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# DETECCIÓN DE MICOTOXINAS REGULADAS, EMERGENTES Y MODIFICADAS EN LA CADENA ALIMENTARIA

Lugo, 2024

**Programa de doctorado en Medicina y Sanidad Veterinaria**

TESIS DOCTORAL

# DETECCIÓN DE MICOTOXINAS REGULADAS, EMERGENTES Y MODIFICADAS EN LA CADENA ALIMENTARIA

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Yo, **Inés Rodríguez Cañás**, doctoranda en el Programa de Medicina y Sanidad Veterinaria declaro no tener ningún conflicto de interés en relación con la tesis doctoral.

Lugo, a 10 de octubre de 2024

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*A mis abuelos, a mis tíos*

*y a padrino*

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## ABREVIATURAS Y ACRÓNIMOS

| | |
|---|---|
| 15-AcDON | 15-acetil-deoxinivalenol |
| 3-AcDON | 3-acetil-deoxinivalenol |
| $AFB_1$ | Aflatoxina $B_1$ |
| AEs | Alcaloides ergóticos |
| AESAN | Agencia Española de Seguridad Alimentaria y Nutrición |
| $AFB_2$ | Aflatoxina $B_2$ |
| $AFG_1$ | Aflatoxina $G_1$ |
| $AFG_2$ | Aflatoxina $G_2$ |
| $AFM_1$ | Aflatoxina $M_1$ |
| $AFM_2$ | Aflatoxina $M_2$ |
| AFs | Aflatoxinas |
| AME | Alternariol metil éter |
| AOH | Alternariol |
| APCI | Ionización química a presión atmosférica |
| ASE | Extracción acelerada con disolventes |
| ATA | Aleuquia Tóxica Alimentaria |
| BEA | Beauvericina |
| CEN | Comité Europeo de Estandarización |
| CPA | Ácido ciclopiazónico |
| CTN | Citrinina |
| DAS | 4,15-diacetoxiscirpenol |
| DOM-1 | Deepoxi deoxinivalenol |
| DON | Deoxinivalenol |
| DON-3-Glu | Glucósido-3-deoxinivalenol |
| d-SPE | Extracción en fase sólida dispersiva |
| EC | Comisión Europea |
| EFSA | Autoridad Europea de Seguridad Alimentaria |
| ELISA | Ensayo por inmunoadsorción ligado a enzimas |
| ENNs | Eniatinas |
| ESI | Ionización por electrospray |
| EU-RL | European Union Reference Laboratory |
| FAO | Organización de las Naciones Unidas para la Agricultura y la Alimentación |
| $FB_1$ | Fumonisina $B_1$ |
| FB2 | Fumonisina $B_2$ |
| $FB_3$ | Fumonisina $B_3$ |
| FBs | Fumonisinas |
| FLD | Detección por fluorescencia |
| FT-ICR | Resonancia ciclotrónica por transformada de Fourier |
| FA | Ácido fusárico |
| FX | Fusarenona X |
| GLIO | Gliotoxina |
| h-$FB_1$ | Fumonisina $B_1$ hidrolizada |
| h-$FB_2$ | Fumonisina $B_2$ hidrolizada |
| h-$FB_3$ | Fumonisina $B_3$ hidrolizada |
| HILIC | Cromatografía líquida de interacciones hidrofílicas |

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| | |
|---|---|
| HPLC | Cromatografía líquida de alta eficacia |
| HT-2 | Toxina HT-2 |
| IAC | Columnas de inmunoafinidad |
| IARC | Agencia Internacional de Investigación contra el Cáncer |
| IT | Trampa de iones |
| IUPAC | International Union of Pure and Applied Chemistry |
| LC | Cromatografía líquida |
| LOD | Límite de detección |
| LOQ | Límite de cuantificación |
| m | pendiente |
| $MgSO_4$ | Sulfato de magnesio anhidro |
| mM | Milimolar |
| MPA | Ácido micofenólico |
| MRM | Monitorizando reacción múltiple |
| MS | Espectrometría de masas |
| MS/MS | Espectrometría de masas en tándem |
| MWE | Extracción con microondas |
| NaCl | Cloruro sódico |
| NEO | Neosolaniol |
| NIV | Nivalenol |
| OMS | Organización Mundial de la Salud |
| OTA | Ocratoxina A |
| OTB | Ocratoxina B |
| OTC | Ocratoxina C |
| PAT | Patulina |
| PDA | Matriz de fotodiodos |
| PDA | Potatoe dextrose agar |
| PSA | Amina primaria/secundaria |
| Q | Cuadrupolo |
| QqQ | Triple cuadrupolo |
| QuEChERS | Quick, Easy, Cheap, Effective, Rugged and Safe |
| $R_A$ | Recuperación aparente |
| $R_E$ | Recuperación |
| ROQC | Roquefortina C |
| $RSD_R$ | Desviación estándar relativa |
| S/N | Señal/ruido |
| SD | Desviación estándar |
| SFE | Extracción con fluidos supercríticos |
| SIM | Scan de iones seleccionados |
| SPE | Extracción en fase sólida |
| SPME | Microextracción en fase sólida |
| SSE | Supresión/sobreestimación de la señal analítica |
| STC | Esterigmatocistina |
| T-2 | Toxina T-2 |
| TMR | Ración total mezclada |
| TOF | Tiempo de vuelo |
| UE | Unión Europea |
| UHPLC | Cromatografía líquida de ultra alto rendimiento |

