are first 'trapped' and then subjected to MS or tandem mass spectrometry (MS/MS) analysis (287).

One of the main advantages of TOF instruments is that they offer mass accuracies in the tens of parts per million (ppm) level and the mass range is theoretically unlimited. On the other hand, Q and IT analysers can measure ions up to 300–4000 *m/z*, depending on the physical characteristics of the quadrupole, and the mass accuracy is generally in the hundreds of ppm level (288).

Hybrid instruments which combine two mass analysers are employed to perform MS/MS. In this mode of work, molecules are ionized and mass-selected in the first stage of analysis, next a chemical reaction occurs (mainly dissociations), and then, the charged products from the reaction are analysed in the second analyser. In this sense, ions can be dissociated through different techniques including collision induced dissociation, which employ a gas such as nitrogen or argon to collide with the selected parent ions, surface-induced dissociation, photodissociation or by interaction with an electron beam. The ions formed as a result of the chemical reaction are known as product or daughter ions. Hybrid instruments are classified in two categories: tandem-in-space or tandem-in-time (289).

- Tandem-in-space instruments so-called beam instruments require a separate analyser for each stage of MS/MS since the analysis occurs sequentially in space.
- Tandem-in-time are typically trapping instruments. This kind of spectometers are able to do multiple stages of MS/MS (MS^n) in the same analyser but separated in time. This can be done by manipulating the ions by changing different voltages during the experiment.

One of the first MS/MS spectrometer was the triple quadrupole (QqQ). In this instrument, the first and third quadrupoles are mass spectrometers, while the second (middle) "quadrupole" acts as the collision cell for CID (Figure 24). Various hybrid instruments were developed including the quadrupole/time-of-flight (Q/TOF), Q-linear ion trap, Q-Orbitrap and ion trap time-of-flight (IT-TOF) (288).

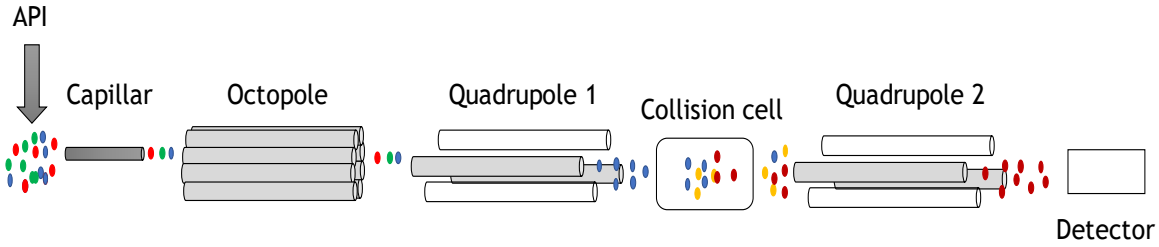


Figure 24. Scheme of a QqQ intrument

The combination of TOF and IT has complementary advantages. In IT-TOF instruments, ions generated in the source are transported first through an ion guide (DQarray) and then through an octopole where they are accumulated. Ions enter into the octopole for a user-defined time (accumulation time) and then are pulsed into the IT. This trap can accumulate ions with wide mass ranges, and it enables multiple stages of MS/MS. The precursor ion can be submitted to CID with neutral gas molecules with different energies. Next, ions are pushed into the reflectron, where they acquire their final energy and arrive in the field-free drift space, where they are separated according to their time of flight in the space (Figure 25) (290). The use of this technology allows to tentatively elucidate the structures of target components by applying the MS^n process (291).

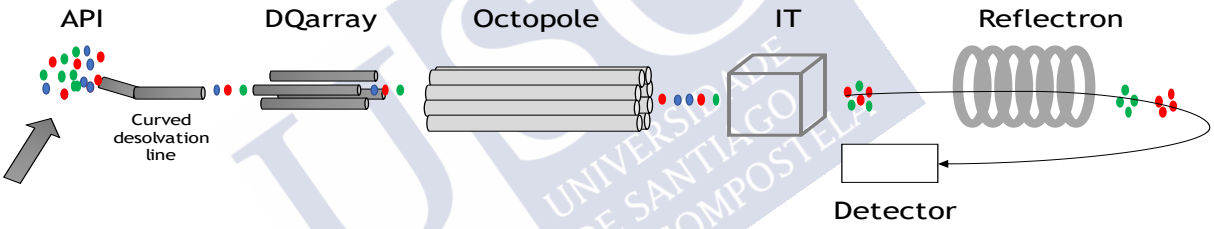


Figure 25. Scheme of an IT-TOF instrument

According to the mode of work of a MS/MS spectrometer, experiments are classified as follows (292, 293):

- Scan: It consist in measuring the complete spectra between two masses several times.
- Product ion scan: A precursor (parent) ion of a chosen *m/z* ratio is submitted to CID and the product ions are identified.
- Precursor ion scan (parent scan). All the precursor ions that produce a selected product are determined.
- Neutral loss scan. In this method, a neutral fragment is selected and all the fragmentations leading to the defined loss are monitored.
- Selected-ion monitoring (SIM). In this mode of analysis only preselected *m/z* values are detected.

- Selected reaction monitoring (SRM). It is similar to SIM, but ions selected by the first mass analyser are only detected when they produce a certain fragment, as consequence of a selected reaction.
- Multiple reaction monitoring (MRM). It is the application of SRM to multiple product ions from one or more precursor ions.

1.4 TOXIN ANALYSIS

Several factors must be considered for the analysis of toxins such as the complex materials in which they are present, the low levels generally encountered in food matrices, and their diversity of chemical structures and properties (294). In order to provide accurate results, the methods of analysis have to be validated. In this sense, different parameters must be determined such as the specificity, recovery, accuracy, precision, repeatability and sensitivity. The objective of the validation is to ensure that the method is acceptable for its intended purpose, and every measurement in routine analysis will be correct (295). In this sense, methods have to be validated for each matrix since toxin recovery may differ, and different compounds are extracted at the same time as target analytes. In this sense, co-eluting compounds may suppress or enhance the ionization of toxins, this drawback is known as matrix effect, and may even affect the method performance parameters (296). The type of method and its purpose indicate which type of validation must be done. In-house validation is the process of checking, within a single laboratory, that a method produces acceptable results. If a method is going to be used by several laboratories, the validation through inter-laboratory studies is preferred following the criteria for collaborative studies established by international organisations such as the Association of Official Analytical Chemists (AOAC). There are specific situations in which a method has to be validated by international collaborative trials like to be used as an official method in the EU. In addition to official methods, legislation specifies reference methods to be employed if the results obtained with other methods are challenged. Moreover, reliable toxin measurement requires the use of certified reference materials for calibration and quality control of the analysis procedure (25, 297, 298).

Sample extraction is a critical step of the analytical process. The contamination level is sometimes low, and toxins can be masked by compounds such as sugars, lipids or salts that interfere with the analysis. Hence, an adequate extraction solvent and several cleansing steps are sometimes necessary for toxin determination. According to the state of the matrix, solid-liquid and liquid-liquid extraction procedures have been traditionally employed for the analysis of solid and liquid samples, respectively.

The purpose of extraction is to remove toxins from the matrix as much as possible into a solvent suitable for subsequent cleanup or direct analysis. The selection of the extraction solvent depends on physical and chemical characteristics of the analyte. In the case solid-liquid extraction procedures, methanol and acetonitrile are the most commonly used solvents although ethanol, acetone, ethyl acetate and chloroform can also be employed. The addition of water enhances the extraction efficiency since it allows the penetration of the solvent in the matrix and the recovery of hydrophilic toxins. On the other hand, the use of acidified solvents is

sometimes necessary to increase the recovery of some analytes such as AFs, FBs and PSPs (299, 300). Therefore, the use of a mixture of solvents is common. For instance, acetonitrile/water/acetic acid 79:20:1 or 49:50:1 (v/v/v) mixtures are regularly employed in the extraction of mycotoxins. Next, the solvent should be separated from the matrix by gravity, filtration or centrifugation. In the case of liquid-liquid extractions, two immiscible phases are used, and the extraction efficiency is determined by the partition coefficient of the toxin between both phases (269, 301).

In some cases, extraction is followed by a cleaning step using different strategies to remove major components of the matrix that may interfere with the detection of the analytes of interest. In this sense, solid phase extraction (SPE) and immunoaffinity columns (IAC) are commonly employed in the analysis of phycotoxins and mycotoxins (302-304). SPE uses the affinity of the compounds for a stationary phase formed by a high-surface-area adsorbent, such as silica gel, octadecyl functionalized silica gel (C18), florisil or ion-exchange materials. Toxins are retained on the stationary phase while other matrix compounds can be washed. Target toxins can be further recovered by rinsing the stationary phase with an appropriate eluent (305-307). IAC are based on the selective binding of toxins by antibodies; hence, they are only useful for the analysis of one group of compounds (308). These methods, SPE and IAC, allow the pre-concentration of trace level of analytes with high cleanup efficiency. However, in some cases, these methods lead to poor recovery, and may produce signal enhancement or suppression, resulting in irreproducible quantitation especially, when lipid components are not sufficiently eliminated during sample preparation (302).

In the last few years, QuEChERS (from Quick, Easy, Cheap, Effective, Rugged, and Safe) methods have been developed for the extraction of mycotoxins and phycotoxins (309, 310). The QuEChERS methodology was initially developed for pesticide analysis and later extended to other residues and contaminants since it enables the elimination of interfering compounds and avoids sample dilution (311). This methodology involves an initial extraction step with acetonitrile-water followed by a liquid-liquid partitioning induced by the addition of inorganic salts (Figure 26). In this sense, anhydrous magnesium sulphate ($MgSO_4$), over the saturation concentration, allows the best salting-out. Moreover, the use of sodium chloride (NaCl) helps to control the polarity of the extraction solvents, which enhances extraction selectivity. However, the use of high amounts of this salt reduce the capability of the acetonitrile layer for polar analyte partition. It is generally considered that the ratio of $MgSO_4$ and NaCl 4:1 is the most effective to elicit phase separation. As a result of the salting-out, some polar components of the matrix remain in the aqueous layer while target analytes are moved into the organic phase (312).

A further dispersive SPE can be employed to reduce other matrix compounds from the organic phase. Some sorbents like graphite, Primary Secondary Amine (PSA) and C18 are commonly employed. However, for toxin analysis this cleanup step is sometimes omitted since some compounds may be trapped by the sorbent reducing the recovery (313-315).

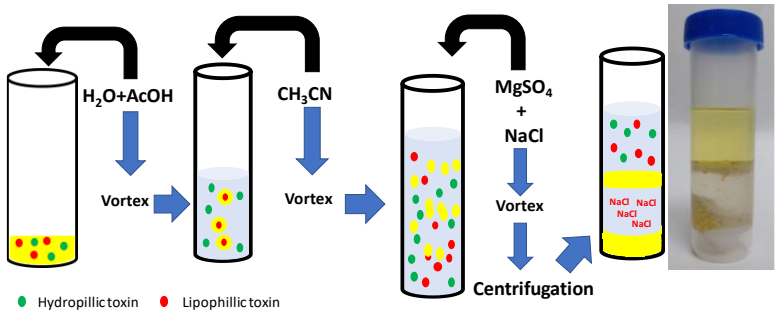


Figure 26. General salting-out step of the QuEChERS procedure for maize samples. Acetic acid (AcOH), acetonitrile (CH_3CN)

In this way, the reduction of matrix-interfering compounds reached with the QuEChERS procedure increases the sensitivity of the method (316).

1.4.1 Analysis of phycotoxins

The mouse bioassay was employed for the detection of phycotoxins for many years since the Commission Regulation (EC) No 2074/2005 established bioassays as the reference method for detecting lipophilic and PSP toxins (307). However, biological assays have some drawbacks concerning animal welfare and ethics. Hence, a wide range of detection methods have been developed for the analysis of phycotoxins, although only a few are employed for the official control of these compounds (308, 309). The use of analytical methods makes necessary to know the TEFs of all toxic analogues to calculate the toxicity of a sample since they can have different toxic potency (317)

The reference method in the EU for the detection of legislated lipophilic marine toxins is the LC-MS/MS. In addition, immunoassays and functional assays, such as the phosphatase inhibition assay, can be used as alternatives or in a supplementary way to the LC-MS/MS method (26). An harmonised protocol was validated under the coordination of the European Union Reference Laboratory for marine biotoxins (EU-RL-MB) for the determination of the OA and DTXs, PTXs, AZAs and YTXs using LC-MS/MS. The proposed procedure allows the quantification of these toxins in different matrices including mussels, clams, razor clams and cockles after extraction with methanol (318).

In 2006, a HPLC-FLD method “Lawrence method” was incorporated into European legislation for the regulatory monitoring of PSP in shellfish (272). From January 2019, this method became the EU reference method for PSP determination, although these toxins can be also detected in accordance with the biological testing method or any other internationally recognised method (271). The Lawrence method is based on the extraction of shellfish with acetic acid and sample boiling, followed by a SPE cleanup step. Next, the sample is submitted to a derivatisation which involves the oxidation of extracts to create fluorescent toxin oxidation products which are subsequently quantified (319). The analysis of PSPs by LC-MS/MS presents some difficulties such as a large number of structural analogues that form this family, including several epimeric pairs. Notwithstanding, some LC-MS/MS methods were in-house validated for the analysis of PSP toxins (320-323). In comparison with the Lawrence method, LC-MS/MS

methods provides unequivocal confirmation of toxin identity, and is potentially more sensitive and straightforward to operate (324).

The total ASP content of edible parts of molluscs must be detected using HPLC using UV detection (25). In this sense, EU-RL-MB has published a harmonised protocol for the determination of DA in shellfish and fish by HPLC-UV after methanol/water (50/50, v/v) extraction (325). In relation to the detection of this toxin by MS, several methods have been published but not validated (54, 326, 327).

Although maximum levels for TTX have not been established in the EU, the EFSA considers necessary to obtain more occurrence data on TTX and its analogues in bivalve molluscs across Europe to provide a reliable exposure assessment (108). Accordingly, EU-RL-MB has proposed LC-MS/MS to evaluate the incidence of this toxin. The method was validated using a Single Laboratory Validation for the analysis of TTX in mussels with the aim of organising a future inter-comparison study (328). LC-MS/MS methods have been developed for the analysis of other emerging phycotoxins including SPXs, PbTXs and pinnatoxins (329, 330). However, the lack of analytical standards prevents the validation of LC-MS/MS and other chemical methods for the analysis of these compounds (331).

1.4.2 Analysis of mycotoxins

According to the legislation, the presence of mycotoxins can be checked by fast and simple screening tools, and with analytical methods for identification and accurate quantification of the amount of each toxin. To determine whether commodities are contaminated, proper sampling procedures and extraction protocols are needed for obtaining reliable results (332).

The design of sampling procedures for mycotoxins has been of international concern for several years. Mycotoxins can be heterogeneously distributed in food products, especially in those with large particle size such as dried figs or groundnuts. In this kind of materials, the number of affected products may be low, but they can contain a high amount of toxin. Hence, for sampling purposes, the number of samples and their volume or weight must be stablished according to the nature of the product (333, 334). To obtain the same representativeness for batches of products the Regulation EC Nº 401/2006 lays down the methods of sampling for the control of the levels of mycotoxins in foodstuffs. This regulation indicates the number of incremental samples to be taken from different places of a lot depending on its weight. In this way, the variability in results due to the sampling procedure is minimized (275).

Several analytical methods have been developed for the analysis of mycotoxins in food and feed. Thin-layer chromatography, HPLC with UV or FD, and enzyme immunoassays were commonly used to analyse these compounds in the past. Recently, LC-MS techniques have become accessible for the qualitative and quantitative determination of mycotoxins. Unlike conventional methods, which commonly deal with a limited number of target analytes, LC-MS/MS allows the simultaneous detection of many mycotoxins with good sensitivity and specificity, and allows to obtain structural information of the analyte. Because of that, LC-MS/MS has become the most promising analytical tool for mycotoxin analysis (335). In fact, several methods have been developed for the multi-mycotoxin analysis (336-338). These

methods are of a special relevance since the co-occurrence of different toxins in contaminated food and feed is common (334).

Although there are no official methods to detect mycotoxins in the EU, only validated methods can be employed for the analysis of these compounds. It is possible to use methods in-house validated for the commodity of interest when these methods fulfil the minimum performance characteristics set out in the regulation EC Nº 401/2006. In this sense, the legislation establishes a range of recoveries acceptable for each mycotoxin according to the level of contamination, the maximum relative standard deviation value for the repeatability (RSDr), namely same sample, same operator, same apparatus, same laboratory, and short interval of time, and the maximum relative standard deviation value for the reproducibility (RSDR). That is, results obtained for an identical material obtained by operators in different laboratories (275). In the case of AFs and CNT, maximum relative standard deviation was fixed as two times the value derived from Horwitz Equation. This equation set a relationship between the precision of an analytical method and the concentration of the analyte as follows: $RSD_R = 2^{(1-0.5\log C)}$, where C is the concentration of the analyte (339). These analysis criteria are summarized in Table 3.

Compound	Level (µg/Kg)	Recovery (%)	RSD_r (%)	RSD_R (%)
AFB_1, AFB_2, AFG_1, AFG_2	<1	50-120	0.66 x RSD_R	Maximum: 2 × value derived from Horwitz Equation
	1-10	70-110		
	>10	80-110		
AFM_1	0.01-0.05	60-120		
	>0.05	70-110		
CTN	All	70-120		
DON	>100-≤ 500	60-110	≤20	≤40
	>500	70-120	≤20	≤40
ZEN	≤50	60-120	≤40	≤50
	>50	70-120	≤25	≤40
FB_1, FB_2	≤500	60-120	≤30	≤60
	>500	70-110	≤20	≤30
T-2, HT-2	15-250	60-130	≤30	≤50
	>250	60-130	≤25	≤40
OTA	<1	50-120	≤40	≤60
	≥1	70-110	≤20	≤30
PAT	<20	50-120	≤30	≤40
	20-50	70-105	≤20	≤30
	>50	75-105	≤15	≤25

Table 3. Performance characteristics for mycotoxin analysis (275).

The acceptability of methods in-house validated can also be determined by using a 'fitness-for-purpose' approach. This criterion evaluates the quality of a method specifying maximum levels of uncertainty in sample measurement. In this sense, methods suitable for official control must produce results with a standard measurement uncertainty (*U*) less than the maximum standard measurement uncertainty (*UF*), calculated as follows:

$$Uf = \sqrt{\left(\frac{LOD}{2}\right)^2 x\ (\alpha\ x\ C)^2}$$

This equation relates the limit of detection (LOD) of the method (μg/L), and the toxin concentration in the sample, while α is a constant that varies according to the value of *C* (275).

The adoption of performance characteristics and the fitness-for-purpose approach for the analysis of mycotoxins ensure that all applicable methods can be employed and do not disincentive the development of new procedures (273, 274). Indeed, methods for mycotoxin detection are in constant change since the analysis of these compounds remains a challenge. In this sense, mycotoxins can undergo modification in plants and microorganisms leading to the formation modified forms. As a result, a variation of physicochemical properties of molecules occurs which may lead to modified chromatographic behaviour and changes in mass of the compound. Hence, modified mycotoxins are not routinely monitored due to the large number of possible compounds and the lack of analytical standards (340).

Although LC-MS/MS is the most employed technique for mycotoxin analysis, it is limited for the detection of modified compounds since only targeted analytes can be detected. On the other hand, high resolution mass spectrometers such as TOF and Orbitrap are full-scan techniques which allow the detention of non-target compounds, novel compound identification, and retrospective data analysis. In addition, the measurement of the accurate mass allows to perform structural elucidation of unknown compounds. Hence, these advantages cause that high-resolution MS are growing in the field of food safety (341).

1.5 DETOXIFICATION PROCEDURES

Numerous approaches have been developed to reduce the impact of phycotoxins and mycotoxins. In the case of phycotoxins, control strategies are mainly limited to eliminate HAB cells by different means like mechanical removement of algae, introduction of biological agents that cause algal mortality or chemicals that promote its precipitation (342). However, in general, harvesting areas are closed until blooms naturally disappear and shellfish detoxification occurs (343). On the other hand, several measures can be applied to reduce the mycotoxins content of food and feed. Fungi growth and mycotoxin formation is controlled by environmental factors, agricultural practices and manufacturing conditions. In this sense, decrease the moisture of agricultural products during storage and certain conditions such as high carbon dioxide concentration prevent fungal development and mycotoxin production. Once mycotoxins contamination occurs, different decontamination strategies have been developed over the years (18, 344).

Traditional decontamination methods include procedures such as sorting and separation, immersing and washing. Sorting and separation of mouldy or poor-quality products significantly reduce mycotoxin levels in final products (345, 346). In this sense, mouldy kernels have lower density than healthy, they can be separated by immersion in water. The subsequent discarding of floating fractions reduce the contamination with mycotoxins, especially with AFs (347). Dehulling can also reduce the mycotoxin content, while milling gain cereals affects each toxin in a different way. For instance, the milling of wheat concentrate ZEN in fiber-rich parts of grains, while DON is distributed among all fractions (348). Certain cooking processes can also reduce mycotoxin content. In this regard, nixtamalization is a traditional process for the preparation of maize to make masa and tortillas. During this procedure, maize is soaked and

cooked in an alkaline solution, usually limewater, washed, and then hulled. In this way, a variable amount of AFs and FBs are removed from mycotoxin-contaminated maize (349).

Sequestering agents are large molecular weight compounds that claim to bind mycotoxins in the gastrointestinal trac, and in this way limit the toxins bioavailability. These agents are divided into mineral and organic adsorbents, and biological adsorbents (350). Mineral and organic adsorbents are aluminosilicates which can be divided into two subclasses, phyllosilicates and tectosilicates. Phyllosilicates such as montmorillonite, bentonite and its processed form, hydrated sodium calcium aluminosilicate (HSCAS), can adsorb substances on their surface or within their interlaminar space. Tectosilicates such as zeolites have a large and specific binding surface since the adsorption is conditionate by the size, shape, and charge of the molecules (351). Other mineral materials including diatomite and talc have been employed as mycotoxin adsorbents (352). Finally, activated carbon (AC) is a highly porous non-soluble powder formed by pyrolysis of organic materials. In aqueous solution, AC adsorbs most of the mycotoxins efficiently, but essential nutrients can also be uptake from treated products (351). Within biological adsorbents, yeast cell walls are the most employed. These cell structures are capable to adsorb a wide variety of compounds from the environment. Although only those yeast containing b-D-glucans, glucomannans and mannan-oligosaccharide as cell wall components are able to adsorb mycotoxins (353). In addition, some strains of bacteria having cell wall peptidoglycans and polysaccharides bind mycotoxins in the small intestine (354). Another strategy is the degradation of mycotoxins by some microorganisms such as bacteria/fungi or enzymes, which are known as biotransforming agents. However, the toxicity of some metabolized compounds is higher than the toxicity of parent mycotoxin (355). Several mycotoxin binders are commercially available. However, up to now, only bentonite has been approved as mycotoxin binder (356). This adsorbent only uptake some mycotoxins, mainly AFs, and has other drawbacks since interferes with antibiotics, may lead to incorrect analytic results about the presence mycotoxins in matrix and its inhalation is a hazard due to its silica content. Therefore, new strategies are needed to deal with mycotoxins (357).

In the last years, different nanotechnology applications have been developed for environmental remediation and food treatment (358-360). In this sense, some studies show magnetic materials and nanoparticles as promising adsorbents in the food and feed industry (361). Nanotechnology is the manipulation and control of matter at the nanoscale, that is, below 100 nanometres. In this way, it is possible to make functional nanomaterials, which, in comparison to bulk materials, have a significantly larger specific surface area. This characteristic increases the chemical reactivity and/or change to the physical properties of the material (362). In general, nanomaterials include materials with any external dimension in the nanoscale or having internal structures or surface structures in the nanoscale length range. The term nano-object comprises materials with one, two or three external dimensions in the nanoscale. If the nano-object has all three dimensions in the nanoscale, it is considered a nanoparticle. A nanostructure is a composition of inter-related constituent parts in which one or more of those parts are in the nanoscale. Finally, nanostructured materials are materials with internal or surface nanostructures (363).

Magnetic nanoparticles (NPs) are composed by magnetic elements such as iron, nickel, cobalt and their oxides. For most applications, particles with a size of around 10-20 nm show the best performance characteristics. In this sense, each nanoparticle acts like a single magnetic domain and shows superparamagnetic behaviour. That is, they are not magnetised after the action of a magnetic file. One additional advantage is that these particles can be used in bio-applications since they are composed by biocompatible materials. However, NPs have some problems; for instance, they are unstable over long periods since they tend to aggregate, resulting in the loss of magnetism and dispersibility. In addition, naked metallic nanoparticles are easily oxidised in contact with the air. To avoid these disadvantages NPs can be coated with different materials which leads to core-shell structures. In this way, the naked NPs constitute a central core which is surrounded by a shell that can be formed by different constituents including surfactants, polymers, silica or carbon (Figure 27A,B). In this sense, NPs can be dispersed in a continuous matrix, coated on larger particles (core-shell) or form agglomerates of individual NPs connected through their shells. Next, nanostructured materials can be formed by incorporating magnetite nanoparticles into biopolymer (chitin, alginate, pectin, agarose) beads (Figure 27C) (364, 365). Some of these materials have been used for the absorption of heavy metals (366, 367).

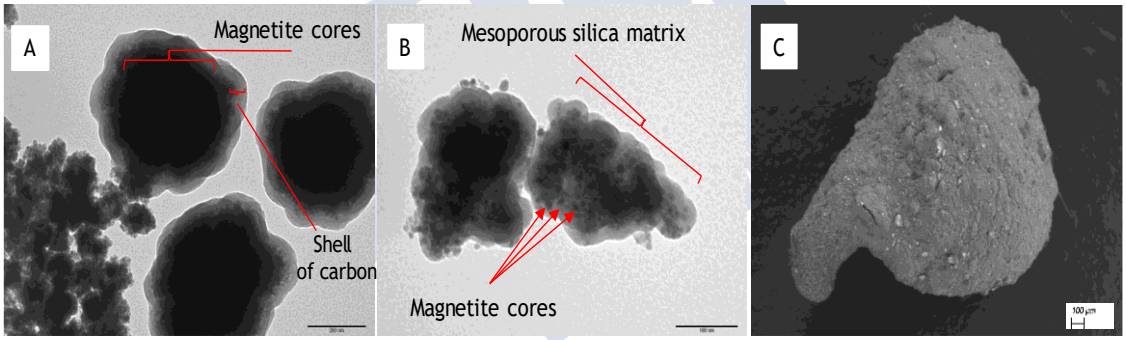


Figure 27. Spherical shell of carbon encapsulating several magnetite (Fe_3O_4) cores (A). Mesoporous silica loaded nanostructures with magnetite nanoparticles anchored on the surface (B). Nanostructured beads composed of magnetite nanoparticles and alginate (C).

Different nanotechnological applications were developed for the removal of toxins from different matrices. Surface Active Maghaemite Nanoparticles, a class of naked superparamagnetic nanoparticles, are an efficient mean for CTN removal from *Monascus*-treated foods (368). Magnetic graphene oxide modified with chitosan particles was developed for the simultaneous removal of AFB_1, OTA and ZEN from water solutions (369). Chitosan-coated magnetite particles can remove PAT from fruit juice, and magnetic carbon nanocomposites were developed for the removal of AFB_1 (370, 371). Chitosan-cellulose composite materials, N-doped TiO_2 nanocomposites, and magnetophoretic nanoparticles of polypyrrole were employed to eliminate different cyanotoxins from water (372-374). The use of nanomaterials to remove phycotoxins has not been widely studied. Reported nanotechnology applications related to these compounds are focused on the development of detection methods, including nanoparticle-based immunoassays and magnetic solid-phase extraction (375). In

addition, the use of covalent organic frameworks for solid-phase adsorption of OA was recently proposed, showing that these nanocomposites are promising candidates in water monitoring devices (376). Similarly, nanotechnology is employed in the detection of mycotoxins. For instance, core-shell NPs have been applied for dispersive solid-phase extraction of different types of mycotoxins (377). Nanomaterials such as magnetic particles, carbon nanomaterials, silica nanomaterials and quantum dots, are increasingly used in the development of nanobiosensors for the detection of mycotoxins (378). Therefore, the nanotechnology not only can be an option for mycotoxins and phycotoxins removal but also is a new approach for the analysis of these compounds.

2 OBJECIVE

The presence of contaminants in products intended for human and animal consumption may pose a risk to public health. The term natural toxins includes secondary metabolites produced by microorganisms. Among them, mycotoxins produced by fungi, phycotoxins mainly produced by dinoflagellates and cyanotoxins produced by bacteria are responsible for the higher number of human and animal poisoning. The increased knowledge of the harmful effects of these compounds makes necessary to develop new methods for their detection as well as for their reduction in final products to avoid intoxications.

In this context, the objective of the present doctoral thesis is to develop tools to ensure food safety through two approaches:

-The use of liquid chromatography coupled to mass spectrometry, low and high resolution, to study the presence of regulated, emerging and modified toxins to optimize sensitive methods for their detection and quantification in several matrices.

-The development of detoxification procedures based on nanotechnology to eliminate natural toxins from water and foodstuffs.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3 PUBLICATIONS

This section shows the results reached in the doctoral thesis, which includes six published articles, three more submitted to journals and one patent. These results include the methodology, explain the novelty of the research and the conclusions obtained in each case. The publications presented are related to the detection and detoxification of phycotoxins and mycotoxins. The results are grouped in the following sections:

SECTION I. ISOLATION OF FUNGAL SPECIES AND IDENTIFICATION OF MYCOTOXINS BY UNTARGETED MASS SPECTROMETRY

This section includes the following publications:

I.1. UPLC−MS−IT−TOF Identification of Circumdatins Produced by *Aspergillus ochraceus*

I.2. Detection of new emerging type-A trichothecenes by untargeted mass spectrometry

I.3. First report of *Fusarium foetens* as a mycotoxin producer

I.4. Incidence, biodiversity and mycotoxin profile of *Fusarium* species naturally occurring on forage maize hybrids harvested for whole-plant silage in North-West Spain

SECTION II. DEVELOPMENT OF QUANTITATIVE METHODS FOR MYCOTOXIN ANALYSIS

This section includes the following publications:

II.1 A QuEChERS based extraction procedure coupled to UPLC-MS/MS detection for mycotoxins analysis in beer

II.2 Evaluation of the occurrence of mycotoxins in feedstuffs, and development of a simple detoxification procedure.

SECTION III. ELIMINATION OF TOXINS BY THE USE OF THE NANOTECHNOLOGY

This section includes the following publications and patent:

III.1. Detoxification agents based on magnetic nanostructured particles as a novel strategy for mycotoxin mitigation in food

III.2. Magnetic nanostructures for marine and freshwater toxins removal

III.3. Method for detoxification of food, feed and water contaminated with natural toxins

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3.1 SECTION I. ISOLATION OF FUNGAL SPECIES AND IDENTIFICATION OF MYCOTOXINS BY UNTARGETED MASS SPECTROMETRY

I.1. UPLC−MS−IT−TOF IDENTIFICATION OF CIRCUMDATINS PRODUCED BY *ASPERGILLUS OCHRACEUS*

Abstract

A method based on the combined use of ultraperformance liquid chromatography coupled to mass spectrometry-ion trap-time-of-flight (UPLC−MS−IT−TOF) detection was employed to identify the metabolite production of *Aspergillus ochraceus*, which is the major cause of food and feed contamination due to ochratoxin A. Under the proposed chromatographic conditions, seven metabolites belonging to the family of circumdatins were separated and identified. Their initial identification was performed through the exact molecular formula, as a function of their accurate mass. Collision-induced dissociation was applied to predict precursor and product ions, and the elemental composition of each compound was obtained. The elimination of nitrogenous groups followed by successive losses of carbonyl groups is the common fragmentation pathway of circumdatins. With the fragmentation data obtained, an UPLC−MS/MS method was created and optimized to detect circumdatins in corn samples.

https://pubs.acs.org/doi/pdf/10.1021/acs.jafc.7b01845

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

I.2. Detection of new emerging type-A trichothecenes by untargeted mass spectrometry

Abstract

Mycotoxins occur naturally as agricultural contaminants all over the world. The toxic effects of some of their metabolites are known and their presence regulated in food and feed. This paper describes two methods for the detection of toxins of type-A trichothecenes group, and their modified forms, using mass spectrometry. Ultra-performance liquid chromatography coupled to mass spectrometry-ion trap-time of flight (UPLC-MS-IT-TOF) was employed to characterize the fragmentation pathways of 10 type-A trichothecenes, and characteristic ions were tentatively identified in scan mode through their accurate masses. Unknown signals were detected in a F. sporotrichioides extract, which afterwards were identified as seven modified forms of neosolaniol (NEO) and T-2 toxin. Then, UPLC coupled to tandem mass spectrometry (MS/MS) was employed to develop a precursor ion scanning method that can be used as a screening tool to detect any modified type-A trichothecenes.

https://www.sciencedirect.com/science/article/abs/pii/S003991401730944X

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

I.3. First report of *Fusarium foetens* as a mycotoxin producer

Abstract

Fusarium foetens, a pathogen of *Begonia* plants, has been recently described as a new fungal species. This *Fusarium* species causes a destructive vascular wilt disease which leads to the death of the plant. Moreover, Fusarium species are known to produce a huge variety of secondary metabolites such as mycotoxins and phytotoxins. Here, we studied the toxicogenic profile of one *F. foetens* strain, isolated from maize, employing two methods based on the use of ultra-performance liquid chromatography coupled to mass spectrometry-ion trap-time of flight detection. The mycotoxins beauvericin and fusaric acid were detected in a pure culture of *F. foetens*. In addition, four fusaric acid analogs (10,11-dihidroxyfusaric acid, hydroxyfusaric acid, dehydrofusaric acid, and a hydroxylated unsaturated fusaric acid analog) were tentatively identified on the basis of their accurate mass and fragmentation patterns. Therefore, these preliminary data indicate that *F. foetens* isolated from maize is able to produce *Fusarium* mycotoxins including beauvericin and fusaric acid.

https://link.springer.com/article/10.1007/s12550-019-00341-3

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

I.4. Incidence, biodiversity and mycotoxin profile of Fusarium species naturally occurring on forage maize hybrids harvested for whole-plant silage in North-West Spain

Abstract

In forage maize, all parts of the plant may be affected by *Fusarium* species, which, besides reducing crop yield, can produce mycotoxins that persist in silage and are detrimental to animal health. In this study, we identified the *Fusarium* species present in six forage maize hybrids grown for silage at three locations in Galicia (NW Spain). Results showed an extended natural infection of stalks, leaves and kernels of the six forage maize hybrids by a complex of mycotoxigenic *Fusarium* species in the three sites. Fungal morphological characteristics and molecular and phylogenetical analysis allowed to identify fifteen species: *F. anthophilum, F. avenaceum, F. cerealis, F. cortaderiae, F. culmorum, F. equiseti, F. graminearum, F. oxysporum, F. poae, F. proliferatum, F. sporotrichioides, F. sterilihyphosum, F. subglutinans, F. temperatum* and *F. verticillioides*. For the first time, the natural infection of kernels, leaves and stalks of forage maize by *Fusarium sterilihyphosum* in the field is reported. In addition, this fungus is first shown to cause seedling blight on forage maize and to produce the mycotoxins beauvericin and moniliformin.

A high incidence of toxigenic *Fusarium* species in stalks, leaves and kernels of six forage maize hybrids was recorded at the three sites in NW Spain, suggesting that whole-plant maize silages could harbor several mycotoxins that could have negative effects on animal health.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3.2 SECTION II. DEVELOPMENT OF QUANTITATIVE METHODS FOR MYCOTOXIN ANALYSIS

II.1. A QuEChERS based extraction procedure coupled to UPLC-MS/MS detection for mycotoxins analysis in beer

Abstract

A new method based on a QuEChERS extraction followed by the ultra-high liquid chromatography tandem mass spectrometry (UPLC-MS/MS) detection has been developed for the analysis of mycotoxin in beer. The method allows the identification and quantification of 23 mycotoxins with different chemical characteristic including regulated, emerging and masked compounds. A sample treatment procedure involving a QuEChERS extraction and dispersive solid-phase clean-up steps was applied. This protocol involves a new approach based on a sample concentration before the extraction. The method was in-house validated in terms of limits of detection (LODs), limits of quantification (LOQs), linearity, repeatability and recoveries. For most compounds, recoveries ranged from 70% to 110% with LOQs (from 0.038 to 30.43 μg/L) lower than the maximum residue levels established in European regulations. In general, acceptable performance characteristics were obtained fulfilling the current legislation. Therefore, the proposed method is appropriate for routine analysis of beer.

https://www.sciencedirect.com/science/article/pii/S0308814618317485

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

II.2. Evaluation of the occurrence of mycotoxins in feedstuffs, and development of a simple detoxification procedure.

Abstract

A new multi-mycotoxin analysis method was developed to identify and quantify 23 mycotoxins in multiple feed matrices. This method is based on a QuEChERS extraction procedure followed by the ultra-high liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) detection. The QuEChERS extraction procedure was optimized for minimizing the matrix effect of maize. Obtained recoveries ranged from 71% to 110% and low LOQs (from 0.22 to 32.64 µg/Kg) for regulated and emerging mycotoxins were obtained. Then, the method was expanded to seven raw materials and eight feedstuffs. In these matrices, the recovery for most mycotoxins was also high enough to fulfil the current legislation. The developed method was used for the analysis of 75 samples obtained from a nearby feed factory. Maize and its byproducts showed the highest incidence of mycotoxins, although always below the legal limit. In addition, the ability of spheres of different composition and size to eliminate mycotoxins from raw materials and feedstuffs was tested. Up to 28% of the mycotoxin content can be removed from matrices by using glass spheres of 2 mm of diameter. Therefore, a new process for the physical removal of mycotoxins was developed.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

3.3 SECTION III. ELIMINATION OF PHYCOTOXINS AND MYCOTOXINS BY THE USE OF THE NANOTECHNOLOGY

III.1. Detoxification agents based on magnetic nanostructured particles as a novel strategy for mycotoxin mitigation in food

Abstract

Mycotoxins are toxic compounds that can be present in feed, food and beverages. In this work, 25 magnetic nanostructured materials were developed to remove the main types of mycotoxins from liquid food matrices. The efficiency for binding mycotoxins from contaminated aqueous solutions was studied. Nanocomposites (diameters lower to 15 μm) composed of mixtures of activated carbon, bentonite and aluminium oxide were able to eliminate up to 87% of mycotoxins with an adsorption efficiency of 450 μg/g. On the other hand, spheres with sizes below 3mm and composed by biopolymers and activated carbon or graphene oxide removed up to 70% of mycotoxins (adsorption of 598 ng/g). These particles were tested for beer detoxification, and spheres composed of alginate and activated carbon or pectin maintain the ability to eliminate toxins from this beverage. Hence, this technology could be a useful tool for the food industry.

https://www.sciencedirect.com/science/article/pii/S0308814619308076

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

III.2. Magnetic nanostructures for marine and freshwater toxins removal

Abstract

Marine and freshwater toxins contaminate water resources, shellfish and aquaculture products, causing a broad range of toxic effects in humans and animals. Different core-shell biocompatible nanoparticles were tested as a new sorbent for removing marine and freshwater toxins from liquid media. Water solutions were contaminated with 20 μg/L of marine toxins and up to 50 μg/L of freshwater toxins and subsequently treated with 250 or 125 mg/L of nanoparticles. Under these conditions, carbon nanoparticles removed around 70% of saxitoxins, spirolides, and azaspiracids, and up to 15% of diarrheic shellfish poisoning toxins. In the case of freshwater toxins, the 85% of microcystin LR was eliminated, other cyclic peptide toxins were also removed in a high percentage. Marine toxins were adsorbed in the first five min of contact, while for freshwater toxins it was necessary 90 min to reach the maximum adsorption. Toxins were recovered by extraction from nanoparticles with different solvents. These results suggest the possibility of using this green methodology in the treatment of contaminated water or in chemical analysis applications.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

III.3. Method for detoxification of food, feed and water contaminated with natural toxins

Abstract

The present invention refers to a method for detoxification of contaminated substances. In addition, the present invention is directed to a composition comprising a contaminated substance and a particulate magnetic composite material and to the use of a particulate magnetic composite material for detoxification of a contaminated substance.