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| | |
|---|---|
| UHPLC-MS/MS | Cromatografía líquida de ultra alto rendimiento acoplada a espectrometría de masas en tándem |
| UHPLC-MS-IT-TOF | Cromatografía líquida de ultra alto rendimiento acoplada a espectrometría de masas de trampa iónica y tiempo de vuelo |
| UV | Radiación ultravioleta-visible |
| ZEA | Zearalenona |
| ZOL/ZAN | Zearalanona |
| α-ZEA | α-Zearalenol |
| α-ZOL | α-Zearalanol |
| β-ZEA | β-Zearalenol |
| β-ZOL | β-Zearalanol |

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# ÍNDICE


**1. INTRODUCCIÓN** ..... 3

**1.1. SEGURIDAD ALIMENTARIA Y LEGISLACIÓN** ..... 3

**1.2. MICOTOXINAS REGULADAS** ..... 7

**1.2.1. Aflatoxinas** ..... 7

**1.2.2. Tricotecenos** ..... 8

**1.2.2.1. Toxina T-2 y HT-2** ..... 9

**1.2.2.2. Deoxinivalenol** ..... 9

**1.2.3. Zearalenona** ..... 10

**1.2.4. Fumonisinas** ..... 11

**1.2.5. Ocratoxina A** ..... 12

**1.2.6. Citrinina** ..... 13

**1.2.7. Otras toxinas reguladas** ..... 13

**1.2.7.1. Patulina** ..... 13

**1.2.7.2. Alcaloides y esclerocios de cornezuelo** ..... 14

**1.3. MICOTOXINAS EMERGENTES** ..... 14

**1.3.1. Beauvericina y eniatinas** ..... 15

**1.3.2. Esterigmatocistina** ..... 15

**1.3.3. Ácido micofenólico** ..... 16

**1.3.4. Roquefortina C** ..... 16

**1.3.5. Gliotoxina** ..... 17

**1.3.6. Toxinas de *Alternaria*** ..... 17

**1.3.7. Diacetoxiscirpenol** ..... 18

**1.3.8. Ácido ciclopiazónico** ..... 18

**1.4. EXTRACCIÓN DE MICOTOXINAS DE MATRICES ALIMENTARIAS** ..... 18

**1.5. DETECCIÓN DE MICOTOXINAS** ..... 20

**1.6. VALIDACIÓN DEL MÉTODO ANALÍTICO** ..... 23

**2. HIPÓTESIS Y OBJETIVOS** ..... 29

**3. MATERIAL Y MÉTODOS** ..... 33

**3.1. REACTIVOS Y MATERIALES** ..... 33

**3.2. INSTRUMENTACIÓN** ..... 34

**3.2.1 Análisis UHPLC-MS/MS** ..... 34

**3.2.2 Análisis UHPLC-MS-IT-TOF** ..... 35

**3.3. PREPARACIÓN DE LA MUESTRA** ..... 36

**3.3.1. Extracción basada en QuEChERS** ..... 36

**3.3.2. Extracción de hongos *Gnomoniopsis smithogilvyi* (*Gnomoniaceae, Diaporthales*)** ..... 37

**3.3.3. Caracterización del perfil metabólico de *G. smithogilvyi*** ..... 37


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**4. RESULTADOS Y PUBLICACIONES** ..... 41

**SECCIÓN 1: MICOTOXINAS EN MATERIAS PRIMAS** ..... 43

**1. Occurrence of mycotoxins in total mixed ration of dairy farms in Portugal and carry-over to milk** ..... 43

**2. First description of adenosine production by *Gnomoniopsis smithogilvyi*, causal agent of chesnut brown rot** ..... 53

**SECCIÓN 2: MICOTOXINAS EN PRODUCTOS ELABORADOS** ..... 65

**3. Detection of mycotoxins in cheese using an optimized analytical method based on a QuEChERS extraction and UHPLC-MS/MS quantification** ..... 65

**4. Identification of mycotoxins in yogurt samples using an optimized QuEChERS extraction and UHPLC-MS/MS detection** ..... 77