Application number: EP18382104.0

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

4 DISCUSSION

Phycotoxins and mycotoxins are contaminants in the food chain that present a risk to public health. Exposure to these compounds leads to different kinds of intoxication. Marine toxins cause acute poisoning, while mycotoxins are characterized by long-term toxic effects, although acute poisoning is also possible. The optimization of new methods for faster detection and quantification of toxins, as well as the identification of emerging and modified forms are a key task for the prevention of human and animal poisoning. Moreover, it is necessary to develop new strategies to ameliorate food quality by reducing the presence of contaminants. The present doctoral thesis was focus on these fields. In this sense, toxin extraction procedures and MS analysis methods have been developed, improving those currently implanted and creating new ones for the detection of emerging and modified mycotoxins. On the other hand, a new detoxification approach for treating food, feed and water contaminated with mycotoxins and phycotoxins was developed using magnetic nanostructured materials.

According to the purpose of the study, different modes of work can be used in MS. Targeted methods such as UHPLC-MS/MS allow to detect specific known compounds in a sample, while untargeted methods like UHPLC coupled to MS-ion trap-time-of-flight (UHPLC-MS-IT-TOF) are full-scan techniques that allow the detection of all compounds, known and unknown. In addition, retrospective data analysis can be done when high resolution technologies are used (341). The UHPLC-MS-IT-TOF perform an accurate mass measurement of the molecular ion ($[M + H]^+$) and their adducts, mainly of sodium ($[M + Na]^+$), potassium ($[M + K]^+$) or ammonium ($[M+NH_4]^+$). Besides, the detected ions can be submitted to a collision induced dissociation process to study their fragmentation process and, in this way, to predict their molecular formulae. This prediction is based on the recorded accurate mass data of fragments and in the fragmentation pathway of each molecule. In this way, it is possible to know the elemental composition of a parent compound and all its product ions with a high degree of confidence (379). The fragmentation data obtained in this way can be further employed in the development of targeted mass spectrometry methods (380). Therefore, we set up several untargeted methods to detect mycotoxins and phycotoxins, which were later employed to study the toxigenic profile of fungi and dinoflagellates.

In order to establish the risk of contamination of food products with toxins, the presence of producing microorganisms in foodstuffs, and the potential of these microorganisms to produce toxins, must be characterized. First, strains of *Aspergillus ochraceus*, *Fusarium sporotrichioides* and *Fusarium foetens* were evaluated by untargeted methods. Species of *Aspergillus* section *Circumdati* such as *A. ochraceus* are known to produce OTA (381). However, less information is available about its capacity to synthesize other toxins. To study the profile of metabolites produced by this fungus, the CBS 108.08 strain was inoculated in maize grains, and incubated at 24 °C in the dark for 3 months. After this period, samples were extracted using a general procedure (382). Next, they were analysed by UHPLC-MS-IT-TOF in scan mode using a method previously optimized for the detection of mycotoxins including

OTA. However, this mycotoxin was not detected in the extract. OTA production is conditionate by different factors such as the nutrient availability and the pH. In addition, this mycotoxin can be degraded by the enzymatic systems present in fungi (383, 384). After ruling out the presence of this mycotoxin, data were reanalysed, and several peaks were found in the extracts. In all cases, the $[M + H]^+$ ion and the $[M + Na]^+$ and $[M + K]^+$ adducts were observed in mass spectrum. The exact masses matched those of several analogues of the circumdatin family. Hence, in order to unequivocally identify these compounds, their fragmentation process was studied. First, a commercial standard of circumdatin A was used to establish the fragmentation pattern of this group of compounds. In this sense, the elimination of nitrogenous groups followed by successive losses of carbonyl groups was found as the main fragmentation pathway of circumdatin A. This pattern was found for all peaks, and the prediction of molecular formulae allowed to identify the compounds produced by the strain as circumdatins A-E, G and I. In this sense, circumdatin I has never been described before as a metabolite produced by *A. ochraceus* nor any other terrestrial fungus (385). Although there is little data on the toxicity of this group of compounds, circumdatins can be considered as mycotoxins based on their *in vitro* toxicity (386, 387). The analysis of circumdatins with the proposed UHPLC-MS-IT-TOF method is possible. However, hybrid instruments are not usually employed in routine analysis. Hence, a UHPLC-MS/MS method was optimized for the multidetection of circumdatins based on the fragmentation data obtained with the IT−TOF. In this sense, a LOD of 0.19 ng/mL and a limit of quantification (LOQ) of 0.39 ng/mL were obtained for circumdatin A. Therefore, the high sensitivity of this new method will allow to study the occurrence of circumdatins in food and feed.

Trichothecenes are the largest group of mycotoxins with more than 180 analogues produced by *Fusarium spp*. Among them, type A trichothecenes including T-2 and HT-2 toxins, are the most toxic. These compounds are primarily produced by *F. sporotrichioides* and *F. poae*. Due to the fact that many different compounds can be synthetized by these strains, including some modified mycotoxins, it is impossible to have standards for all of them, and therefore they are undetectable by conventional identification methods (388). To characterize the mycotoxigenic profile of *F. sporotrichoides*, one strain was grown in PDA for 7 days in the dark at 25 °C. After incubation, the agar was extracted using an acetonitrile/water/acetic acid (79:20:1, v/v/v) solution, and the extract was subsequently analysed by UHPLC-MS-IT-TOF. To identify the production of mycotoxins, analytical standards of the major type-A trichothecenes, NEO, T-2 toxin and HT-2 toxin were used to set up retention times and the exact mass of the $[M+NH_4]^+$ ion, usually selected to monitor these compounds (388). Next, NEO, T-2 toxin and HT-2 toxin were searched in the extract and five peaks were detected. The exact mass and the RT of three of those peaks matched with the standards, while the other two were unknown compounds related to the exact mass of NEO and HT-2. In order to characterize these peaks, the fragmentation pathway of both standards and unknow compounds was studied. In all cases, the fragmentation of $[M+NH_4]^+$ and $[M+Na]^+$ adducts yielded losses of the isovaleryl group, acetic acid, formaldehyde and water. Based on the accurate mass data of parent and product ions the formula predictor software was applied to know elemental composition of the detected compounds. In this way, iso-NEO and NEO monoacetate were

identified in the extract together with NEO, T-2 and HT-2 toxins. Several shared product ions were found in the fragmentation pathway of the five trichothecenes including the ions at *m/z* 245.1172 ($C_{15}H_{17}O_3$), *m/z* 215.1067 ($C_{14}H_{15}O_2$), and *m/z* 197.0961 ($C_{14}H_{13}O$). These ions were monitoring in MS^1 scan, and eleven peaks were obtained, five of them corresponding to the previously identified compounds. The unknown peaks were subsequently studied following the same approach. That is, establishing the fragmentation pathway and predicting molecular formulae. In this way, five new trichothecenes were detected including NEO 8-propionate, NEO 8-isobutyrate, 3′-Hydroxy T-2, 3-acetyl T-2 toxin, and the α,β T-2 analogue or β, γ T-2 analogue, which has not been previously described as a natural product. Next, a MRM method was optimized for the detection of these compounds. Analytical conditions including product ions, collision energies and fragmentor voltages were optimized and two transitions per compound were selected. In this way, a MRM method was developed that allows the detection of all these toxins with a LOD of 1.25 ng/mL for NEO, 0.75 ng/mL for T-2 toxin and 3.25 ng/mL for HT-2 toxin. However, the optimized MRM will not detect other type-A trichothecenes. Hence, in order to detect any analog of this group of mycotoxins with a QqQ instrument, we developed a precursor ion scanning method based on the shared product ions identified in the fragmentation study. In this mode of work, a LOD of 20 ng/mL for NEO and T-2 toxin and 80 ng/mL for HT-2 toxin was obtained. Therefore, three different methods were developed to detect regulated and emerging type-A trichothecenes.

Fusarium foetens has been recently described as a new fungal species. Although it is genetically related with the mycotoxigenic fungi *F. oxysporum*, the capacity of *F. foetens* to produce secondary metabolites has not been established. Therefore, we studied the toxicogenic profile of one *F. foetens* strain, isolated from maize. In order to elucidate if this species was able to produce mycotoxins, the isolate was incubated in PDA at 25 °C for a week in the dark. Next, the culture was extracted and analysed by UHPLC-MS-IT-TOF in scan mode. On the basis of the accurate mass, BEA was tentatively identified in the extract, which was further confirmed with the use of an analytical standard. In addition, a high intense peak was detected, the extract ion chromatogram showed the ion at *m/z* 180.1013, which may correspond to fusaric acid (FA). However, chromatographic conditions were not suitable to analyse this compound since the peak showed a long tail and the retention time was not reproducible. Hence, analytical column, mobile phases, and elution gradient were optimized using an analytical standard of FA. With the new conditions, the tail of the peak was eliminated, and retention time become reproducible. Next, the fragmentation of FA was studied using the standard, which consisted in water and carbonyl losses. The coincidence of the exact mass of the $[M+H]^+$ ion, the retention time and fragmentation pathway of the standard and the compound detected in the extract confirmed that *F. foetens* produce FA. In order to elucidate if this fungal strain synthesize some FA analogue, ions showing neutral losses of water were searched in MS^1 and, in addition to FA, 4 peaks showed this pattern. The $[M+H]^+$ ions of the unknown peaks were fragmented and the same fragmentation was found. Therefore, the match of fragmentation patterns points out that the detected ions were analogues of FA. The formula predictor software was applied to elucidate the element composition by using the exact mass of parent and product ions. In this way, the 10,11-dihidroxyfusaric acid, hydroxyfusaric acid, dehydrofusaric acid, and a

hydroxylated unsaturated FA analogue were tentatively identified. Therefore, a new method was developed for the identification of the FA and their analogues. Finally, in order to provide a more stable basis for the production of these compounds, the strain was grown in different culture media including PDA amended with chloramphenicol or streptomycin, malt extract agar, *Komada* medium, Spezieller Nährstoffarmer agar, yeast extract sucrose agar, and dichloran Rose-Bengal chloramphenicol agar. In general, BEA, FA and its analogues were found in all cultures with the exception of Spezieller Nährstoffarmer agar. Therefore, low nutrient media avoid de production of mycotoxins. In this work, we demonstrate the mycotoxigenic profile of *F. foetens,* and since the toxins are highly toxic to several plants this could explain the high phytopathogenicity of this fungus.

Similarly, we developed untargeted methods for the detection of EU emerging PbTXs. The common fragmentation pathway of this group of phycotoxins was studied by UHPLC-MS-IT-TOF employing PbTXs standards. In this way, a characteristic mass pattern with $[M+Na]^+$ adducts, doubly charged ions and several water losses was identified. After that, cultures of the dinoflagellate *K. brevis* were analysed, and the recorded data allowed to identify several PbTXs analogues in samples according to exacts masses and fragmentation pathways. Although these results were not included in an article, they were presented at the 10th European Conference on Marine Natural Products held in Kolymbari, Greece in September of 2017.

On the other hand, incidence of fungi was studied in forage maize is another product with high relevance in the economic activity of Galicia since livestock farming systems usually employ silage of maize for feed animals (389). Maize plants can be infected by pathogenic species of *Fusarium spp.,* to estimate the risk of maize silage contamination with mycotoxins, the incidence and toxigenic profile of *Fusarium* species occurring in the field were studied. In this sense, six forage maize hybrids from three locations with mild temperate climate were obtained. *Fusarium spp.* was found in practically all grains, stalks and leaves. A total of 1411 isolates were obtained, among them, the species more frequently isolated were: *F. anthophilum*, *F. avenaceum*, *F. cerealis*, *F. culmorum*, *F. cortaderiae*, *F. equiseti*, *F. graminearum*, *F. oxysporum*, *F. poae*, *F. proliferatum*, *F. sporotrichioides*, *F. subglutinans*, *F. sterilihyphosum*, *F. temperatum* and *F. verticillioides*. In order to stablish the potential of these species to produce mycotoxins, strains were grown on PDA and in wheat, unlike previous studies in which the production of toxins was tested in a single medium (390-392). In this sense, representative monosporic isolates of each species were incubated in sterilized wheat grains at 24 ºC in the dark for three weeks, and on PDA at 24 ºC in the dark for one week. Next, wheat and PDA samples were extracted using previously validated protocols, and samples were analyzed by UHPLC-MS-IT-TOF in scan mode (393, 394). Toxins were identified on the basis of the accurate mass of $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ and $[M+K]^+$ ions, and the retention time in those compounds whose analytical standard was available. The emerging mycotoxin BEA and MON showed the higher incidence since they were detected in the 50% of the tested species. Type-A trichothecenes were produced by *F. sporotrichioides*, *F. equiseti* and *F. poae*. Type-B trichothecenes, including acetyl derivatives of DON, were detected in *F. cortaderiae*, *F. cerealis*, *F. culmorum* and *F. graminearum* extracts. ZEN and its analogues were produced by *F. culmorum*, *F. graminearum*, *F. cerealis* and *F. cortaderiae*. As expected, *F. verticillioides*

and *F. proliferatum* produce FBs. Regarding other emerging toxins, ENNs were detected in *F. avenaceum, F. oxysporum* and *F. poae* cultures, equisetin was exclusively produced by *F. equiseti*, and fusarin C was detected in several species. Therefore, all strains were mycotoxigenic, but toxin production was conditioned by the substrate. In comparison with wheat samples, a higher variety of mycotoxins was found in PDA. For instance, *F cortaderiae* produced type B trichothecenes, ZEN, and fusarin C in PDA, while none of these mycotoxins were found in wheat.

In addition to the untargeted methods before exposed for the identification of modified and unknown toxins, it is also necessary to have methods to provide accurate quantitative results. In this doctoral thesis, different methods of extraction and analysis were developed for the quantification of toxins in beverages, raw materials, food and feed.

Beer is one of the most widely consumed drinks in the world and it is usually contaminated with mycotoxins. These compounds are not eliminated by technological treatments employed during beer production, and even an increase of DON content can occur during malting (395, 396). In order to analyse regulated, emerging and modified mycotoxins a new UHPLC-MS/MS method was developed allowing the identification and quantification of 23 mycotoxins in a single run of 13 min. All toxins can be separated and identified except for 3Ac-DON and 15Ac-DON, in this case a single peak for both compounds was obtained, and therefore these toxins were quantified as the sum of the isomers as in previous works (397). Two transitions were selected per compound, hence, according to the European legislation, an unequivocal identification of toxins was made (270). In order to maximize the sensitivity, the acquisition was performed in the dynamic mode. In this way, transitions were just monitored for a specific period of time, which increase the sensitivity of the method (338). Different solvent mixtures were evaluated for standards and sample analysis. In general, high amounts of water decrease the sensitivity to detect BEA and ENN, while the use of high proportions of organic solvents causes the formation of a peak tail of DON. In addition, the use of acetic acid increases the signal of FBs. Hence, the acetonitrile/water/acetic acid mixture 49/50/1(v/v/v) was chosen for UHPLC-MS/MS analysis. In this solvent, calibration curves with correlation coefficients >0.995 were obtained in a wide lineal range, with LOQs below to 1 ng/mL for most toxins. In addition, good repeatability within-batch was observed with a maximum variation of 4.5%. Hence, the developed method was appropriate for analysing samples with different levels of contamination. In beer, there are a wide range of compounds (sugars, pigments or organic acids) that interfere in the quantification since they cause a significant matrix effect (398). To avoid these interactions, most of the published multi-mycotoxin methods use a sample dilution, which increases the LOD, and, in many cases, the matrix effect is not significantly minimized (399). Hence, we developed a QuEChERS method to eliminate interfering compounds using a novel approach that avoids sample dilution. In this case, 15 mL of beer were first centrifuged to eliminate insoluble matter and the resultant supernatant was evaporated. Next, samples were extracted with 2 mL of a 2% acetic acid solution and 1 mL of acetonitrile. To elicit phase separation, 1 g of anhydrous $MgSO_4$ and 0.25 g of NaCl were added. Different amounts of sorbent were tested for the cleanup step, and 400 mg of $MgSO_4$ and 100 mg of C18 were chosen

since higher amount of this sorbent decreased the recovery of BEA and ENN. The use of other sorbents like PSA or graphite was discarded since a number of mycotoxins are trapped by those sorbents, reducing recoveries (311-313). The extraction protocol led to an extract 1.25 times concentrated, which is an advantage over previously published methods that dilute the sample (396, 398, 399). This extraction procedure yielded a matrix effect that varied from a signal suppression/enhancement value of 10 for DON to 109% for ENNA, with values above 100% indicating matrix enhancement while under 100% matrix suppression. Recoveries for regulated mycotoxins ranged from 70% for AFB_1 to 115% for HT-2 toxin, which are in compliance with the criteria established in the European legislation. In addition to specific performance criteria, the Regalement 401/2006 also includes a 'fitness-for-purpose' approach for the validation of methods employed for mycotoxin analysis (275). According to this criterion, the method was suitable for the analysis of all toxins with the exception of MON. Therefore, the novel developed method of extraction and analysis allows the quantification of regulated, emerging and modified mycotoxins in a run time of 13 min with enough sensitivity and repeatability to fulfil the EU legislation.