**5. Application of a multi-toxin detect method to analyze mycotoxins occurrence in plant-based beverages** ..... 90

**5. DISCUSIÓN** ..... 103

**6. CONCLUSIONES** ..... 115

**7. BIBLIOGRAFÍA** ..... 119

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# RESUMEN

Las micotoxinas son metabolitos secundarios producidos por ciertos hongos filamentosos, que presentan toxicidad para humanos y animales. Los principales hongos productores de micotoxinas pertenecen a los géneros *Aspergillus*, *Penicillium* y *Fusarium*. Estos hongos pueden contaminar materias primas, como los cereales, tanto durante el cultivo como después de la cosecha, en las etapas de procesamiento, transporte o almacenamiento. Con el fin de evitar intoxicaciones, la Unión Europea (UE) establece niveles máximos permitidos de ciertas micotoxinas en productos alimenticios, pero no propone métodos oficiales para su detección. En el caso de estos contaminantes, establece criterios de funcionamiento para los métodos analíticos destinados al análisis de toxinas reguladas, los cuales deben ser validados para las distintas matrices de interés. Además de las micotoxinas reguladas, existen formas modificadas de estas y toxinas emergentes que no están contempladas en la legislación debido a la falta de datos toxicológicos y de incidencia, pero que pueden estar presentes en materias primas y productos derivados, lo que podría suponer un riesgo.

En la presente tesis doctoral se han optimizado métodos analíticos que incluyen métodos de extracción basados en la metodología QuEChERS y métodos de detección por cromatografía líquida de ultra alto rendimiento acoplada a espectrometría de masas en tándem (UHPLC-MS/MS) para analizar de forma simultánea micotoxinas reguladas, emergentes y modificadas. Los métodos fueron validados en diversas matrices, incluyendo materias primas destinadas a alimentación animal, concretamente TMR (ración total mezclada), productos lácteos como queso y yogur, y bebidas vegetales. La validación de los métodos se realizó en términos de linealidad, límites de detección y cuantificación, exactitud y precisión, cumpliendo con los criterios de funcionamiento establecidos en la legislación para métodos confirmatorios destinados al análisis de micotoxinas. Además, se estudió el perfil metabólico del hongo *Gnomoniopsis smithogilvy* aislado de castañas, alimento habitual de los animales domésticos en Galicia. Para ello se empleó un método UHPLC acoplado a espectrometría de masas de alta resolución, utilizando un analizador de trampa de iones y tiempo de vuelo (UHPLC-MS-IT-TOF). De esta forma, se descartó que el hongo produjera micotoxinas; sin embargo, se identificaron tres metabolitos, adenosina, oxasetina y fitosfingosina, con interesantes propiedades farmacológicas y fitotoxicológicas.

Una vez validados, los métodos analíticos se emplearon para estudiar la incidencia de las micotoxinas a lo largo de la cadena alimentaria. Se observó que se produce un arrastre de las micotoxinas emergentes eniatinas, beauvericina y roquefortina C desde el TMR, utilizado en la alimentación de ganado vacuno, hasta la leche. Además, las eniatinas y la beauvericina se detectaron en queso y yogur. Los hongos empleados en la maduración del queso son responsables de la contaminación adicional de este alimento con toxinas como la roquefortina C y el ácido ciclopiazónico. En el queso también se observó la presencia de toxinas no producidas por los hongos de maduración, como la ocratoxina A, que se encontró esporádicamente, pero en niveles que podrían representar un riesgo para la salud humana. Esta toxina también se detectó en yogures naturales. Por otra parte, se evidenció que la adición de avena a los yogures incrementa la cantidad de micotoxinas, como el neosolaniol o la toxina HT-2, que son arrastradas del cereal. En el caso de bebidas vegetales, se identificaron las toxinas T-2 y HT-2 en productos a base de avena, mientras que las aflatoxinas se observaron en bebidas de arroz y almendra. Nuevamente, las micotoxinas emergentes eniatinas y beauvericina se detectaron en todos los tipos de bebidas.

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# INTRODUCCIÓN

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# 1. INTRODUCCIÓN

## 1.1. SEGURIDAD ALIMENTARIA Y LEGISLACIÓN

La seguridad alimentaria está definida por la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO) como la *"situación que se da cuando todas las personas tienen, en todo momento, acceso físico, social y económico a suficientes alimentos inocuos y nutritivos para satisfacer sus necesidades alimenticias y sus preferencias en cuanto a los alimentos a fin de llevar una vida activa y sana*" (1). La FAO y la Organización Mundial de la Salud (OMS) trabajan para garantizar la inocuidad de los alimentos y de esta forma proteger la salud de los consumidores, así como, proporcionar ayuda de calidad a los trabajadores del sector agroalimentario para que puedan producir, procesar y distribuir alimentos inocuos (2).

Tras las fuertes crisis alimentarias de los años 90, en el año 2000, la Comisión Europea (CE) publica el *"Libro Blanco sobre Seguridad Alimentaria".* En él se explica la estrategia para alcanzar los niveles más altos en términos de seguridad alimentaria dentro de la Unión Europea (UE), basándose en el principio general *"de la granja a la mesa"*, considerando de forma integral la cadena alimentaria, es decir, desde la producción primaria hasta llegar al consumo del alimento.