Maximum amounts of mycotoxins are legislated in foods intended for human consumption worldwide. However, legislation regarding the presence of mycotoxins for animal feed is very lax. Consequently, livestock animals are the most affected by mycotoxins, and, in general, there is a lack of multi-mycotoxin analysis methods for feedstuffs (400). To overcome this situation, we have developed a detection method applicable to multiple raw materials and feeds. In this sense, the chromatographic and detection conditions were previously optimized for the analysis of mycotoxins in beer (401). The extraction procedure was optimized to reduce matrix effect using maize as a representative matrix. First, a blank sample was extracted using a QuEChERS extraction procedure. In this sense, 2.5 g of sample were mixed with 10 mL of a 2% acetic acid solution and 10 mL of acetonitrile. Next, 4 g of anhydrous $MgSO_4$ and 1 g of NaCl were added to elicit phase partitioning. Matrix-matched calibration curves were constructed in solvent and in the extract 0, 2, 4, 8 and 10 times diluted to calculate the matrix effect in each condition. In this sense, when the sample was not diluted, there was an important suppression of signal, and the use of higher dilution factors led to lower matrix effect. However, when the sample was diluted more than 8 times, no significant reductions in signal suppression were observed. Next, the applicability of dispersive SPE cleanup was studied using C18 as sorbent. However, this step did not significantly decrease the matrix effect, and consequently the dispersive SPE cleanup was ruled out, and a sample 8 times diluted was chosen for the analysis. Using the optimized procedure, the recovery was around 80%, and the obtained RSDr values were, in general, lower to 10%. Therefore, the extraction procedure is according to European regulation (275). Next, the method was in-house validated for other raw materials commonly used by the feed manufacturing industry. In this sense, matrix effect and recoveries were studied for wheat, wheat bran, barley, soybeans, alfalfa, sunflower and distillers dried grains with solubles (DDGS). Wheat, wheat bran, barley and soybeans showed similar results with low matrix effect, varying between 70% and 120%, while alfalfa and sunflower caused the greatest signal suppression, especially for DON and its modified forms. In general, recoveries between 80% and 110% were obtained. For some matrix such as soybeans, the obtained recoveries were much

higher than those previously reported by other methods (402). The developed procedure was employed to monitor raw materials employed by a local company. In this sense, 61 samples were analysed. The most contaminated feedstuffs were DDGS, maize and wheat, while sunflower, soybeans and barley contained low levels of mycotoxins. In all cases, detected levels were lower to the maximum allowed or recommended by the EU. Next, the method was in-house validated for feedstuffs of some of the main livestock animals, namely, cow, calf, rabbit, hen, broiler and swine. DON was the most affected toxin by all matrix and, in general, rabbit feedstuffs caused the higher matrix effect. The overall recovery was in the range of 80% to 120%. Hence, the developed extraction procedure was also adequate for the analysis of mycotoxins in feedstuffs. The presence of this contaminates was checked in 14 products finding a high prevalence of FBs, ENNs, DON and ZEN, although these mycotoxins were detected in low amounts. Hence, the combined use of the QuEChERS extraction with the UHPLC-MS/MS detection allowed the identification and quantification of regulated, emerging and modified mycotoxins in a run time of 13 min fulfilling the current legislation for most mycotoxins and matrices.

In addition, different methods were optimized for the quantification of phycotoxins and cyanotoxins. In this sense, an UHPLC-MS/MS method was developed for the simultaneous quantification of 14 toxins of STX group, DA and TTXs, and further improved to analyse 15 lipophilic toxins. In this way, all EU regulated toxins can be quantified in a single run. Both methods have been validated at the same level than other published methods (323). These methods were employed in the detoxification assays carried out in this doctoral thesis.

In order to reduce the contamination of toxins in products intended for human and animal consumption, many strategies have been developed. In this way, we studied in depth two approaches, spheres with different composition and sizes, and nanotechnology in order to eliminate toxins from different matrices.

As it has been shown, feed is usually contaminated with mycotoxins. Consequently, feed producing companies routinely add mycotoxin sequestrants such as yeast cell walls and clays to their products, in order to decrease the effect of mycotoxins in animals, although reduction of symptoms associated with these toxins is reported very infrequently (403). Therefore, new strategies are needed to reduce the impact of mycotoxins on animal feed. In this sense, for mycotoxin analysis, it is necessary the use of deactivated glass material since some interactions can occur between glass and mycotoxins (404). Thus, we studied the ability of 5 beads of different diameters and composition, glass and aluminium hydroxide, to eliminate mycotoxins from raw materials and feedstuffs. Materials and naturally contaminated products were placed in tubes, then they were submitted to mechanical agitation for 180 min by using an overhead mixer. After treatment, materials and products were separated using a sieve and the concentration of toxin in the matrix was studied before and after treatment employing the developed extraction and analysis methods. In this sense, glass beads of 2 mm of diameters could be useful to minimize the impact of mycotoxins since allow the physical removal of up to 28% of the mycotoxin content of raw materials and feedstuffs including AFs, FBs, ZEN and trichothecenes.

Nanotechnology have recently been implemented in several fields, including medicine, cosmetics, agriculture and food. Convergence of nanotechnology with other technologies is also leading to further innovations that are expected to make a major impact on production, traceability, safety and security of food (405). In the present doctoral thesis, we explore the possibility of using the nanotechnology for the detoxification of liquid matrices contaminated with mycotoxins, phycotoxins and cyanotoxins.

First, the adsorption capacity of 25 magnetic nanostructured particles, with different sizes and compositions, was determined for the main types of naturally occurring mycotoxins, namely DON, ZEN, FB_1, and AFs. The adsorption capacity of particles was tested in an aqueous medium by incubating a fixed amount of adsorbent and toxins for 180 min. The particles were classified in small, medium and large size. In this sense, 226 mg/L of small and medium size sorbents, and 10 large size particles were employed for the detoxification experiments. Small size particles were core-shell structures with a size lower than 400 nanometres and formed by a core of Fe_3O_4 and shells of carbon, mesoporous silica or different proportions of aluminium hydroxide. In general, all these particles were highly effective in removing FB_1. Silica composites were also able to eliminate ZEN (15%), and carbon particles removed DON (15%), AFs (up to 80%) and ZEN (94%). Medium size materials were formed by aglomerates of 15 μm of Fe_3O_4, bentonite, activated carbon, aluminium oxide and gelatin. Particles without gelatin adsorbed about 400 ng of toxins per mg of particles, removing up to 20% of DON and 90% of other toxins. On the other hand, the same amount of particles with 9% of gelatin only removed around 65 ng of mycotoxins. These results suggest that gelatin may cover the accessible locations where the active compounds (bentonite, activated carbon, and aluminium oxide) bind the toxins. Finally, large size particles consisted of macroscopic size spheres composed by different combinations of biopolymers (alginate, humic acid), activated carbon and sodium sulphite were used. Particles composed of alginate were able to remove up to 90% of ZEN and AFs. In addition to these toxins, DON (54%) and FB_1 (71%) were removed when activated carbon was included in particles. The substitution of activated carbon by biopolymers led to different results although in general, the adsorption capacity was diminished. Nanostructured materials were superparamagnetic; hence, they can be separated from the matrix by an external magnet after the detoxification process. In addition, toxins were recovered from particles by extraction with an organic solution, and particles had not suffered any structural damage. Hence, they can be reused in subsequent detoxification cycles. Next, two medium-sized and two large-sized particles with the higher efficacy in reducing toxins from the aqueous medium were chosen to establish their adsorption efficacy in beer. This product is usually contaminated with different mycotoxins such as DON, ZEN or FB_1 (406). Hence, the adsorption efficiency was tested as a multi-mycotoxin system containing DON, ZEN, FB_1, OTA and AFs (AFB_1, AFB_2, AFG_1 and AFG_2) above the usual levels of these toxins in beer. Medium size particles adsorbed less than 40% of mycotoxins, while large size particles, formed by mixtures of alginate and activated carbon, reduced 20% of DON, 50% of FB_1, 57% of ZEN, around 80% of AFs and 90% of OTA. Some compounds (yeast, aluminosilicates, bentonite, humic acid or grape pomace) have been previously studied as mycotoxin adsorbents. Their ability to eliminate DON is negligible, and yeast products adsorb low amounts of AFB_1 (up to

29%) and ZEN (up to 68%) (407-409). In addition to the higher adsorption efficacy of nanostructured materials, they can be eliminated from the matrix using a magnet and they can be reused since adsorbed toxins are removed with a simple extraction. These published results have already been improved. In this sense, we have tested particles composed by the adsorbents that showed the better results. In this way, particles formed by mixtures of Fe_3O_4, alginate, activated carbon, and esterified pectin allowed the absorption of 59% of DON and more than the 90% of the other toxins from aqueous solutions, while from beer these particles removed the 20% of DON, 65% of FB_1 and more than 90% of ZEN, OTA and AFs.

Similarly, the two small size particles with the greatest capacity to adsorb mycotoxins were tested for the elimination of phycotoxins and cyanotoxins from contaminated solutions. Eutrophication and climate change might enhance the frequency and magnitude of HABs, and consequently the risk of human exposure to phycotoxins and cyanotoxins (410). Several detoxification strategies have been developed to eliminate toxin producing organisms. However, control methods currently employed may cause the rupture of cells, which leads to an increase in the amount of toxins in the water. Therefore, additional steps to eliminate dissolved toxins are needed. For this reason, the capacity of small size particles to remove phycotoxins and cyanotoxins was studied. Particles were developed combining Fe_3O_4 as the magnetic phase, and carbon or mesoporous silica to facilitate chemical affinity with the toxins. Initially, the absorption of phycotoxins was studied by treating a set of aqueous solutions contaminated with a mixture of hydrophilic or lipophilic compounds with 125 mg/L of particles. Under these conditions, carbon particles attained reductions near to 45% of hydrophilic toxins (STX, dcSTX and NEO), and about 90% for lipophilic compounds (AZAs and SPX20G), while silica particles only showed an adsorption capacity higher than carbon in the case of YTX removing a 43% of this compound. Next, the adsorption of phycotoxins was studied independently. Carbon particles were chosen due to their greater capacity to eliminate toxins. Water was artificially contaminated with 20 µg/L of regulated and emerging toxins including PSPs, DSPs, AZAs, YTXs and SPXs, and subsequently treated with 125 and 250 mg/L of particles. The use of 250 mg/L yielded toxin reductions between 58% and 72% for STXs (STX, NEO, dcSTX, dcNEO). In the case of C and GTX toxins reductions lower to 15% were reached, these type of PSP toxins have sulphate side groups in their structure, and it was previously reported that molecules with sulphate groups are weakly adsorbed by activated carbon (411). The reduction in the number of particles from 250 mg/L to 125 mg/L caused a proportional decrease in the percentage of the eliminated toxin. For instance, the adsorption of STX decreased from 72% to 44%. Hence, the amount of toxin adsorbed was proportional to the number of particles. Taking advantage of this, the removement of lipophilic toxins was studied using 125 mg/L of carbon particles. The incubation for 60 min led to reductions of up to 8.5% of YTXs, 16% of DSPs, 68% of AZAs and 71.1% of SPXs. Again, toxins with sulphate groups in their structure, namely YTXs, were the less absorbed. The World Health Organization has recommended a maximum of 1µg/L for MC-LR in drinking water (412). Hence, the ability of carbon particles to eliminate freshwater toxins was also determined. To do this, water was contaminated with a mixture of ATX-a, NOD, CYN, MC-LR, and MC-RR, and treated with 125 mg/L of particles. The incubation for 60 min removed 81.90% of MC-RR, 60.68% of MC-

LR and 29.57% of NOD, while reductions lower to 10% were obtained for ATX-a and CYN. Hence, each gram of NPs can remove up to 376.72 µg of freshwater toxins. The adsorption of these compounds depends on their structure since cyclic peptides, MCs and NOD, where highly removed, while only low amounts of the alkaloids ATX-a and CYN were eliminated from solutions. These results improve the elimination of MC-LR achieved by using iron oxide particles (413). Marine toxins were adsorbed in the first five min of contact, while for freshwater toxins it was necessary 90 min to reach the maximum adsorption. The short period of time in which toxin removal occurs suppose an advantage over bulk materials. For instance, oyster shells and chitin materials were tested as STX adsorbents, these materials need around 40 h to reach the maximum absorption, which was far inferior to that of the studied materials (414). In addition, toxins can be recovered from particles by extraction with different solvents, which enable the recycling of composites for successive treatments.

In summary, multiple methods for the analysis of toxins have been developed in this doctoral thesis. In this sense, novel procedures based on the QuEChERS extraction were optimized and in-house validated. Different target (QqQ) and untargeted (TOF) MS methods were developed for the detection of regulated, modified and emerging toxins. In addition, we have demonstrated that the nanotechnology is a promising tool in the field of food, and water remediation

5 CONCLUSIONS

1. *Aspergillus ochraceus* produce circumdatins A-E, G and I. These compounds are identified through the losses of nitrogenous and carbonyl groups.
2. Circumdatin I is present in terrestrial fungus.
3. The ions *m/z* 245.1172, *m/z* 215.1067 and *m/z* 197.0961 allow to develop methods to identify any type A trichothecenes.
4. *Fusarium foetens* is a mycotoxigenic fungi able to produce BEA, FA and four analogues.
5. In the field, *Fusarium spp.* contaminate most of grains, stalks and leaves of maize. Species of this genera produce a higher variety of mycotoxins in PDA than in wheat.
6. The method of extraction and analysis of mycotoxins in beer allows the detection of 23 mycotoxins with low limit of quantification in 13 minutes.
7. The method developed to analyse mycotoxins in solid matrices allow the simultaneous quantification of regulated, emerging and modified compounds in several feedstuffs.
8. Magnetic nanostructured materials allow the removal of up to 90% of several mycotoxins from contaminated beverages.
9. Magnetic nanoparticles with carbon shells are able to remove up to 70% of regulated and emerging phycotoxins from water, as well as up to 85% of cyanotoxins.
10. Nanomaterials can be reused in subsequent detoxification cycles after one washing step without suffer any structural damage.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

6 Bibliography

1. Halkier B, Holm L. Shifting responsibilities for food safety in Europe: an introduction. Appetite. 2006;47(2):127-33.
2. European Commission. Commission Green Paper. The General Principles of Food Law in the European Union. Brussels, 1997.
3. European Commission. Commission White Paper on food safety. Brussels, 2000.
4. Bergeaud-Blackler F, Paola Ferretti M. More politics, stronger consumers? A new division of responsibility for food in the European Union. Appetite. 2006;47(2):134-42.
5. Regulation (EC) No 852/2004 of the European Parliament and of the Council of 29 April 2004 on the hygiene of foodstuffs. Official Journal of the European Communities, L 139, p. 1-54.
6. Regulation (EC) No 853/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific hygiene rules for food of animal origin. Official Journal of the European Communities, L 139, p. 55-205.
7. Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. Official Journal of the European Communities, L 139, p. 206-320.
8. Regulation (EC) No 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. Official Journal, L 31, p. 1-34.
9. Aune T. Risk assessment of marine toxins. In: Botana LM, editor. Seafood and freshwater toxins. New York, CRC Press; 2008. p. 21-38.
10. EFSA. Guidance on human health risk-benefit assessment of foods. EFSA J. 2010;8(7):1673.
11. Council Regulation (EEC) No 315/93 of 8 February 1993 laying down Community procedures for contaminants in food. Official Journal, L 37, p. 1-5.
12. WHO. WHO global strategy for food safety: safer food for better health. Geneva, 2002.
13. EFSA. Summary of the 2016 Data Collection on Contaminant Occurrence Data. EFSA J. 2017;14(5):1217-497.
14. Ramos A, Sanchis V, Marin S. The prehistory of mycotoxins: related cases from ancient times to the discovery of aflatoxins. World Mycotoxin J. 2011;4(2):101-12.
15. Wright JL. Dealing with seafood toxins: present approaches and future options. Food Res Int. 1995;28(4):347-58.
16. van Egmond HP. Natural toxins: risks, regulations and the analytical situation in Europe. Anal Bioanal Chem. 2004;378(5):1152-60.
17. Reboreda A, Lago J, Chapela M-J, Vieites JM, Botana LM, Alfonso A, et al. Decrease of marine toxin content in bivalves by industrial processes. Toxicon. 2010;55(2):235-43.

18. Luo Y, Liu X, Li J. Updating techniques on controlling mycotoxins - A review. Food Control. 2018;89:123-32.
19. Rossini GP, Hess P. Phycotoxins: chemistry, mechanisms of action and shellfish poisoning. In: Luch A, editor. Molecular, Clinical and Environmental Toxicology, Basel; 2010. p. 65-122.
20. Botana LM, Alfonso A, Vale C, Vilariño N, Rubiolo J, Alonso E, et al. The Mechanistic Complexities of Phycotoxins: Toxicology of Azaspiracids and Yessotoxins. Adv Mol Toxicol. 2014;8:1-33.
21. Alonso E, Alvariño R. Marine Toxins. In: Botana LM, editor. Environmental Toxicology. Berlin, De Gruyter; 2018.
22. Gerssen A, Pol-Hofstad IE, Poelman M, Mulder PP, van den Top HJ, de Boer J. Marine toxins: chemistry, toxicity, occurrence and detection, with special reference to the Dutch situation. Toxins. 2010;2(4):878-904.
23. Commission Regulation (EU) No 786/2013 of 16 August 2013 amending Annex III to Regulation (EC) No 853/2004 of the European Parliament and of the Council as regards the permitted limits of yessotoxins in live bivalve molluscs. Official Journal of the European Union. L 220, p. 14.
24. EFSA. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on Marine Biotoxins in Shellfish – Saxitoxin Group. EFSA J. 2009;1019:1-76.
25. Commission Regulation (EC) No 2074/2005 of 5 December 2005 laying down implementing measures for certain products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the organisation of official controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. Official Journal of the European Union. L 338, p. 27-59.
26. Commission Regulation (EU) No 15/2011 of 10 January 2011 amending Regulation (EC) No 2074/2005 as regards recognised testing methods for detecting marine biotoxins in live bivalve molluscs. L 6, p. 3-6.
27. Paerl HW, Valdes LM, Pinckney JL, Piehler MF, Dyble J, Moisander PH. Phytoplankton Photopigments as Indicators of Estuarine and Coastal Eutrophication. BioScience. 2003;53(10):953-64.
28. Hernandez-Becerril DU, Alonso-Rodriguez R, Alvarez-Gongora C, Baron-Campis SA, Ceballos-Corona G, Herrera-Silveira J, et al. Toxic and harmful marine phytoplankton and microalgae (HABs) in Mexican Coasts. J Environ Sci Health. 2007;42(10):1349-63.
29. Sellner KG, Doucette GJ, Kirkpatrick GJ. Harmful algal blooms: causes, impacts and detection. J Ind Microbiol Biotechnol. 2003;30(7):383-406.
30. Anderson CR, Moore SK, Tomlinson MC, Silke J, Cusack CK. Living with Harmful Algal Blooms in a Changing World: Strategies for Modeling and Mitigating Their Effects in Coastal Marine Ecosystems. In: Shroder JF, Ellis JT, Sherman DJ, editors. Coastal and Marine Hazards, Risks, and Disasters. Boston, Elsevier; 2015. p. 495-561.
31. Botana LM. Toxicological Perspective on Climate Change: Aquatic Toxins. Chem Res Toxicol. 2016;29(4):619-25.
32. Shumway SE. A Review of the Effects of Algal Blooms on Shellfish and Aquaculture. J World Aquacult Soc. 1990;21(2):65-104.