Como resultado del plan de acción sobre seguridad alimentaria, se aprueba el Reglamento (CE) Nº 178/2002 por el que se establecen los principios y los requisitos generales de la legislación alimentaria, y se crea la Agencia Europea de Seguridad Alimentaria (European Food Safety Authority, EFSA) (3). La EFSA es una agencia europea independiente que cuenta con paneles de expertos altamente cualificados que brindan asesoramiento científico y comunicación sobre los riesgos asociados a la seguridad de alimentos y piensos dentro de la cadena alimentaria. La evaluación de los riesgos da lugar a la elaboración de dictámenes científicos y consejos para colaborar con la CE, el Parlamento Europeo y los estados miembros de la UE en la toma de decisiones de gestión de riesgos que comprometan la inocuidad de los alimentos. En este sentido, se establece que se debe llevar a cabo el análisis del riesgo: evaluación del riesgo, gestión del riesgo y, por último, comunicación del riesgo.

En el año 2001, se crea la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) que pertenece actualmente al Ministerio de Derechos Sociales, Consumo y Agenda 2030. El objetivo principal de AESAN es garantizar la seguridad alimentaria y ofrecer información objetiva y de calidad, tanto a los consumidores como al sector agroalimentario.

En el año 2004, se publica la legislación acerca de la higiene general de los productos alimenticios, conocido como paquete de higiene, en el que se incluyen los Reglamentos (CE) Nº 852/2004, 853/2004 y 854/2004 modificados por el Reglamento (CE) 2074/2005 (4). En el año 2017, se dicta el Reglamento (UE) 2017/625 para asegurar la aplicación de los controles oficiales y normas sobre la salud humana y animal, así como, la protección del medio ambiente (5, 6).

Una de las funciones de AESAN es gestionar el riesgo en alimentos, es decir, aplicar medidas regidas por normativas nacionales y de la UE que logren prevenir, minimizar o eliminar el riesgo. De esta forma, se garantiza el grado más alto en términos de seguridad alimentaria. Dentro de la gestión de riesgos se diferencian la seguridad biológica, la seguridad nutricional y la seguridad química. La seguridad biológica trata los riesgos que tienen relación con bacterias, virus o priones presentes en alimentos, especialmente, en los productos alimenticios que proceden de animales. La seguridad nutricional se enfoca en garantizar la protección dentro del mercado interior de alimentos, especialmente, en establecer el correcto etiquetado de los alimentos para poner a disposición del consumidor información clara y transparente acerca de la composición nutricional

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y de los ingredientes. Por otro lado, la seguridad química se centra en la producción y la distribución de los alimentos, consiguiendo una mejora gracias a la adición intencionada de ciertos compuestos, como los productos fitosanitarios o medicamentos de uso veterinario para obtener mejor producción de los cultivos o del ganado, la adición de aditivos en alimentos para mejorar características organolépticas o sensoriales, o el uso de materiales para el envasado o embalaje del alimento, facilitando de esta manera su distribución y mejorando su aspecto. El uso de sustancias químicas puede conllevar a un arrastre hacia los alimentos, por lo tanto, es necesario garantizar a través de controles de calidad que son inocuos para el consumidor. Por otro lado, existen sustancias que no se han añadido de manera intencionada y también se consideran un peligro químico, denominadas contaminantes. Dentro de este grupo se incluyen metales, dioxinas, nitratos, hidrocarburos aromáticos policíclicos, toxinas marinas y micotoxinas.

A finales de la década de los 50, principios de los 60, ocurrió una crisis alimentaria como consecuencia de una enfermedad inicialmente desconocida que provocó la muerte de 100.000 pavos en Reino Unido. Posteriormente, se descubrió que la causa residía en el pienso contaminado con micotoxinas, en concreto, con aflatoxinas (AFs). Paralelamente, se produjo una epizootia de cáncer de hígado en truchas arcoíris de piscifactoría en California. Se identificó que el pienso con el que se alimentaban los peces también estaba contaminado con AFs (7, 8). La palabra micotoxina proviene del griego "*mycos*" (hongo) y del latín "toxicum" (tóxico). Las micotoxinas son metabolitos secundarios de bajo peso molecular con potencial tóxico, producidos por ciertos hongos filamentosos (mohos), que suelen formarse al final de la fase exponencial o en la fase estacionaria del crecimiento del hongo. La mayor parte de micotoxinas pertenecen al género *Aspergillus, Fusarium* y *Penicillium.* Por otro lado, los géneros *Alternaria* y *Claviceps* también producen micotoxinas, aunque en menor proporción (9, 10). Estos metabolitos pueden contaminar alimentos o piensos, suponiendo un riesgo para la salud humana y animal, ya que la ingesta, inhalación o contacto directo provoca intoxicaciones conocidas como micotoxicosis. Se ha demostrado que ciertas micotoxinas son carcinógenas, genotóxicas, nefrotóxicas y/o mutagénicas. Además, una única especie fúngica es capaz de producir múltiples micotoxinas y por lo tanto es común detectar varias micotoxinas en un mismo producto alimenticio. A raíz de estos sucesos la UE empezó a controlar la presencia de micotoxinas en alimentos y piensos.