33. Hackett JD, Wisecaver JH, Brosnahan ML, Kulis DM, Anderson DM, Bhattacharya D, et al. Evolution of saxitoxin synthesis in cyanobacteria and dinoflagellates. Mol Biol Evol. 2013;30(1):70-8.
34. Sommer H, Meyer K. Paralytic Shell-Fish Poisoning. Arch Pathol Lab Med. 1937;24:560-98.
35. Schantz EJ, Mold JD, Stanger DW, Shavel J, Riel FJ, Bowden JP, et al. Paralytic shellfish poison. A procedure for the isolation and purification of the poison from toxic clam and mussel tissues. J Am Chem Soc. 1957;79(19):5230-5.
36. Wang DZ. Neurotoxins from marine dinoflagellates: a brief review. Mar Drugs. 2008;6(2):349-71.
37. Suarez-Isla BA. Saxitoxin and Other Paralytic Toxins: Toxicological Profile. In: Gopalakrishnakone P, Haddad Jr V, Tubaro A, Kim E, Kem WR, editors. Marine and Freshwater Toxins. Dordrecht, Springer 2016. p. 23-41.
38. Wiese M, D'Agostino PM, Mihali TK, Moffitt MC, Neilan BA. Neurotoxic alkaloids: saxitoxin and its analogs. Mar Drugs. 2010;8(7):2185-211.
39. Negri A, Stirling D, Quilliam M, Blackburn S, Bolch C, Burton I, et al. Three novel hydroxybenzoate saxitoxin analogues isolated from the dinoflagellate Gymnodinium catenatum. Chem Res Toxicol. 2003;16(8):1029-33.
40. Luckas B, Hummert C, Oshima Y. Analytical methods for paralytic shellfish poisons. In: Hallegraeff GM, Anderson DM, Cembella AD, editors. Manual on Harmful Marine Microalgae. Paris, United Nations 2003. p. 191-209.
41. Kao CY. Paralytic Shellfish Poisoning. In: Falconer IR, editor. Algal Toxins in Seafood and Drinking Water. San Diego, Academic Press; 1993. p. 75-86.
42. Garcia C, del Carmen Bravo M, Lagos M, Lagos N. Paralytic shellfish poisoning: post-mortem analysis of tissue and body fluid samples from human victims in the Patagonia fjords. Toxicon. 2004;43(2):149-58.
43. Andrinolo D, Gomes P, Fraga S, Soares-da-Silva P, Lagos N. Transport of the organic cations gonyautoxin 2/3 epimers, a paralytic shellfish poison toxin, through the human and rat intestinal epitheliums. Toxicon. 2002;40(10):1389-97.
44. Gessner BD, Bell P, Doucette GJ, Moczydlowski E, Poli MA, Van Dolah F, et al. Hypertension and identification of toxin in human urine and serum following a cluster of mussel-associated paralytic shellfish poisoning outbreaks. Toxicon. 1997;35(5):711-22.
45. Faber S. Saxitoxin and the induction of paralytic shellfish poisoning. J Young Investig. 2012;23(1):1-7.
46. Perez S, Vale C, Botana AM, Alonso E, Vieytes MR, Botana LM. Determination of toxicity equivalent factors for paralytic shellfish toxins by electrophysiological measurements in cultured neurons. Chem Res Toxicol. 2011;24(7):1153-7.
47. Toyofuku H. Joint FAO/WHO/IOC activities to provide scientific advice on marine biotoxins. Marine pollution bulletin. 2006;52(12):1735-45.
48. Levin RE. Paralytic shellfish toxins: their origin, characteristics and methods of detection: a review. J Food Biochem. 1991;15(6):405-17.
49. Alonso E, Alfonso A, Vieytes MR, Botana LM. Evaluation of toxicity equivalent factors of paralytic shellfish poisoning toxins in seven human sodium channels types by an automated high throughput electrophysiology system. Arch Toxicol. 2016;90(2):479-88.
50. Takemoto T, Daigo K. Constituents of *Chondria armata*. Chem Pharm Bull. 1958;6(5):578-80.

51. Perl TM, Bédard L, Kosatsky T, Hockin JC, Todd EC, Remis RS. An outbreak of toxic encephalopathy caused by eating mussels contaminated with domoic acid. N Engl J Med. 1990;322(25):1775-80.
52. Bates S, Bird CJ, Freitas Ad, Foxall R, Gilgan M, Hanic LA, et al. Pennate diatom *Nitzschia* pungens as the primary source of domoic acid, a toxin in shellfish from eastern Prince Edward Island, Canada. Can J Fish Aquat Sci. 1989;46(7):1203-15.
53. Jeffery B, Barlow T, Moizer K, Paul S, Boyle C. Amnesic shellfish poison. Food Chem Toxicol. 2004;42(4):545-57.
54. Beach DG, Walsh CM, McCarron P. High-throughput quantitative analysis of domoic acid directly from mussel tissue using Laser Ablation Electrospray Ionization - tandem mass spectrometry. Toxicon. 2014;92:75-80.
55. Jing J, Petroff R, Shum S, Crouthamel B, Topletz AR, Grant KS, et al. Toxicokinetics and physiologically based pharmacokinetic modeling of the shellfish toxin domoic acid in nonhuman primates. Drug Metab Dispos. 2018;46(2):155-65.
56. Lefebvre KA, Robertson A. Domoic acid and human exposure risks: a review. Toxicon. 2010;56(2):218-30.
57. Vale C. Domoic acid: Chemestry and, pharmacology. In: Botana LM, editor. Seafood and freshwater toxins: pharmacology, physiology, and detection. 3th ed. Boca Raton CRC Press; 2014. p. 875-94.
58. Pulido OM. Domoic acid toxicologic pathology: a review. Mar Drugs. 2008;6(2):180-219.
59. EFSA. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish – domoic acid. EFSA J. 2009;1181:1-61.
60. Valdiglesias V, Prego-Faraldo MV, Pasaro E, Mendez J, Laffon B. Okadaic acid: more than a diarrheic toxin. Mar Drugs. 2013;11(11):4328-49.
61. Tachibana K, Scheuer PJ, Tsukitani Y, Kikuchi H, Van Engen D, Clardy J, et al. Okadaic acid, a cytotoxic polyether from two marine sponges of the genus Halichondria. J Am Chem Soc. 1981;103(9):2469-71.
62. Murata M, Shimatani M, Sugitani H, Oshima Y, Yasumoto T. Isolation and Structural Elucidation of the Causative Toxin of the Diarrhetic Shellfish Poisoning. Nippon Suisan Gakkaishi. 1982;48(4):549-52.
63. Kumagai M, Yanagi T, Murata M, Yasumoto T, Kat M, Lassus P, et al. Okadaic Acid as the Causative Toxin of Diarrhetic Shellfish Poisoning in Europe. Agric Biol Chem. 1986;50(11):2853-7.
64. Quilliam MA, Ross NW. Analysis of Diarrhetic Shellfish Poisoning Toxins and Metabolites in Plankton and Shellfish by Ion-Spray Liquid Chromatography-Mass Spectrometry. Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry. ACS Symposium Series. 619, American Chemical Society; 1996. p. 351-64.
65. Matias WG, Traore A, Creppy EE. Variations in the distribution of okadaic acid in organs and biological fluids of mice related to diarrhoeic syndrome. Hum Exp Toxicol. 1999;18(5):345-50.
66. Ito E, Yasumoto T, Takai A, Imanishi S, Harada K. Investigation of the distribution and excretion of okadaic acid in mice using immunostaining method. Toxicon. 2002;40(2):159-65.
67. Bialojan C, Takai A. Inhibitory effect of a marine-sponge toxin, okadaic acid, on protein phosphatases. Specificity and kinetics. Biochem J. 1988;256(1):283-90.

68. Vieira AC, Rubiolo JA, Lopez-Alonso H, Cifuentes JM, Alfonso A, Bermudez R, et al. Oral toxicity of okadaic acid in mice: study of lethality, organ damage, distribution and effects on detoxifying gene expression. Toxins. 2013;5(11):2093-108.
69. Vale P, de Sampayo MA. First confirmation of human diarrhoeic poisonings by okadaic acid esters after ingestion of razor clams (*Solen marginatus*) and green crabs (*Carcinus maenas*) in Aveiro lagoon, Portugal and detection of okadaic acid esters in phytoplankton. Toxicon. 2002;40(7):989-96.
70. Abal P, Louzao MC, Suzuki T, Watanabe R, Vilarino N, Carrera C, et al. Toxic Action Reevaluation of Okadaic Acid, Dinophysistoxin-1 and Dinophysistoxin-2: Toxicity Equivalency Factors Based on the Oral Toxicity Study. Cell Physiol Biochem. 2018;49(2):743-57.
71. Abal P, Louzao MC, Cifuentes JM, Vilarino N, Rodriguez I, Alfonso A, et al. Characterization of the dinophysistoxin-2 acute oral toxicity in mice to define the Toxicity Equivalency Factor. Food Chem Toxicol. 2017;102:166-75.
72. Fujiki H, Suganuma M. Unique features of the okadaic acid activity class of tumor promoters. J Cancer Res Clin Oncol. 1999;125(3-4):150-5.
73. EFSA. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – okadaic acid and analogues. EFSA J. 2008;589:1-62.
74. Reguera B, Blanco J. *Dinophysis* Toxins: Distribution, Fate in Shellfish and Impacts. Toxins. 2019;11(7).
75. Yasumoto T, Murata M, Oshima Y, Sano M, Matsumoto GK, Clardy J. Diarrhetic shellfish toxins. Tetrahedron. 1985;41(6):1019-25.
76. Allingham JS, Miles CO, Rayment I. A structural basis for regulation of actin polymerization by pectenotoxins. J Mol Biol. 2007;371(4):959-70.
77. EFSA. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission on marine biotoxins in shellfish – pectenotoxin group. EFSA J. 2009;1109:1-47.
78. Espina B, Louzao MC, Ares IR, Cagide E, Vieytes MR, Vega FV, et al. Cytoskeletal toxicity of pectenotoxins in hepatic cells. Br J Pharmacol. 2008;155(6):934-44.
79. Espina B, Louzao MC, Ares IR, Fonfria ES, Vilarino N, Vieytes MR, et al. Impact of the pectenotoxin C-43 oxidation degree on its cytotoxic effect on rat hepatocytes. Chem Res Toxicol. 2010;23(3):504-15.
80. Ito E, Suzuki T, Oshima Y, Yasumoto T. Studies of diarrhetic activity on pectenotoxin-6 in the mouse and rat. Toxicon. 2008;51(4):707-16.
81. Munday JS. Toxicology of the pectenotoxins. In: Botana LM, editor. Seafood and freshwater toxins: pharmacology, physiology, and detection. 2nd ed. Boca Raton, CRC Press; 2008. p. 371-80.
82. Murata M, Kumagai M, Lee JS, Yasumoto T. Isolation and structure of yessotoxin, a novel polyether compound implicated in diarrhetic shellfish poisoning. Tetrahedron Lett. 1987;28(47):5869-72.
83. Tubaro A, Dell'Ovo V, Sosa S, Florio C. Yessotoxins: A toxicological overview. Toxicon. 2010;56(2):163-72.
84. Miles CO, Samdal IA, Aasen JAG, Jensen DJ, Quilliam MA, Petersen D, et al. Evidence for numerous analogs of yessotoxin in *Protoceratium reticulatum*. Harmful Algae. 2005;4(6):1075-91.
85. Alfonso A, Vieytes MR, Botana LM. Yessotoxin, a Promising Therapeutic Tool. Mar Drugs. 2016;14(2).

86. Fernandez-Araujo A, Alfonso A, Vieytes MR, Botana LM. Key role of phosphodiesterase 4A (PDE4A) in autophagy triggered by yessotoxin. Toxicology. 2015;329:60-72.
87. Pazos MJ, Alfonso A, Vieytes MR, Yasumoto T, Botana LM. Kinetic analysis of the interaction between yessotoxin and analogues and immobilized phosphodiesterases using a resonant mirror optical biosensor. Chem Res Toxicol. 2005;18(7):1155-60.
88. Tobio A, Fernandez-Araujo A, Alfonso A, Botana LM. Role of yessotoxin in calcium and cAMP-crosstalks in primary and K-562 human lymphocytes: the effect is mediated by anchor kinase A mitochondrial proteins. J Cell Biochem. 2012;113(12):3752-61.
89. EFSA. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – yessotoxin group. EFSA J. 2008;907:1-62.
90. Rossi R, Dell'Aversano C, Krock B, Ciminiello P, Percopo I, Tillmann U, et al. Mediterranean *Azadinium dexteroporum* (Dinophyceae) produces six novel azaspiracids and azaspiracid-35: a structural study by a multi-platform mass spectrometry approach. Anal Bioanal Chem. 2017;409(4):1121-34.
91. Miles CO, Kilcoyne J, McCarron P, Giddings SD, Waaler T, Rundberget T, et al. Selective Extraction and Purification of Azaspiracids from Blue Mussels (*Mytilus edulis*) Using Boric Acid Gel. J Agric Food Chem. 2018;66(11):2962-9.
92. Tillmann U, Borel CM, Barrera F, Lara R, Krock B, Almandoz GO, et al. *Azadinium poporum* from the Argentine Continental Shelf, Southwestern Atlantic, produces azaspiracid-2 and azaspiracid-2 phosphate. Harmful Algae. 2016;51:40-55.
93. Satake M, Ofuji K, Naoki H, James KJ, Furey A, McMahon T, et al. Azaspiracid, a new marine toxin having unique spiro ring assemblies, isolated from Irish mussels, *Mytilus edulis*. J Am Chem Soc. 1998;120(38):9967-8.
94. Riobó P, Franco JM, López E. Chemical methods for detecting phycotoxins: LC and LC/MS/MS. In: Cabado AG, Vieites JM, editors. New Trends in Marine and Freshwater Toxins: Food and Safety Concerns, Nova Science; 2011.
95. Furey A, Moroney C, Brana-Magdalena A, Saez MJ, Lehane M, James KJ. Geographical, temporal, and species variation of the polyether toxins, azaspiracids, in shellfish. Environ Sci Technol. 2003;37(14):3078-84.
96. Aasen JAB, Espenes A, Hess P, Aune T. Sub-lethal dosing of azaspiracid-1 in female NMRI mice. Toxicon. 2010;56(8):1419-25.
97. Pelin M, Kilcoyne J, Nulty C, Crain S, Hess P, Tubaro A, et al. Toxic equivalency factors (TEFs) after acute oral exposure of azaspiracid 1, -2 and -3 in mice. Toxicol Lett. 2018;282:136-46.
98. Roman Y, Alfonso A, Vieytes MR, Ofuji K, Satake M, Yasumoto T, et al. Effects of Azaspiracids 2 and 3 on intracellular cAMP, [Ca2+], and pH. Chem Res Toxicol. 2004;17(10):1338-49.
99. Vale C, Wandscheer C, Nicolaou KC, Frederick MO, Alfonso C, Vieytes MR, et al. Cytotoxic effect of azaspiracid-2 and azaspiracid-2-methyl ester in cultured neurons: involvement of the c-Jun N-terminal kinase. J Neurosci Res. 2008;86(13):2952-62.
100. Ferreiro SF, Vilarino N, Carrera C, Louzao MC, Santamarina G, Cantalapiedra AG, et al. In vivo cardiomyocyte response to YTX- and AZA-1-induced damage: autophagy versus apoptosis. Arch Toxicol. 2017;91(4):1859-70.
101. EFSA. Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on marine biotoxins in shellfish – azaspiracids. EFSA J. 2008;723:1-52.

102. Gobler CJ, Doherty OM, Hattenrath-Lehmann TK, Griffith AW, Kang Y, Litaker RW. Ocean warming since 1982 has expanded the niche of toxic algal blooms in the North Atlantic and North Pacific oceans. Proc Natl Acad Sci U S A. 2017;114(19):4975-80.
103. Bravo J, Suárez F, Ramírez A, Acosta F. Ciguatera, an emerging human poisoning in Europe. J Aquac Mar Biol. 2015;3:00053.
104. EFSA. Scientific Opinion on marine biotoxins in shellfish– Emerging toxins: Brevetoxin group. EFSA J. 2010;8(7):1677-706.
105. Tubaro A, Favero G, Pelin M, Bignami G, Poli M. Palytoxin and analogues: biological effects and detection. In: Botana LM, editor. Seafood and freshwater toxins: pharmacology, physiology, and detection. Boca Raton, CRC Press; 2014.
106. Rodriguez P, Alfonso A, Vale C, Alfonso C, Vale P, Tellez A, et al. First toxicity report of tetrodotoxin and 5,6,11-trideoxyTTX in the trumpet shell *Charonia lampas* in Europe. Anal Chem. 2008;80(14):5622-9.
107. Hwang DF, Noguchi T. Tetrodotoxin Poisoning. Advances in Food and Nutrition Research. 52, Academic Press; 2007. p. 141-236.
108. EFSA. Scientific opinion on the risks for public health related to the presence of tetrodotoxin (TTX) and TTX analogues in marine bivalves and gastropods. EFSA J. 2017;15(4):4752-817.
109. Perez-Arellano JL, Luzardo OP, Perez Brito A, Hernandez Cabrera M, Zumbado M, Carranza C, et al. Ciguatera fish poisoning, Canary Islands. Emerg Infect Dis. 2005;11(12):1981-2.
110. Otero P, Perez S, Alfonso A, Vale C, Rodriguez P, Gouveia NN, et al. First toxin profile of ciguateric fish in Madeira Arquipelago (Europe). Anal Chem. 2010;82(14):6032-9.
111. EFSA. Scientific Opinion on marine biotoxins in shellfish–Emerging toxins: Ciguatoxin group. EFSA J. 2010;8(6):1627.
112. Seki T, Satake M, Mackenzie L, Kaspar HF, Yasumoto T. Gymnodimine, a new marine toxin of unprecedented structure isolated from New Zealand oysters and the dinoflagellate, *Gymnodinium sp*. Tetrahedron Letters. 1995;36(39):7093-6.
113. Campbell K, Vilariño N, Botana LM, Elliott CT. A European perspective on progress in moving away from the mouse bioassay for marine-toxin analysis. TrAC, Trends Anal Chem. 2011;30(2):239-53.
114. Otero A, Chapela MJ, Atanassova M, Vieites JM, Cabado AG. Cyclic imines: chemistry and mechanism of action: a review. Chem Res Toxicol. 2011;24(11):1817-29.
115. Molgo J, Marchot P, Araoz R, Benoit E, Iorga BI, Zakarian A, et al. Cyclic imine toxins from dinoflagellates: a growing family of potent antagonists of the nicotinic acetylcholine receptors. J Neurochem. 2017;142 Suppl 2:41-51.
116. Silva M, Pratheepa VK, Botana LM, Vasconcelos V. Emergent toxins in North Atlantic temperate waters: a challenge for monitoring programs and legislation. Toxins. 2015;7(3):859-85.
117. Manning SR, Nobles DR. Impact of global warming on water toxicity: cyanotoxins. Curr Opin Food Sci. 2017;18:14-20.
118. Merel S, Walker D, Chicana R, Snyder S, Baurès E, Thomas O. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environment International. 2013;59:303-27.
119. Testai E, Buratti FM, Funari E, Manganelli M, Vichi S, Arnich N, et al. Review and analysis of occurrence, exposure and toxicity of cyanobacteria toxins in food. EFSA Supporting Publications. 2016;13(2):998E.