En el año 2006, se publica el Reglamento (CE) Nº 1881/2006 que establece el contenido máximo de determinados contaminantes, incluyendo a las micotoxinas, en los productos alimenticios (11). Tras el aumento de la incidencia de micotoxinas en alimentos, el reglamento sufre una serie de modificaciones. En 2015, se modifica a través del Reglamento (UE) 2015/1137 que establece un contenido máximo de ocratoxina A (OTA) en las especias Capsicum spp. (15-20 µg/kg) (12). En 2021, sufre una modificación a través del Reglamento (UE) 2021/1399 y se establecen niveles máximos de esclerocios de cornezuelo (0,2 µg/kg) y alcaloides de cornezuelo (20-400 µg/kg) en cereales y productos derivados (13). En 2022, el Reglamento (UE) 2022/1370 añade nuevos datos en relación con el contenido máximo de OTA en productos alimenticios, como las semillas de soja en las que se fija un nivel de 5 µg/kg, así como semillas de girasol, calabaza, melón, sandía y cáñamo. Finalmente, tras varias modificaciones realizadas, el Reglamento (CE) Nº 1881/2006 queda derogado por el actual Reglamento (UE) Nº 2023/915 (Tabla 1) (14). Por otro lado, la legislación de alimentación animal establece niveles máximos de aflatoxina $B_1$ ($AFB_1$) y cornezuelo de centeno según la Directiva 2002/32/CE (15) y niveles recomendados de deoxinivalenol (DON), zearalenona (ZEA), OTA, fumonisina $B_1$ ($FB_1$) y fumonisina $B_2$ ($FB_2$), toxina HT-2 (HT-2) y toxina T-2 (T-2) según la Recomendación 2006/579/CE (16).

| Micotoxinas | Productos alimenticios | Nivel máximo permitido (µg/kg) | Niveles permitidos [1] o recomendados [2] (µg/kg) en pienso |
|---|---|---|---|
| | | Reglamento (UE) Nº 2023/915 | Directiva 2002/32/CE [1] Recomendación 2006/576/CE [2] |
| AFs | Cereales | 4 | 5-20 ($AFB_1$) |
| $AFM_1$ | Leche cruda y preparados para lactantes | 0,025-0,05 | - |
| OTA | Granos de cereales, productos derivados de cereales | 3-5 | 10-250 |
| | Alimentos infantiles elaborados a base de cereales | 0,50 | - |
| DON | Granos de cereales, productos derivados de cereales | 500-1250 | 900-12000 |
| | Alimentos infantiles elaborados a base de cereales | 200 | - |
| ZEA | Granos de cereales, productos derivados de cereales | 50-100 | 100-3000 |
| | Alimentos infantiles elaborados a base de cereales | 20 | - |
| FBs | Granos de maíz, productos derivados de maíz | 800-4000 | 500-6000 |
| | Alimentos infantiles elaborados a base de maíz | 200 | - |
| CTN | Complementos alimenticios a base de arroz fermentado | 100 | - |
| T-2 y HT-2 | Granos de cereales, salvado de cereales, productos derivados de panadería | 20-50 | 50 |
| | Granos de avena sin transformar | 1250 | - |
| | Copos de avena, productos derivados de avena | 100 | - |
| | Alimentos infantiles elaborados a base de cereales | 10 | - |

**Tabla 1**. Resumen de niveles máximos permitidos (µg/kg) de las principales micotoxinas según el Reglamento (UE) Nº 2023/915 que deroga el Reglamento (CE) Nº 1881/2006, que establece el contenido máximo de contaminantes en determinados productos alimenticios. Niveles permitidos o recomendados (µg/kg) en piensos según la Directiva 2002/32/CE y según la Recomendación 2006/579/CE. Aflatoxina $M_1$ ($AFM_1$), fumonisinas (FBs), citrinina (CTN).

La contaminación por hongos micotoxigénicos se suele desarrollar en una amplia variedad de cultivos, antes de la cosecha o después, es decir, durante el procesado, el transporte o el almacenamiento de las materias primas. No obstante, la presencia de hongos no siempre conlleva a una contaminación por micotoxinas, ya que los factores que afectan a la producción de micotoxinas no son los mismos que afectan a la proliferación de hongos. Por otro lado, la ausencia del hongo no garantiza la ausencia de micotoxinas ya que se trata de metabolitos que son termoestables y estables químicamente. Su producción va a depender de factores como la

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temperatura, la actividad de agua, la humedad, el pH, el sustrato y el tipo de cepa. Además, los hongos pueden regular el pH del entorno a través de la secreción de ácidos residuales, siendo el pH ácido adecuado para la producción de micotoxinas en la mayoría de los casos (17). Además, la contaminación de micotoxinas en productos agrícolas genera un gran impacto económico-social debido a las pérdidas de producción del campo (18).

Las condiciones climáticas son de vital importancia tanto en la producción de los hongos como de las micotoxinas. Los hongos *Fusarium* crecen en zonas de elevada humedad, siendo habituales durante la precosecha. En cambio, los hongos *Aspergillus* y *Penicillium* crecen en entornos con humedades relativas al 80 % o menos, y suelen aparecer en etapas posteriores a la cosecha. Existe una preocupación actual debido al cambio climático y al impacto en la contaminación de micotoxinas ya que se ha detectado un aumento de la incidencia en nuevas áreas geográficas. En el caso de las AFs, que suelen detectarse en regiones como el sur de África, también se espera que ocurran con mayor frecuencia en países del mediterráneo como Italia y España, debido al aumento de temperatura y de épocas de sequía (17, 19).

Actualmente, se conocen aproximadamente más de 400 metabolitos secundarios identificados como micotoxinas, sin embargo, solo están reguladas en la UE las que se mencionan a continuación: las AFs, OTA, patulina (PAT), DON, ZEA, $FB_1$ y $FB_2$, CTN, T-2 y HT-2, esclerocios y alcaloides de cornezuelo (20). En la Tabla 1 se indican los niveles máximos permitidos en alimentos y piensos según el reglamento actual, así como, los niveles de concentración recomendados en productos destinados a la alimentación animal.

Las micotoxinas pueden sufrir modificaciones en su estructura química, tras la acción metabólica de animales, plantas u hongos. Estas micotoxinas se conocen bajo el concepto de "micotoxinas modificadas" adoptado por la EFSA en una opinión científica del año 2014 (21). Son aquellas que han sufrido alteraciones en la estructura de las toxinas precursoras o libres, definidas como los compuestos secundarios que se han producido por el metabolismo de ciertos hongos filamentosos. Las micotoxinas modificadas pueden encontrarse en niveles similares a las micotoxinas libres, y en ciertos casos, la concentración será superior en alimentos procesados (22). Por ello, la evaluación de riesgos en la cadena alimentaria debe tener en cuenta a las formas modificadas ya que pueden contribuir a la toxicidad. Estas modificaciones se producen como consecuencia del mecanismo de defensa de plantas infestadas, tras sufrir reacciones de hidrólisis, oxidación o reducción (fase I) o tras la conjugación con moléculas orgánicas (fase II) en plantas, en el metabolismo de ciertos microorganismos (hongos o bacterias) o del animal mamífero. Los metabolitos formados en la fase II de las plantas también se denominan micotoxinas enmascaradas, y suelen conjugarse con moléculas polares de bajo peso molecular como la glucosa (23). Por ejemplo, las micotoxinas α-ZEA y β-ZEA han sido modificadas tras la acción del metabolismo de las plantas en la fase I. No obstante, existe la posibilidad de que sean metabolitos modificados por el mecanismo de defensa de hongos o bacterias. Por otro lado, las formas modificadas pueden producirse tras procesos físicos o químicos donde intervienen parámetros como el pH, la presión, la temperatura o la radiación. En general, las micotoxinas son estables al procesado de alimentos, sin embargo, pueden sufrir modificaciones transformándose en un análogo o asociándose a algún componente de la matriz alimentaria, y, por lo tanto, el nivel de la micotoxina precursora o libre va a disminuir en el alimento (22). El deepoxi deoxinivalenol (DOM-1) es un análogo del DON que puede producirse tras la acción metabólica de microorganismos, pero también por degradación del DON tras un proceso de calor (24).

Por otro lado, existe un amplio grupo denominado micotoxinas emergentes que no están monitorizadas en análisis rutinarios, por lo que no están reguladas debido a la falta de datos toxicológicos y de presencia en alimentos (25). Sin embargo, existen evidencias de su alta

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incidencia en productos alimenticios (25). Alguna de las micotoxinas emergentes más importantes son las eniatinas (ENNs), la beauvericina (BEA), el ácido fusárico (FA), la esterigmatocistina (STC), el alternariol (AOH), el alternariol metil éter (AME), el ácido micofenólico (MPA), la roquefortina C (ROQC) y la gliotoxina (GLIO). Cabe destacar que, en la industria alimentaria, se emplean cepas de hongos no micotoxigénicos en etapas de producción, como el uso de *Penicillium roqueforti* para elaborar queso denominación de origen Roquefort (26). Sin embargo, la ROQC está producida por esta especie, pudiendo aparecer en queso (27).