120. Corbel S, Mougin C, Nelieu S, Delarue G, Bouaicha N. Evaluation of the transfer and the accumulation of microcystins in tomato (*Solanum lycopersicum* cultivar MicroTom) tissues using a cyanobacterial extract containing microcystins and the radiolabeled microcystin-LR ((14)C-MC-LR). Sci. Total Environ. 2016;541:1052-8.
121. Vichi S, Lavorini P, Funari E, Scardala S, Testai E. Contamination by Microcystis and microcystins of blue-green algae food supplements (BGAS) on the Italian market and possible risk for the exposed population. Food Chem. Toxicol. 2012;50(12):4493-9.
122. WHO. Cyanobacterial toxins: microcystin-LR. Guidelines for drinking water quality. World Health Organization, Geneva, 1998.
123. Burch MD. Effective doses, guidelines & regulations. Adv Exp Med Biol. 2008;619:831-53.
124. Chorus I. Current approaches to Cyanotoxin risk assessment, risk management and regulations in different countries. Federal Environment Agency, 2012.
125. Merel S, Walker D, Chicana R, Snyder S, Baures E, Thomas O. State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ Int. 2013;59:303-27.
126. Bennett JW, Klich M. Mycotoxins. Clin Microbiol Rev. 2003;16(3):497-516.
127. Sainz MJ, Alfonso A, Botana LM. Considerations about international mycotoxin legislation, food security, and climate change. In: Botana LM, Sainz MJ, editors. Climate Change and Mycotoxins. Berlín, De Gruyter; 2015.
128. Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union, L 364, p. 5-24.
129. Commission Regulation (EU) No 212/2014 of 6 March 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of the contaminant citrinin in food supplements based on rice fermented with red yeast Monascus purpureus. Official Journal of the European Union. L 67, p. 3-4.
130. Commission Regulation (EU) 2015/1940 of 28 October 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of ergot sclerotia in certain unprocessed cereals and the provisions on monitoring and reporting. Official Journal of the European Union, L 283, p. 3-6.
131. Commission Recommendation (2013/165/EU) of 27 March 2013 on the presence of T-2 and HT-2 toxin in cereals and cereal products. Official Journal of the European Union, L 91, p. 12-15.
132. Directive 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed. Official Journal, L 140, p. 10-22.
133. Commission Recommendation (2006/576/EC) of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Official Journal of the European Union. L 229, p. 7-9.
134. Commission Regulation (EU) No 574/2011 of 16 June 2011 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council as regards maximum levels for nitrite, melamine, *Ambrosia spp*. and carry-over of certain coccidiostats and histomonostats and consolidating Annexes I and II thereto. Official Journal of the European Union, L 159, p. 7-24.
135. EFSA. Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. EFSA J. 2014;12(12):3916.

136. Rychlik M, Humpf HU, Marko D, Danicke S, Mally A, Berthiller F, et al. Proposal of a comprehensive definition of modified and other forms of mycotoxins including "masked" mycotoxins. Mycotoxin research. 2014;30(4):197-205.
137. Berthiller F, Schuhmacher R, Adam G, Krska R. Formation, determination and significance of masked and other conjugated mycotoxins. Anal Bioanal Chem. 2009;395(5):1243-52.
138. Berthiller F, Crews C, Dall'Asta C, Saeger SD, Haesaert G, Karlovsky P, et al. Masked mycotoxins: a review. Mol Nutr Food Res. 2013;57(1):165-86.
139. Jestoi M. Emerging *Fusarium*-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin: a review. Crit Rev Food Sci Nutr. 2008;48(1):21-49.
140. Gruber-Dorninger C, Novak B, Nagl V, Berthiller F. Emerging Mycotoxins: Beyond Traditionally Determined Food Contaminants. J Agric Food Chem. 2017;65(33):7052-70.
141. Walker GM, White NA. Introduction to fungal physiology. In: Kavanagh K, editor. Fungi: biology and applications, John Wiley & Sons; 2017. p. 1-35.
142. Sanchis V, Magan N. Environmental conditions affecting mycotoxins. In: Magan N, Olsen M, editors. Mycotoxins in food: Detection and control. Cambridge, Woodhead 2004. p. 174-89.
143. Kirk P, Cannon P, Minter D, Stalpers J. Dictionary of the fungi 10th edition. CAB International, Wallingford, UK. 2008.
144. Mannaa M, Kim KD. Influence of Temperature and Water Activity on Deleterious Fungi and Mycotoxin Production during Grain Storage. Mycobiology. 2017;45(4):240-54.
145. Sillué SM, Sanchis Almenar V, Ramos Girona AJ. Factores ecofisiológicos que condicionan el crecimiento fúngico y la producción de micotoxinas. In: Ramos JA, editor. Micotoxinas y micotoxicosis. Madrid AMV ediciones 2011.
146. Brase S, Encinas A, Keck J, Nising CF. Chemistry and biology of mycotoxins and related fungal metabolites. Chem Rev. 2009;109(9):3903-90.
147. Ismaiel A, Papenbrock J. Mycotoxins: Producing fungi and mechanisms of phytotoxicity. Agriculture. 2015;5(3):492-537.
148. Calvo AM, Wilson RA, Bok JW, Keller NP. Relationship between secondary metabolism and fungal development. Microbiol Mol Biol Rev. 2002;66(3):447-59.
149. Phoku JZ, Barnard TG, Potgieter N, Dutton MF. Mycotoxigenic potentials of the genera: *Aspergillus*, *Fusarium* and *Penicillium* isolated from houseflies (Musca domestica L.). Acta Trop. 2017;168:29-36.
150. Leslie JF, Summerell BA. The *Fusarium* laboratory manual, John Wiley & Sons; 2008.
151. Sainz M, J., González-Jartín JM, Aguín O, Mansilla JP, Botana LM. Isolation, characterization, and identification of mycotoxin-producing fungi. In: Botana LM, editor. Environmental Toxicology, De Gruyter; 2018.
152. Chandler EA, Simpson DR, Thomsett MA, Nicholson P. Development of PCR assays to Tri7 and Tri13 trichothecene biosynthetic genes, and characterisation of chemotypes of *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium cerealis*. Physiol Mol Plant Pathol. 2003;62(6):355-67.
153. Frisvad JC, Andersen B, Thrane U. The use of secondary metabolite profiling in chemotaxonomy of filamentous fungi. Mycol Res. 2008;112(Pt 2):231-40.
154. Frisvad JC, Larsen TO, de Vries R, Meijer M, Houbraken J, Cabanes FJ, et al. Secondary metabolite profiling, growth profiles and other tools for species recognition and important *Aspergillus* mycotoxins. Stud Mycol. 2007;59:31-7.

155. Sainz MJ, Alfonso A, Botana LM. Climate change and mycotoxins. In: Botana LM, Sainz MJ, editors. Climate change and mycotoxins, De Gruyter; 2015. p. 153–80.
156. Filazi A, Tansel Sireli U. Occurrence of Aflatoxins in Food. In: Razzaghi-Abyaneh M, editor. Aflatoxins - Recent Advances and Future Prospects. Rijeka, InTech; 2013.
157. EC. The Rapid Alert System for Food and Feed - 2017 annual report. Luxembourg, Publications Office of the European Union; 2018.
158. Marimón Sibaja KV, de Oliveira Garcia S, Feltrin ACP, Diaz Remedi R, Cerqueira MBR, Badiale-Furlong E, et al. Aflatoxin biotransformation by commercial peroxidase and its application in contaminated food. J Chem Technol Biotechnol. 2019;94(4):1187-94.
159. Bbosa GS, Kitya D, Odda J, Ogwal-Okeng J. Aflatoxins metabolism, effects on epigenetic mechanisms and their role in carcinogenesis. Health. 2013;5(10):14-34.
160. EFSA. Scientific Opinion of the Panel on Contaminants in the Food Chain on a request from the European Commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. EFSA J. 2007;446:1-127.
161. Fink-Gremmels J. Mycotoxins: their implications for human and animal health. The Veterinary quarterly. 1999;21(4):115-20.
162. Lewis L, Onsongo M, Njapau H, Schurz-Rogers H, Luber G, Kieszak S, et al. Aflatoxin contamination of commercial maize products during an outbreak of acute aflatoxicosis in eastern and central Kenya. Environ Health Perspect. 2005;113(12):1763-7.
163. Coppock RW, Christian RG, Jacobsen BJ. Aflatoxins. In: Gupta RC, editor. Veterinary Toxicology Academic Press; 2018. p. 983-94.
164. Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. Am J Clin Nutr. 2004;80(5):1106-22.
165. IARC. Monographs on the evaluation of carcinogenic risks to humans: Overall evaluations of carcinogenicity: An updating of IARC Monographs. Lyon, France, 1987. 1–403 p.
166. IARC. Chemical Agents and Related Occupations. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Lyon, 2012.
167. Van de Perre E, Jacxsens L, Lachat C, El Tahan F, De Meulenaer B. Impact of maximum levels in European legislation on exposure of mycotoxins in dried products: case of aflatoxin B1 and ochratoxin A in nuts and dried fruits. Food Chem Toxicol. 2015;75:112-7.
168. Freeman GG, Morrison RI. Trichothecin: an Antifungal Metabolic Product of Trichothecium roseum Link. Nature. 1948;162(4105):30-.
169. Moss MO. Secondary metabolism and food intoxication - moulds. J Appl Bacteriol. 1992;73(21):80-8.
170. Fung F, Clark RF. Health effects of mycotoxins: a toxicological overview. J Toxicol Clin Toxicol. 2004;42(2):217-34.
171. Creppy EE. Update of survey, regulation and toxic effects of mycotoxins in Europe. Toxicol Lett. 2002;127(1-3):19-28.
172. Rocha O, Ansari K, Doohan FM. Effects of trichothecene mycotoxins on eukaryotic cells: a review. Food Addit Contam. 2005;22(4):369-78.
173. Shank RA, Foroud NA, Hazendonk P, Eudes F, Blackwell BA. Current and future experimental strategies for structural analysis of trichothecene mycotoxins-a prospectus. Toxins. 2011;3(12):1518-53.

174. Dohnal V, Jezkova A, Jun D, Kuca K. Metabolic pathways of T-2 toxin. Curr Drug Metab. 2008;9(1):77-82.
175. Wu Q, Dohnal V, Huang L, Kuca K, Yuan Z. Metabolic pathways of trichothecenes. Drug Metab Rev. 2010;42(2):250-67.
176. Pestka JJ, Smolinski AT. Deoxynivalenol: toxicology and potential effects on humans. J Toxicol Environ Health B Crit Rev. 2005;8(1):39-69.
177. Nagl V, Woechtl B, Schwartz-Zimmermann HE, Hennig-Pauka I, Moll WD, Adam G, et al. Metabolism of the masked mycotoxin deoxynivalenol-3-glucoside in pigs. Toxicol Lett. 2014;229(1):190-7.
178. Le Drean G, Auffret M, Batina P, Arnold F, Sibiril Y, Arzur D, et al. Myelotoxicity of trichothecenes and apoptosis: an in vitro study on human cord blood CD34+ hematopoietic progenitor. Toxicol In Vitro. 2005;19(8):1015-24.
179. Lutsky, II, Mor N. Alimentary toxic aleukia (septic angina, endemic panmyelotoxicosis, alimentary hemorrhagic aleukia): t-2 toxin-induced intoxication of cats. Am J Pathol. 1981;104(2):189-91.
180. Joffe AZ. *Fusarium* species: their biology and toxicology, John Wiley & Sons; 1986.
181. Meissonnier GM, Laffitte J, Raymond I, Benoit E, Cossalter AM, Pinton P, et al. Subclinical doses of T-2 toxin impair acquired immune response and liver cytochrome P450 in pigs. Toxicology. 2008;247(1):46-54.
182. EFSA. Deoxynivalenol in food and feed: occurrence and exposure. EFSA J. 2013;11(10):3379-435.
183. EFSA. Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. EFSA J. 2011;9(12):2481-668.
184. Galbenu P, Damiescu L, Trif A. Zearalenone occurrence in cereal and cereal-based foodstuffs marketed in Timis County. Res J Agric Sci. 2011;43(1):43-9.
185. Stob M, Baldwin RS, Tuite J, Andrews FN, Gillette KG. Isolation of an Anabolic, Uterotrophic Compound from Corn infected with Gibberella zeae. Nature. 1962;196(4861):1318-.
186. Urry WH, Wehrmeister HL, Hodge EB, Hidy PH. The structure of zearalenone. Tetrahedron Lett. 1966;7(27):3109-14.
187. Shier WT, Shier AC, Xie W, Mirocha CJ. Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. Toxicon. 2001;39(9):1435-8.
188. Shin BS, Hong SH, Bulitta JB, Hwang SW, Kim HJ, Lee JB, et al. Disposition, oral bioavailability, and tissue distribution of zearalenone in rats at various dose levels. J Toxicol Environ Health A. 2009;72(21-22):1406-11.
189. Malekinejad H, Maas-Bakker R, Fink-Gremmels J. Species differences in the hepatic biotransformation of zearalenone. Vet J. 2006;172(1):96-102.
190. Mirocha CJ, Pathre SV, Robison TS. Comparative metabolism of zearalenone and transmission into bovine milk. Food Cosmet Toxicol. 1981;19(1):25-30.
191. Minervini F, Dell'Aquila ME, Maritato F, Minoia P, Visconti A. Toxic effects of the mycotoxin zearalenone and its derivatives on in vitro maturation of bovine oocytes and 17 beta-estradiol levels in mural granulosa cell cultures. Toxicol In Vitro. 2001;15(4-5):489-95.
192. Program NT. Carcinogenesis Bioassay of Zearalenone (CAS No. 17924-92-4) in F344/N Rats and B6C3F1 Mice (Feed Study). Natl Toxicol Program Tech Rep Ser. 1982;235:1-155.

193. Turcotte JC, Hunt PJ, Blaustein JD. Estrogenic effects of zearalenone on the expression of progestin receptors and sexual behavior in female rats. Horm Behav. 2005;47(2):178-84.
194. EFSA. Scientific Opinion on the risks for public health related to the presence of zearalenone in food. EFSA J. 2011;9(6):2197-321.
195. Rheeder JP, Marasas WFO, Vismer HF. Production of Fumonisin Analogs by *Fusarium* Species. Appl Environ Microbiol. 2002;68(5):2101-5.
196. Janse van Rensburg B, McLaren NW, Flett BC. Grain colonization by fumonisin-producing *Fusarium spp*. and fumonisin synthesis in South African commercial maize in relation to prevailing weather conditions. Crop Prot. 2017;102:129-36.
197. Gelderblom WC, Jaskiewicz K, Marasas WF, Thiel PG, Horak RM, Vleggaar R, et al. Fumonisins-novel mycotoxins with cancer-promoting activity produced by *Fusarium moniliforme*. Appl Environ Microbiol. 1988;54(7):1806-11.
198. EFSA. Risks for animal health related to the presence of fumonisins, their modified forms and hidden forms in feed. EFSA J. 2018;16(5):e05242.
199. Shier WT. The fumonisin paradox: a review of research on oral bioavailability of fumonisin B1, a mycotoxin produced by *Fusarium moniliforme*. J Toxicol Toxin Rev. 2000;19(2):161-87.
200. Norred WP, Plattner RD, Chamberlain WJ. Distribution and excretion of [14C] fumonisin B1 in male Sprague-Dawley rats. Nat Toxins. 1993;1(6):341-6.
201. Wang E, Ross PF, Wilson TM, Riley RT, Merrill AH, Jr. Increases in serum sphingosine and sphinganine and decreases in complex sphingolipids in ponies given feed containing fumonisins, mycotoxins produced by *Fusarium moniliforme*. J Nutr. 1992;122(8):1706-16.
202. Merrill AH, Jr., Sullards MC, Wang E, Voss KA, Riley RT. Sphingolipid metabolism: roles in signal transduction and disruption by fumonisins. Environ Health Perspect. 2001;109 (2):283-9.
203. Marasas WF, Riley RT, Hendricks KA, Stevens VL, Sadler TW, Gelineau-van Waes J, et al. Fumonisins disrupt sphingolipid metabolism, folate transport, and neural tube development in embryo culture and in vivo: a potential risk factor for human neural tube defects among populations consuming fumonisin-contaminated maize. J Nutr. 2004;134(4):711-6.
204. Marasas WF, Kellerman TS, Gelderblom WC, Coetzer JA, Thiel PG, van der Lugt JJ. Leukoencephalomalacia in a horse induced by fumonisin B1 isolated from *Fusarium moniliforme*. Onderstepoort J Vet Res. 1988;55(4):197-203.
205. Smith GW, Constable PD, Eppley RM, Tumbleson ME, Gumprecht LA, Haschek-Hock WM. Purified fumonisin B(1) decreases cardiovascular function but does not alter pulmonary capillary permeability in swine. Toxicl Sci. 2000;56(1):240-9.
206. Stockmann-Juvala H, Savolainen K. A review of the toxic effects and mechanisms of action of fumonisin B1. Hum Exp Toxicol. 2008;27(11):799-809.
207. Chu FS, Li GY. Simultaneous occurrence of fumonisin B1 and other mycotoxins in moldy corn collected from the People's Republic of China in regions with high incidences of esophageal cancer. Appl Environ Microbiol. 1994;60(3):847-52.
208. FAO/WHO. Fumonisins. Safety evaluation of certain food additives and contaminants Food Additives Series 65. Geneva, WHO 2012.
209. Chiotta ML, Ponsone ML, Combina M, Torres AM, Chulze SN. Aspergillus section *Nigri* species isolated from different wine-grape growing regions in Argentina. Int J Food Microbiol. 2009;136(1):137-41.

210. Abarca ML, Accensi F, Bragulat MR, Castella G, Cabanes FJ. *Aspergillus carbonarius* as the main source of ochratoxin A contamination in dried vine fruits from the Spanish market. J Food Prot. 2003;66(3):504-6.
211. van der Merwe KJ, Steyn PS, Fourie L, Scott DB, Theron JJ. Ochratoxin A, a toxic metabolite produced by *Aspergillus ochraceus* Wilh. Nature. 1965;205(976):1112-3.
212. Remiro R, Ibanez-Vea M, Gonzalez-Penas E, Lizarraga E. Validation of a liquid chromatography method for the simultaneous quantification of ochratoxin A and its analogues in red wines. J Chromatogr A. 2010;1217(52):8249-56.
213. Hagelberg S, Hult K, Fuchs R. Toxicokinetics of ochratoxin A in several species and its plasma-binding properties. J Appl Toxicol. 1989;9(2):91-6.
214. Petzinger E, Ziegler K. Ochratoxin A from a toxicological perspective. J Vet Pharmacol Ther. 2000;23(2):91-8.
215. Gross-Steinmeyer K, Weymann J, Hege HG, Metzler M. Metabolism and lack of DNA reactivity of the mycotoxin ochratoxin A in cultured rat and human primary hepatocytes. J Agric Food Chem. 2002;50(4):938-45.
216. Mally A, Keim-Heusler H, Amberg A, Kurz M, Zepnik H, Mantle P, et al. Biotransformation and nephrotoxicity of ochratoxin B in rats. Toxicol Appl Pharmacol. 2005;206(1):43-53.
217. Creppy EE, Roschenthaler R, Dirheimer G. Inhibition of protein synthesis in mice by ochratoxin A and its prevention by phenylalanine. Food Chem Toxicol. 1984;22(11):883-6.
218. Omar RF, Rahimtula AD, Bartsch H. Role of cytochrome P-450 in ochratoxin A-stimulated lipid peroxidation. J Biochem Toxicol. 1991;6(3):203-9.
219. Ringot D, Chango A, Schneider YJ, Larondelle Y. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chem Biol Interact. 2006;159(1):18-46.
220. Walker R, Larsen JC. Ochratoxin A: previous risk assessments and issues arising. Food Addit Contam. 2005;22 (1):6-9.
221. Pfohl-Leszkowicz A, Manderville RA. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res. 2007;51(1):61-99.
222. Al-Anati L, Petzinger E. Immunotoxic activity of ochratoxin A. J Vet Pharmacol Ther. 2006;29(2):79-90.
223. Gayathri L, Dhivya R, Dhanasekaran D, Periasamy VS, Alshatwi AA, Akbarsha MA. Hepatotoxic effect of ochratoxin A and citrinin, alone and in combination, and protective effect of vitamin E: In vitro study in HepG2 cell. Food Chem Toxicol. 2015;83:151-63.
224. Patil RD, Dwivedi P, Sharma AK. Critical period and minimum single oral dose of ochratoxin A for inducing developmental toxicity in pregnant Wistar rats. Reprod Toxicol. 2006;22(4):679-87.
225. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occuring Substances: Food Items and Costituents, Heterocyclic Aromatic Amines and Mycotoxins. Lyon, France, 1993.
226. Ostry V, Malir F, Toman J, Grosse Y. Mycotoxins as human carcinogens-the IARC Monographs classification. Mycotoxin Res. 2017;33(1):65-73.
227. EFSA. Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to ochratoxin A in food. EFSA J. 2006;365:1-36.
228. Doughari J. The occurrence, properties and significance of citrinin mycotoxin. J Plant Pathol Microbiol. 2015;6(11):2.