## 1.2. MICOTOXINAS REGULADAS

### 1.2.1. Aflatoxinas

Las AFs son un grupo de 18 compuestos de bisfuranocumarina, aunque las más conocidas hasta el momento son aflatoxina $B_1$ ($AFB_1$), $B_2$ ($AFB_2$), $G_1$ ($AFG_1$), $G_2$ ($AFG_2$), $AFM_1$ y $M_2$ ($AFM_2$) (28). La estructura química de cada una se muestra en la Fig. 1. Son sustancias cristalinas, solubles en agua y en disolventes orgánicos como el metanol. Son sensibles a pH muy ácidos o fuertemente alcalinos. En estado sólido son estables en ausencia de luz (29). La $AFM_1$ y $AFM_2$ son los compuestos hidroxilados de sus micotoxinas precursoras $AFB_1$ y $AFB_2$. La reacción de hidroxilación tiene lugar en el metabolismo del animal que ingiere el alimento contaminado con $AFB_1$ y/o $AFB_2$. No obstante, la evaluación de riesgos excluye a la $AFM_2$ porque no hay datos de incidencia en alimentos.

Las AFs son un grupo de compuestos producidos por diferentes especies del género *Aspergillus*, *A. flavus* y *A. parasiticus*, consideradas patógenos para los humanos y los animales (30). Se suelen desarrollar en climas húmedos y cálidos pudiendo crecer en regiones tropicales e infestar cultivos durante la cosecha o durante el almacenamiento. Las AFs de tipo B están producidas por ambas especies, mientras que las de tipo G son específicas de *A. parasiticus*. La "B" y la "G" hacen referencia a los colores azul (blue) y verde (green) que se manifiestan frente a la luz ultravioleta. Las AFs son genotóxicas, mutagénicas y consideradas carcinógenos hepáticos por la Agencia Internacional de Investigación contra el Cáncer (IARC). La $AFB_1$, se considera el análogo más tóxico, ya que está clasificada como carcinógeno del grupo 1 lo que significa que su carcinogenidad está demostrada en humanos (31).

Las AFs suelen aparecer en cereales y productos derivados, frutos secos, arroz, especias y leche. El cambio climático juega un papel fundamental en el incremento de los niveles de AFs en los países europeos en diversos cultivos (32). El aumento de contaminación de $AFB_1$ en maíz, u otras materias primas, está relacionada con el aumento en los niveles de $AFM_1$ en la leche, ya que este cereal es la base de la alimentación animal y el análogo hidroxilado ($AFM_1$) es capaz de transferirse a los tejidos y fluidos del animal (33). La $AFM_1$ es resistente a altas temperaturas, por lo tanto, los procesos de industria alimentaria para transformar la leche en productos derivados, como la uperización no asegurarían la eliminación de este contaminante (34).

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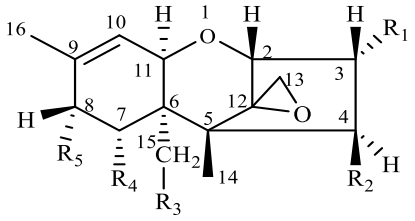


| | $AFB_1$ | $AFB_2$ | $AFM_1$ | $AFG_1$ | $AFG_2$ |
|---|---|---|---|---|---|
| Fórmula molecular | $C_{17}H_{12}O_6$ | $C_{17}H_{14}O_6$ | $C_{17}H_{12}O_7$ | $C_{17}H_{12}O_7$ | $C_{17}H_{14}O_7$ |
| Peso molecular (g/mol) | 312,3 | 314,3 | 328,3 | 328,3 | 330,3 |

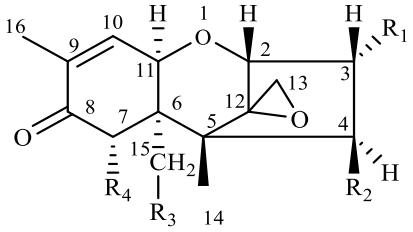
**Fig. 1.** Estructura química de las principales AFs

### 1.2.2. Tricotecenos

Los tricotecenos son una familia formada por aproximadamente 18 compuestos cíclicos denominados sesquiterpenoides, divididos en cuatro grupos (A, B, C y D) según su estructura química, diferenciándose en los distintos radicales ($R_1$, $R_2$, $R_3$, $R_4$, $R_5$) que están enlazados a la estructura cíclica (12,13-epoxitricotec-9-eno) común a todos ellos (Fig. 2). Están producidos por diferentes especies de *Fusarium, Myrothecium, Cephalosporum, Verticimonosporum, Trichoderma, Trichothecium* y *Stachybotrys.*