229. Hetherington AC, Raistrick H. On the Production and Chemical Constitution of a New Yellow Colouring Matter, Citrinin, Produced from Glucose by *Penicillium citrinum* Thom. Philos Trans R Soc Lond B Biol Sci. 1931;220:269-95.
230. Ambrose A, De Eds F. Some toxicological and pharmacological properties of citrinin. J Pharmacol Exp Ther. 1946;88(2):173-86.
231. Chrevatidis A. Mycotoxins. Occurrence and determination. In: Caballero B, editor. Encyclopedia of Food Sciences and Nutrition 2ed. Oxford, Academic Press; 2003. p. 4089-96.
232. de Oliveira Filho JWG, Islam MT, Ali ES, Uddin SJ, Santos JVO, de Alencar M, et al. A comprehensive review on biological properties of citrinin. Food Chem Toxicol. 2017;110:130-41.
233. Phillips RD, Berndt WO, Hayes AW. Distribution and excretion of [14C]citrinin in rats. Toxicology. 1979;12(3):285-98.
234. EFSA. Scientific Opinion on the risks for public and animal health related to the presence of citrinin in food and feed. EFSA J. 2012;10(3):2605-87.
235. Mehdi NA, Carlton WW, Tuite J. Acute toxicity of citrinin in turkeys and ducklings. Avian Pathol. 1983;12(2):221-33.
236. Flajs D, Peraica M. Toxicological properties of citrinin. Arch Ind Hyg Toxicol 2009;60(4):457-64.
237. Arai M, Hibino T. Tumorigenicity of citrinin in male F344 rats. Cancer letters. 1983;17(3):281-7.
238. Chan WH. Citrinin induces apoptosis via a mitochondria-dependent pathway and inhibition of survival signals in embryonic stem cells, and causes developmental injury in blastocysts. Biochem J. 2007;404(2):317-26.
239. Qingqing H, Linbo Y, Yunqian G, Shuqiang L. Toxic effects of citrinin on the male reproductive system in mice. Exp Toxicol Pathol. 2012;64(5):465-9.
240. Dombrink-Kurtzman MA, Blackburn JA. Evaluation of several culture media for production of patulin by Penicillium species. Int J Food Microbiol. 2005;98(3):241-8.
241. Birkinshaw JH, Bracken A, Michael SE, Raistrick H. Biochemistry and Chemistry. The Lancet. 1943;242(6273):625-30.
242. Guray T, Tuncel M, Uysal UD. A rapid determination of patulin using capillary zone electrophoresis and its application to analysis of apple juices. J Chromatogr Sci. 2013;51(4):310-7.
243. Dailey RE, Blaschka AM, Brouwer EA. Absorption, distribution, and excretion of [14C] patulin by rats. J Toxicol Environ Health. 1977;3(3):479-89.
244. Puel O, Galtier P, Oswald IP. Biosynthesis and toxicological effects of patulin. Toxins. 2010;2(4):613-31.
245. Saleh I, Goktepe I. The characteristics, occurrence, and toxicological effects of patulin. Food Chem Toxicol. 2019;129:301-11.
246. Jin H, Yin S, Song X, Zhang E, Fan L, Hu H. p53 activation contributes to patulin-induced nephrotoxicity via modulation of reactive oxygen species generation. Sci Rep. 2016;6.
247. McKinley ER, Carlton WW, Boon GD. Patulin mycotoxicosis in the rat: toxicology, pathology and clinical pathology. Food Chem Toxicol. 1982;20(3):289-300.
248. Assuncao R, Alvito P, Kleiveland CR, Lea TE. Characterization of in vitro effects of patulin on intestinal epithelial and immune cells. Toxicol Lett. 2016;250-251:47-56.
249. Moake MM, Padilla-Zakour OI, Worobo RW. Comprehensive review of patulin control methods in foods. Compr Rev Food Sci Food Saf. 2005;4(1):8-21.

250. Robert H, Payros D, Pinton P, Theodorou V, Mercier-Bonin M, Oswald IP. Impact of mycotoxins on the intestine: are mucus and microbiota new targets? J Toxicol Environ Health B Crit Rev. 2017;20(5):249-75.
251. Glaser N, Stopper H. Patulin: Mechanism of genotoxicity. Food Chem Toxicol. 2012;50(5):1796-801.
252. WHO. Evaluation of certain food additives and contaminants: forty-fourth report of the Joint FAO/WHO Expert Committee on Food Additives. Geneva, World Health Organization; 1995.
253. Lorenz N, Wilson EV, Machado C, Schardl CL, Tudzynski P. Comparison of ergot alkaloid biosynthesis gene clusters in *Claviceps* species indicates loss of late pathway steps in evolution of *C. fusiformis*. Appl Environ Microbiol. 2007;73(22):7185-91.
254. Tudzynski P, Correia T, Keller U. Biotechnology and genetics of ergot alkaloids. Appl Microbiol Biotechnol. 2001;57(5-6):593-605.
255. EFSA. Scientific Opinion on Ergot alkaloids in food and feed. EFSA J. 2012;10(7):2798-956.
256. Aellig WH, Nuesch E. Comparative pharmacokinetic investigations with tritium-labeled ergot alkaloids after oral and intravenous administration man. Int J Clin Pharmacol Biopharm. 1977;15(3):106-12.
257. Griffith RW, Grauwiler J, Hodel C, Leist KH, Matter B. Toxicologic considerations. In: Berde B, Schild HO, editors. Ergot alkaloids and related compounds. Berlin, Springer; 1978. p. 805-51.
258. Logrieco A, Moretti A, Castella G, Kostecki M, Golinski P, Ritieni A, et al. Beauvericin Production by *Fusarium* Species. Appl Environ Microbiol. 1998;64(8):3084-8.
259. Mallebrera B, Prosperini A, Font G, Ruiz MJ. In vitro mechanisms of Beauvericin toxicity: A review. Food Chem Toxicol. 2018;111:537-45.
260. Mallebrera B, Juan-Garcia A, Font G, Ruiz MJ. Mechanisms of beauvericin toxicity and antioxidant cellular defense. Toxicol Lett. 2016;246:28-34.
261. EFSA. Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. EFSA J. 2014;12(8):3802-949.
262. Blesa J, Marín R, Lino CM, Mañes J. Evaluation of enniatins A, A1, B, B1 and beauvericin in Portuguese cereal-based foods. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2012;29(11):1727-35.
263. Sy-Cordero AA, Pearce CJ, Oberlies NH. Revisiting the enniatins: a review of their isolation, biosynthesis, structure determination and biological activities. J Antibiot (Tokyo). 2012;65(11):541-9.
264. Ivanova L, Skjerve E, Eriksen GS, Uhlig S. Cytotoxicity of enniatins A, A1, B, B1, B2 and B3 from *Fusarium avenaceum*. Toxicon. 2006;47(8):868-76.
265. Juan C, Manyes L, Font G, Juan-García A. Evaluation of immunologic effect of Enniatin A and quantitative determination in feces, urine and serum on treated Wistar rats. Toxicon. 2014;87:45-53.
266. Hallas-Moller M, Nielsen KF, Frisvad JC. Production of the *Fusarium* Mycotoxin Moniliformin by *Penicillium melanoconidium*. J Agric Food Chem. 2016;64(22):4505-10.
267. Toro PCD, Arévalo FJ, Fumero MV, Zon MA, Fernández H. Very sensitive electrochemical sensor for moniliformin detection in maize samples. Sens Actuators B Chem. 2016;225:384-90.
268. EFSA. Risks to human and animal health related to the presence of moniliformin in food and feed. EFSA J. 2018;16(3):5082-177.

269. Rodríguez I, Alfonso A. Analytical instrumentation and principles. In: Botana Luis M, editor. Environmental Toxicology. Berlin De Gruyter; 2018. p. 17-57.
270. Commission Decision 2002/657/EC (2002) implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Official Journal, L 221, p. 8-36.
271. Commission Regulation (EU) 2017/1980 of 31 October 2017 amending Annex III to Regulation (EC) No 2074/2005 as regards paralytic shellfish poison (PSP) detection method. L 285, p. 8-9.
272. Commission Regulation (EC) No 1664/2006 of 6 November 2006 amending Regulation (EC) No 2074/2005 as regards implementing measures for certain products of animal origin intended for human consumption and repealing certain implementing measures. L 320, p. 13-45.
273. Thompson M, Wood R. Using uncertainty functions to predict and specify the performance of analytical methods. Accredit Qual Assur. 2006;10(9):471-8.
274. Lattanzio VMT. Toward Harmonization of Performance Criteria for Mycotoxin Screening Methods: The EU Perspective. J AOAC Int. 2016;99(4):906-13.
275. Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. Official Journal of the European Union. L 70, p. 12-34.
276. Careri M, Mangia A, Musci M. Applications of liquid chromatography-mass spectrometry interfacing systems in food analysis: pesticide, drug and toxic substance residues. J Chromatogr A. 1996;727(2):153-84.
277. Fekete S, Schappler J, Veuthey J-L, Guillarme D. Current and future trends in UHPLC. Trends Analyt Chem. 2014;63:2-13.
278. Bélanger JMR, Jocelyn Paré JR, Sigouin M. Chapter 2 High performance liquid chromatography (HPLC): Principles and applications. In: Paré JRJ, Bélanger JMR, editors. Techniques and Instrumentation in Analytical Chemistry. 18, Elsevier; 1997. p. 37-59.
279. Zhang B, Li X, Yan B. Advances in HPLC detection-towards universal detection. Anal Bioanal Chem. 2008;390(1):299-301.
280. Rouessac F, Rouessac A. Chemical analysis: modern instrumentation methods and techniques, John Wiley & Sons; 2013.
281. Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. Anal Bioanal Chem. 2012;402(1):231-47.
282. Torres-Lapasió JR, García-Álvarez-Coque MC. Levels in the interpretive optimisation of selectivity in high-performance liquid chromatography: A magical mystery tour. J Chromatogr A. 2006;1120(1):308-21.
283. Mass Spectrometry/ Chromatography Coupling Mass Spectrometr. In: De Hoffmann E, Stroobant V, editors. Mass Spectrometry: Principles and Applications. 3 ed. England Wiley 2007.
284. Niessen WMA. Progress in liquid chromatography–mass spectrometry instrumentation and its impact on high-throughput screening. J Chromatogr A. 2003;1000(1):413-36.
285. El-Aneed A, Cohen A, Banoub J. Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers. Appl Spectrosc Rev. 2009;44(3):210-30.
286. Voo ACC, Ng R, Tunstall JJ, Taylor S. Transmission through the quadrupole mass spectrometer mass filter: The effect of aperture and harmonics. J Vac Sci Technol A. 1997;15(4):2276-81.

287. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422(6928):198-207.
288. Glish GL, Vachet RW. The basics of mass spectrometry in the twenty-first century. Nat Rev Drug Discov. 2003;2(2):140-50.
289. Glish GL. Multiple stage mass spectrometry: the next generation tandem mass spectrometry experiment. Analyst. 1994;119(4):533-7.
290. Liu Z-Y. An introduction to hybrid ion trap/time-of-flight mass spectrometry coupled with liquid chromatography applied to drug metabolism studies. J Mass Spectrom. 2012;47(12):1627-42.
291. Chen XF, Wu HT, Tan GG, Zhu ZY, Chai YF. Liquid chromatography coupled with time-of-flight and ion trap mass spectrometry for qualitative analysis of herbal medicines. J Pharm Anal. 2011;1(4):235-45.
292. Tandem mass spectrometry. In: De Hoffmann E, Stroobant V, editors. Mass Spectrometry: Principles and Applications. 3 ed. England Wiley 2007.
293. Kondrat RW, McClusky GA, Cooks RG. Multiple reaction monitoring in mass spectrometry/mass spectrometry for direct analysis of complex mixtures. Anal Chem. 1978;50(14):2017-21.
294. Shephard GS. Current Status of Mycotoxin Analysis: A Critical Review. J AOAC Int. 2016;99(4):842-8.
295. Trullols E, Ruisanchez I, Rius FX. Validation of qualitative analytical methods. TrAC, Trends Anal Chem. 2004;23(2):137-45.
296. Rogatsky E, Stein D. Evaluation of matrix effect and chromatography efficiency: new parameters for validation of method development. Journal of the American Society for Mass Spectrometry. 2005;16(11):1757-9.
297. Wood R. How to validate analytical methods. TrAC, Trends Anal Chem. 1999;18(9-10):624-32.
298. Rambla-Alegre M, Esteve-Romero J, Carda-Broch S. Is it really necessary to validate an analytical method or not? That is the question. J Chromatogr A. 2012;1232:101-9.
299. Zhang L, Dou XW, Zhang C, Logrieco AF, Yang MH. A Review of Current Methods for Analysis of Mycotoxins in Herbal Medicines. Toxins. 2018;10(2):65-104.
300. Turner AD, Hatfield RG, Rapkova M, Higman W, Algoet M, Suarez-Isla BA, et al. Comparison of AOAC 2005.06 LC official method with other methodologies for the quantitation of paralytic shellfish poisoning toxins in UK shellfish species. Anal Bioanal Chem. 2011;399(3):1257-70.
301. Man Y, Liang G, Li A, Pan L. Analytical Methods for the Determination of Alternaria Mycotoxins. Chromatographia. 2017;80(1):9-22.
302. Lee ME, Ko K-h, Park N-H, Lee W, Yoo HH, Lee J, et al. Reliable quantification of trace diarrhetic shellfish poisoning toxins in high-lipid bivalves by UHPLC-ESI-MS/MS in time segment polarity switching mode: Comparison of three extraction methods. Microchem J. 2018;143:441-9.
303. Wu F, Xu C, Jiang N, Wang J, Ding C-F. Poly (methacrylic acid-co-diethenyl-benzene) monolithic microextraction column and its application to simultaneous enrichment and analysis of mycotoxins. Talanta. 2018;178:1-8.
304. Regueiro J, Rossignoli AE, Álvarez G, Blanco J. Automated on-line solid-phase extraction coupled to liquid chromatography–tandem mass spectrometry for determination of lipophilic marine toxins in shellfish. Food Chem. 2011;129(2):533-40.
305. Jin PG, Han Z, Cai ZX, Wu YJ, Ren YP. Simultaneous determination of 10 mycotoxins in grain by ultra-high-performance liquid chromatography–tandem mass spectrometry

using 13C15-deoxynivalenol as internal standard. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2010;27(12):1701-13.
306. These A, Scholz J, Preiss-Weigert A. Sensitive method for the determination of lipophilic marine biotoxins in extracts of mussels and processed shellfish by high-performance liquid chromatography–tandem mass spectrometry based on enrichment by solid-phase extraction. J Chromatogr A. 2009;1216(21):4529-38.
307. Berrueta LA, Gallo B, Vicente F. A review of solid phase extraction: Basic principles and new developments. Chromatographia. 1995;40(7):474-83.
308. Moser AC, Hage DS. Immunoaffinity chromatography: an introduction to applications and recent developments. Bioanalysis. 2010;2(4):769-90.
309. Mattarozzi M, Milioli M, Bianchi F, Cavazza A, Pigozzi S, Milandri A, et al. Optimization of a rapid QuEChERS sample treatment method for HILIC-MS2 analysis of paralytic shellfish poisoning (PSP) toxins in mussels. Food Control. 2016;60:138-45.
310. Koesukwiwat U, Sanguankaew K, Leepipatpiboon N. Evaluation of a modified QuEChERS method for analysis of mycotoxins in rice. Food Chem. 2014;153:44-51.
311. Perestrelo R, Silva P, Porto-Figueira P, Pereira JAM, Silva C, Medina S, et al. QuEChERS - Fundamentals, relevant improvements, applications and future trends. Anal Chim Acta. 2019;1070:1-28.
312. González-Curbelo MÁ, Socas-Rodríguez B, Herrera-Herrera AV, González-Sálamo J, Hernández-Borges J, Rodríguez-Delgado MÁ. Evolution and applications of the QuEChERS method. Trends Analyt Chem. 2015;71:169-85.
313. Wang L, Shi X, Zhao Q, Sun A, Li D, Zhao J. Determination of lipophilic marine toxins in fresh and processed shellfish using modified QuEChERS and ultra-high-performance liquid chromatography–tandem mass spectrometry. Food Chem. 2019;272:427-33.
314. Arroyo-Manzanares N, García-Campaña AM, Gámiz-Gracia L. 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. J Chromatogr A. 2013;1282:11-9.
315. Liu Y, Han S, Lu M, Wang P, Han J, Wang J. Modified QuEChERS method combined with ultra-high performance liquid chromatography tandem mass spectrometry for the simultaneous determination of 26 mycotoxins in sesame butter. J Chromatogr B 2014;970:68-76.
316. Zachariasova M, Lacina O, Malachova A, Kostelanska M, Poustka J, Godula M, et al. Novel approaches in analysis of *Fusarium* mycotoxins in cereals employing ultra performance liquid chromatography coupled with high resolution mass spectrometry. Anal Chim Acta. 2010;662(1):51-61.
317. Holland PT. Analysis of Marine Toxins -Techniques, Method Validation, Calibration Standards, and Screening Methods. In: Botana LM, editor. Seafood and Freshwater Toxins. 2nd ed. Boca Raton, CRC Press; 2008. p. 39-68.
318. EU-RL-MB. EU-harmonised standard operating procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS. 2015.
319. Lawrence JF, Niedzwiadek B, Menard C. Quantitative determination of paralytic shellfish poisoning toxins in shellfish using prechromatographic oxidation and liquid chromatography with fluorescence detection: collaborative study. J AOAC Int. 2005;88(6):1714-32.
320. Boundy MJ, Selwood AI, Harwood DT, McNabb PS, Turner AD. Development of a sensitive and selective liquid chromatography–mass spectrometry method for high

throughput analysis of paralytic shellfish toxins using graphitised carbon solid phase extraction. J Chromatogr A. 2015;1387:1-12.
321. Jansson D, Åstot C. Analysis of paralytic shellfish toxins, potential chemical threat agents, in food using hydrophilic interaction liquid chromatography–mass spectrometry. J Chromatogr A. 2015;1417:41-8.
322. Turner AD, McNabb PS, Harwood DT, Selwood AI, Boundy MJ. Single-Laboratory Validation of a Multitoxin Ultra-Performance LC-Hydrophilic Interaction LC-MS/MS Method for Quantitation of Paralytic Shellfish Toxins in Bivalve Shellfish. J AOAC Int. 2015;98(3):609-21.
323. Rodríguez I, Alfonso A, González-Jartín JM, Vieytes MR, Botana LM. A single run UPLC-MS/MS method for detection of all EU-regulated marine toxins. Talanta. 2018;189:622-8.
324. Thomas KM, Beach DG, Reeves KL, Gibbs RS, Kerrin ES, McCarron P, et al. Hydrophilic interaction liquid chromatography-tandem mass spectrometry for quantitation of paralytic shellfish toxins: validation and application to reference materials. Anal Bioanal Chem. 2017;409(24):5675-87.
325. EU-RL. EU-harmonised standard operating procedure for determination of lipophilic marine biotoxins in molluscs by LC-MS/MS. 2015.
326. Mafra LL, Léger C, Bates SS, Quilliam MA. Analysis of trace levels of domoic acid in seawater and plankton by liquid chromatography without derivatization, using UV or mass spectrometry detection. J Chromatogr A. 2009;1216(32):6003-11.
327. Wang Z, Maucher-Fuquay J, Fire SE, Mikulski CM, Haynes B, Doucette GJ, et al. Optimization of solid-phase extraction and liquid chromatography–tandem mass spectrometry for the determination of domoic acid in seawater, phytoplankton, and mammalian fluids and tissues. Anal Chim Acta. 2012;715:71-9.
328. EU-RL-MB. Determination of Tetrodotoxin by HILIC-MS/MS. 2017.
329. Garcia-Altares M, Casanova A, Bane V, Diogene J, Furey A, de la Iglesia P. Confirmation of pinnatoxins and spirolides in shellfish and passive samplers from Catalonia (Spain) by liquid chromatography coupled with triple quadrupole and high-resolution hybrid tandem mass spectrometry. Mar Drugs. 2014;12(6):3706-32.
330. Shin C, Hwang J-Y, Yoon J-H, Kim S-H, Kang G-J. Simultaneous determination of neurotoxic shellfish toxins (brevetoxins) in commercial shellfish by liquid chromatography tandem mass spectrometry. Food Control. 2018;91:365-71.
331. Rodríguez I, Vieytes MR, Alfonso A. Analytical challenges for regulated marine toxins. Detection methods. Curr Opin Food Sci. 2017;18:29-36.
332. Zheng MZ, Richard JL, Binder J. A review of rapid methods for the analysis of mycotoxins. Mycopathologia. 2006;161(5):261-73.
333. Whitaker TB. Sampling Foods for Mycotoxins. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2006;23(1):50-61.
334. Krska R, Schubert-Ullrich P, Molinelli A, Sulyok M, MacDonald S, Crews C. Mycotoxin analysis: An update. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2008;25(2):152-63.
335. Rahmani A, Jinap S, Soleimany F. Qualitative and Quantitative Analysis of Mycotoxins. Compr Rev Food Sci Food Saf. 2009;8(3):202-51.
336. Rasmussen RR, Storm IMLD, Rasmussen PH, Smedsgaard J, Nielsen KF. Multi-mycotoxin analysis of maize silage by LC-MS/MS. Anal Bioanal Chem. 2010;397(2):765-76.

337. Monbaliu S, Van Poucke C, Detavernier Cl, Dumoulin F, Van De Velde M, Schoeters E, et al. Occurrence of mycotoxins in feed as analyzed by a multi-mycotoxin LC-MS/MS method. J Agric Food Chem. 2010;58(1):66-71.
338. Varga E, Glauner T, Berthiller F, Krska R, Schuhmacher R, Sulyok M. Development and validation of a (semi-)quantitative UHPLC-MS/MS method for the determination of 191 mycotoxins and other fungal metabolites in almonds, hazelnuts, peanuts and pistachios. Anal Bioanal Chem. 2013;405(15):5087-104.
339. Horwitz W, Kamps LR, Boyer KW. Quality assurance in the analysis of foods and trace constituents. J Assoc Off Anal Chem. 1980;63(6):1344-54.
340. Freire L, Sant'Ana AS. Modified mycotoxins: An updated review on their formation, detection, occurrence, and toxic effects. Food Chem Toxicol. 2018;111:189-205.
341. Righetti L, Paglia G, Galaverna G, Dall'Asta C. Recent Advances and Future Challenges in Modified Mycotoxin Analysis: Why HRMS Has Become a Key Instrument in Food Contaminant Research. Toxins. 2016;8(12-34).
342. Anderson DM. Approaches to monitoring, control and management of harmful algal blooms (HABs). Ocean Coast Manag. 2009;52(7):342.
343. Reboreda A, Lago J, Chapela MJ, Vieites JM, Botana LM, Alfonso A, et al. Decrease of marine toxin content in bivalves by industrial processes. Toxicon. 2010;55(2-3):235-43.
344. Driehuis F, Oude Elferink SJ. The impact of the quality of silage on animal health and food safety: a review. The Veterinary quarterly. 2000;22(4):212-6.
345. van der Westhuizen L, Shephard GS, Rheeder JP, Burger HM, Gelderblom WCA, Wild CP, et al. Optimising sorting and washing of home-grown maize to reduce fumonisin contamination under laboratory-controlled conditions. Food Control. 2011;22(3):396-400.
346. Beretta B, Gaiaschi A, Galli CL, Restani P. Patulin in apple-based foods: occurrence and safety evaluation. Food Addit Contam. 2000;17(5):399-406.
347. Fandohan P, Zoumenou D, Hounhouigan DJ, Marasas WFO, Wingfield MJ, Hell K. Fate of aflatoxins and fumonisins during the processing of maize into food products in Benin. Int J Food Microbiol. 2005;98(3):249-59.
348. Karlovsky P, Suman M, Berthiller F, De Meester J, Eisenbrand G, Perrin I, et al. Impact of food processing and detoxification treatments on mycotoxin contamination. Mycotoxin Res. 2016;32(4):179-205.
349. Dombrink-Kurtzman MA, Dvorak TJ, Barron ME, Rooney LW. Effect of nixtamalization (Alkaline cooking) on fumonisin-contaminated corn for production of masa and tortillas. J Agric Food Chem. 2000;48(11):5781-6.
350. Jard G, Liboz T, Mathieu F, Guyonvarc'h A, Lebrihi A. Review of mycotoxin reduction in food and feed: from prevention in the field to detoxification by adsorption or transformation. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2011;28(11):1590-609.
351. Huwig A, Freimund S, Kappeli O, Dutler H. Mycotoxin detoxication of animal feed by different adsorbents. Toxicol Lett. 2001;122(2):179-88.
352. Sprynskyy M, Gadzala-Kopciuch R, Nowak K, Buszewski B. Removal of zearalenone toxin from synthetics gastric and body fluids using talc and diatomite: a batch kinetic study. Colloids Surf B Biointerfaces. 2012;94:7-14.
353. Pfliegler WP, Pusztahelyi T, Pócsi I. Mycotoxins – prevention and decontamination by yeasts. J Basic Microbiol. 2015;55(7):805-18.

354. Kabak B, Dobson AD, Var I. Strategies to prevent mycotoxin contamination of food and animal feed: a review. Crit Rev Food Sci Nutr. 2006;46(8):593-619.
355. Loi M, Fanelli F, Liuzzi VC, Logrieco AF, Mule G. Mycotoxin Biotransformation by Native and Commercial Enzymes: Present and Future Perspectives. Toxins 2017;9(4):111-150.
356. Commission Implementing Regulation (EU) No 1060/2013 of 29 October 2013 concerning the authorisation of bentonite as a feed additive for all animal species.Official Journal of the European Union, L 289, p. 33-37.
357. EFSA. Safety and efficacy of bentonite as a feed additive for all animal species EFSA J. 2017;15(12):5096-109.
358. Fosso-Kankeu E, Mishra AK. Photocatalytic degradation and adsorption techniques involving nanomaterials for biotoxins removal from drinking water. In: Grumezescu AM, editor. Water Purification. London, Academic Press; 2017. p. 323-54.
359. Shannon MA, Bohn PW, Elimelech M, Georgiadis JG, Mariñas BJ, Mayes AM. Science and technology for water purification in the coming decades. Nature. 2008;452:301.
360. Vandermeersch G, Lourenço HM, Alvarez-Muñoz D, Cunha S, Diogène J, Cano-Sancho G, et al. Environmental contaminants of emerging concern in seafood – European database on contaminant levels. Environ Res. 2015;143:29-45.
361. Çelik K. The efficacy of mycotoxin-detoxifying and biotransforming agents in animal nutrition. In: Rai M, Abd-Elsalam KA, editors. Nanomycotoxicology, Academic Press; 2020. p. 271-84.
362. Santos CSC, Gabriel B, Blanchy M, Menes O, García D, Blanco M, et al. Industrial Applications of Nanoparticles – A Prospective Overview. Mater Today Proc. 2015;2(1):456-65.
363. Nanotechnologies I. Vocabulary–Part 1: Core Terms. International Stanardisation Organisation (ISO), Technical Specification ISO/TS. 2015:80004-1.
364. Sanvicens N, Marco MP. Multifunctional nanoparticles – properties and prospects for their use in human medicine. Trends Biotechnol. 2008;26(8):425-33.
365. Lu A-H, Salabas EL, Schüth F. Magnetic Nanoparticles: Synthesis, Protection, Functionalization, and Application. Angew Chem Int Ed. 2007;46(8):1222-44.
366. de Castro Alves L, Yanez-Vilar S, Pineiro-Redondo Y, Rivas J. Novel Magnetic Nanostructured Beads for Cadmium(II) Removal. Nanomaterials. 2019;9(3):356-71.
367. Gleiter H. Nanostructured materials: basic concepts and microstructure. Acta Materialia. 2000;48(1):1-29.
368. Magro M, Moritz DE, Bonaiuto E, Baratella D, Terzo M, Jakubec P, et al. Citrinin mycotoxin recognition and removal by naked magnetic nanoparticles. Food Chem. 2016;203:505-12.
369. Abbasi Pirouz A, Abedi Karjiban R, Abu Bakar F, Selamat J. A Novel Adsorbent Magnetic Graphene Oxide Modified with Chitosan for the Simultaneous Reduction of Mycotoxins. Toxins. 2018;10(9):361-74.
370. Luo Y, Zhou Z, Yue T. Synthesis and characterization of nontoxic chitosan-coated Fe3O4 particles for patulin adsorption in a juice-pH simulation aqueous. Food Chem. 2017;221:317-23.
371. Zahoor M, Ali Khan F. Aflatoxin B1 detoxification by magnetic carbon nanostructures prepared from maize straw. Desalination Water Treat. 2016;57(25):11893-903.
372. Tran CD, Duri S, Delneri A, Franko M. Chitosan-cellulose composite materials: Preparation, Characterization and application for removal of microcystin. J Hazard Mater. 2013;252:355-66.

373. Pelaez M, Baruwati B, Varma RS, Luque R, Dionysiou DD. Microcystin-LR removal from aqueous solutions using a magnetically separable N-doped TiO2 nanocomposite under visible light irradiation. Chem Commun (Camb). 2013;49(86):10118-20.
374. Hena S, Rozi R, Tabassum S, Huda A. Simultaneous removal of potent cyanotoxins from water using magnetophoretic nanoparticle of polypyrrole: adsorption kinetic and isotherm study. Environ Sci Pollut Res Int. 2016;23(15):14868-80.
375. Zhang W, Yan Z, Gao J, Tong P, Liu W, Zhang L. Metal–organic framework UiO-66 modified magnetite@silica core–shell magnetic microspheres for magnetic solid-phase extraction of domoic acid from shellfish samples. J Chromatogr A. 2015;1400:10-8.
376. Salonen LM, Pinela SR, Fernandes SPS, Louçano J, Carbó-Argibay E, Sarriá MP, et al. Adsorption of marine phycotoxin okadaic acid on a covalent organic framework. J Chromatogr A. 2017;1525:17-22.
377. González-Sálamo J, Socas-Rodríguez B, Hernández-Borges J, Rodríguez-Delgado MÁ. Core-shell poly(dopamine) magnetic nanoparticles for the extraction of estrogenic mycotoxins from milk and yogurt prior to LC–MS analysis. Food Chem. 2017;215:362-8.
378. Rai M, Jogee PS, Ingle AP. Emerging nanotechnology for detection of mycotoxins in food and feed. Int J Food Sci Nutr. 2015;66(4):363-70.
379. Hopfgartner G, Chernushevich IV, Covey T, Plomley JB, Bonner R. Exact mass measurement of product ions for the structural elucidation of drug metabolites with a tandem quadrupole orthogonal-acceleration time-of-flight mass spectrometer. J Am Soc Mass Spectrom. 1999;10(12):1305-14.
380. Hoffmann T, Krug D, Huttel S, Muller R. Improving natural products identification through targeted LC-MS/MS in an untargeted secondary metabolomics workflow. Anal Chem. 2014;86(21):10780-8.
381. Gil-Serna J, Vázquez C, Sardiñas N, González-Jaén MT, Patiño B. Revision of ochratoxin a production capacity by the main species of *Aspergillus* section *Circumdati*. *Aspergillus steynii* revealed as the main risk of OTA contamination. Food Control. 2011;22(2):343-5.
382. Sulyok M, Berthiller F, Krska R, Schuhmacher R. Development and validation of a liquid chromatography/tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Commun Mass Spectrom. 2006;20(18):2649-59.
383. Wu Q, Dohnal V, Huang L, Kuca K, Wang X, Chen G, et al. Metabolic pathways of ochratoxin A. Curr Drug Metab. 2011;12(1):1-10.
384. O'Callaghan J, Stapleton PC, Dobson AD. Ochratoxin A biosynthetic genes in *Aspergillus ochraceus* are differentially regulated by pH and nutritional stimuli. Fungal Genet Biol. 2006;43(4):213-21.
385. Zhang D, Yang X, Kang JS, Choi HD, Son BW. Circumdatin I, a new ultraviolet-A protecting benzodiazepine alkaloid from a marine isolate of the fungus *Exophiala*. J Antibiot (Tokyo). 2008;61(1):40-2.
386. Lopez-Gresa MP, Gonzalez MC, Primo J, Moya P, Romero V, Estornell E. Circumdatin H, a new inhibitor of mitochondrial NADH oxidase, from *Aspergillus ochraceus*. J Antibiot (Tokyo). 2005;58(6):416-9.
387. Taevernier L, Wynendaele E, De Vreese L, Burvenich C, De Spiegeleer B. The mycotoxin definition reconsidered towards fungal cyclic depsipeptides. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2016;34(2):114-35.

388. Nakagawa H, Sakamoto S, Sago Y, Kushiro M, Nagashima H. Detection of masked mycotoxins derived from type A trichothecenes in corn by high-resolution LC-Orbitrap mass spectrometer. Food Addit Contam, Part A. 2013;30(8):1407-14.
389. Sainz M, Aguín O, Bande M, Pintos C, Mansilla J. Biodiversity of *Fusarium* species in stalks of forage maize in Galicia (NW Spain). Pastos. 2012;42(1):51-66.
390. Ferrer I, Thurman EM. Liquid chromatography/time-of-flight/mass spectrometry (LC/TOF/MS) for the analysis of emerging contaminants. TrAC, Trends Anal Chem. 2003;22(10):750-6.
391. Jimenez M, Huerta T, Mateo R. Mycotoxin production by *Fusarium* species isolated from bananas. Appl Environ Microbiol. 1997;63(2):364-9.
392. Logrieco A, Moretti A, Castella G, Kostecki M, Golinski P, Ritieni A, et al. Beauvericin Production by *Fusarium* Species. Appl Environ Microbiol. 1998;64(8):3084-8.
393. Streit E, Schwab C, Sulyok M, Naehrer K, Krska R, Schatzmayr G. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. Toxins. 2013;5(3):504-23.
394. Smedsgaard J. Micro-scale extraction procedure for standardized screening of fungal metabolite production in cultures. J Chromatogr A. 1997;760(2):264-70.
395. Zachariasova M, Vaclavikova M, Lacina O, Vaclavik L, Hajslova J. Deoxynivalenol oligoglycosides: new "masked" *Fusarium* toxins occurring in malt, beer, and breadstuff. J Agric Food Chem. 2012;60(36):9280-91.
396. Rodriguez-Carrasco Y, Fattore M, Albrizio S, Berrada H, Manes J. Occurrence of *Fusarium* mycotoxins and their dietary intake through beer consumption by the European population. Food Chem. 2015;178:149-55.
397. Berthiller F, Schuhmacher R, Buttinger G, Krska R. Rapid simultaneous determination of major type A- and B-trichothecenes as well as zearalenone in maize by high performance liquid chromatography-tandem mass spectrometry. J Chromatogr A. 2005;1062(2):209-16.
398. Zachariasova M, Cajka T, Godula M, Malachova A, Veprikova Z, Hajslova J. Analysis of multiple mycotoxins in beer employing (ultra)-high-resolution mass spectrometry. Rapid Commun Mass Spectrom. 2010;24(22):3357-67.
399. Al-Taher F, Banaszewski K, Jackson L, Zweigenbaum J, Ryu D, Cappozzo J. Rapid method for the determination of multiple mycotoxins in wines and beers by LC-MS/MS using a stable isotope dilution assay. J Agric Food Chem. 2013;61(10):2378-84.
400. Binder EM. Managing the risk of mycotoxins in modern feed production. Anim Feed Sci Technol. 2007;133(1):149-66.
401. González-Jartín JM, Alfonso A, Rodríguez I, Sainz MJ, Vieytes MR, Botana LM. A QuEChERS based extraction procedure coupled to UPLC-MS/MS detection for mycotoxins analysis in beer. Food Chem. 2019;275:703-10.
402. Abia WA, Warth B, Sulyok M, Krska R, Tchana AN, Njobeh PB, et al. Determination of multi-mycotoxin occurrence in cereals, nuts and their products in Cameroon by liquid chromatography tandem mass spectrometry (LC-MS/MS). Food Control. 2013;31(2):438-53.
403. Wielogórska E, MacDonald S, Elliott C. A review of the efficacy of mycotoxin detoxifying agents used in feed in light of changing global environment and legislation. World Mycotoxin J. 2016;9(3):419-33.
404. Turcotte AM, Scott PM, Tague B. Analysis of cocoa products for ochratoxin A and aflatoxins. Mycotoxin research. 2013;29(3):193-201.

405. Sekhon BS. Nanotechnology in agri-food production: an overview. Nanotechnol Sci Appl. 2014;7:31-53.
406. Pascari X, Ramos AJ, Marin S, Sanchis V. Mycotoxins and beer. Impact of beer production process on mycotoxin contamination. A review. Food Res Int. 2018;103:121-9.
407. Avantaggiato G, Greco D, Damascelli A, Solfrizzo M, Visconti A. Assessment of multi-mycotoxin adsorption efficacy of grape pomace. J Agric Food Chem. 2014;62(2):497-507.
408. Joannis-Cassan C, Tozlovanu M, Hadjeba-Medjdoub K, Ballet N, Pfohl-Leszkowicz A. Binding of zearalenone, aflatoxin B1, and ochratoxin A by yeast-based products: a method for quantification of adsorption performance. J Food Prot. 2011;74(7):1175-85.
409. Santos RR, Vermeulen S, Haritova A, Fink-Gremmels J. Isotherm modeling of organic activated bentonite and humic acid polymer used as mycotoxin adsorbents. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2011;28(11):1578-89.
410. Anderson DM. Approaches to monitoring, control and management of harmful algal blooms (HABs). Ocean Coast Manag. 2009;52(7):342-7.
411. Duman O, Ayranci E. Adsorption Characteristics of Benzaldehyde, Sulphanilic acid, and p-Phenolsulfonate from Water, Acid, or Base Solutions onto Activated Carbon Cloth. Sep Sci Technol. 2006;41(16):3673-92.
412. WHO. Guideline values for chemicals that are of health significance in drinking-water. Guidelines for Drinking-Water Quality: Fourth Edition Incorporating the First Addendum. WHO Guidelines Approved by the Guidelines Review Committee. Geneva, 2017.
413. Gao YQ, Gao NY, Deng Y, Gu JS, Shen YC, Wang SX. Adsorption of microcystin-LR from water with iron oxide nanoparticles. Water Environ Res. 2012;84(7):562-8.
414. Melegari SP, Matias WG. Preliminary assessment of the performance of oyster shells and chitin materials as adsorbents in the removal of saxitoxin in aqueous solutions. Chem Cent J. 2012;6(1):86.