Tricoteceno A



Tricoteceno B

| Micotoxina | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Fórmula molecular | Peso molecular |
|---|---|---|---|---|---|---|---|
| **Tricoteceno A** | | | | | | | |
| T-2 | OH | $OCOCH_3$ | $OCOCH_3$ | H | $OCOCH_2CH(CH_3)_2$ | $C_{24}H_{34}O_9$ | 466,2 |
| HT-2 | OH | OH | $OCOCH_3$ | H | $OCOCH_2CH(CH_3)_2$ | $C_{22}H_{32}O_8$ | 424,2 |
| NEO | OH | $OCOCH_3$ | $OCOCH_3$ | H | OH | $C_{19}H_{26}O_8$ | 382,2 |
| DAS | OH | $OCOCH_3$ | $OCOCH_3$ | H | H | $C_{19}H_{26}O_7$ | 366,2 |
| T2 triol | OH | OH | OH | H | $OCOCH_2CH(CH_3)_2$ | $C_{20}H_{30}O_7$ | 382,2 |
| **Tricoteceno B** | | | | | | | |
| **Micotoxina** | **$R_1$** | **$R_2$** | **$R_3$** | **$R_4$** | **$R_5$** | **Fórmula molecular** | **Peso molecular** |
| DON | OH | H | OH | OH | - | $C_{15}H_{20}O_6$ | 296,3 |
| 3-AcDON | $OCOCH_3$ | H | OH | OH | - | $C_{17}H_{22}O_7$ | 338,4 |
| 15-AcDON | OH | H | $OCOCH_3$ | OH | - | $C_{17}H_{22}O_7$ | 338,4 |
| DON-3-Glu | $C_6H_{11}O_6$ | H | OH | OH | - | $C_{21}H_{20}O_{11}$ | 448,1 |
| Nivalenol | OH | $OCOCH_3$ | OH | H | - | $C_{15}H_{20}O_7$ | 312,1 |

**Fig. 2.** Estructura química de tricotecenos A y B

Los tricotecenos A, B y C se diferencian en el grupo funcional posicionado en el carbono 8. Los del grupo A incluyen un grupo hidroxilo o un grupo éster, mientras que los de tipo B poseen un grupo carbonilo. Por otra parte, los de tipo C poseen un grupo epóxido en la posición del carbono 7 y carbono 8, o carbono 9 y carbono 10. Por último, los de tipo D tienen un anillo macrocíclico unido al carbono 4 y al carbono 15. Los tricotecenos más importantes son los de tipo A y B, debido a una mayor incidencia en cereales. Los tricotecenos A incluyen T-2, HT-2,

neosolaniol (NEO), 4,15-diacetoxiscirpenol (DAS) y T2 triol. Los tricotecenos B engloban al DON, 3-acetil-deoxinivalenol (3-AcDON), 15-deoxinivalenol (15-AcDON), glucósido-3-deoxinivalenol (DON-3-Glu), fusarenona X (FX) y nivalenol (NIV).
Los efectos tóxicos de los tricotecenos se dieron a conocen tras la "Aleuquia Tóxica Alimentaria" (ATA) producida en Rusia en el 1980, caracterizada por la aparición de hemorragias y la disminución de plaquetas (35). La exposición a estas micotoxinas puede causar problemas inmunológicos, dermatitis o hemorragias internas (9).

#### 1.2.2.1. Toxina T-2 y HT-2

La T-2 es la abreviatura de (3α,4β,8α)-12,13-epoxitricotec-9-eno-3,4,8,15-tetro-4,15-diacetato 8-(3-metilbutirato). La HT-2 es la abreviatura de (3α,4β,8α)-12,13-epoxitricotec-9-eno-3,4,8,15-tetrol-15-acetato 8-(3-metilbutirato). Ambas toxinas difieren en el grupo funcional del carbono 4 (ver Fig. 2) (36). La HT-2 es un producto hidroxilado de la T-2 que se obtiene tras la acción del metabolismo animal o a través del de plantas y hongos. El punto de ebullición de la T-2 es de 151-152 ºC y el de la HT-2 es similar a su precursora, alrededor de 151,5 ºC. A temperatura ambiente tienen aspecto aceitoso de color amarillo o bien, pueden formar cristales si el compuesto está puro. Son solubles en disolventes orgánicos como el metanol, acetato de etilo o acetonitrilo y tienen baja solubilidad en agua. Son compuestos no volátiles y estables en pH neutro y ácido. Los tricotecenos A son menos polares que los tricotecenos B, debido al grupo funcional del carbono 8. En general, están producidos por especies de *Fusarium* como *F. sporotrichioides*, *F. poae* y *F. langsethiae***.** Suelen estar presentes en alimentos de cultivo, como la avena, cebada, trigo o maíz, y pueden persistir en las etapas posteriores a la poscosecha, como en la molienda (37).

#### 1.2.2.2. Deoxinivalenol

El DON y sus análogos 3-AcDON y 15-AcDON son micotoxinas producidas por especies de hongos *Fusarium*, y se consideran tricotecenos de tipo B. DON es la abreviatura de tricotec-9-en-8-ona,12,13-epoxi-3,7,15-trihidroxi-(3α,7α). En su estado puro puede formar cristales en forma de agujas de color. Es estable a altas temperaturas (120-180 ºC). Es soluble en agua y en algunos disolventes orgánicos polares como el acetonitrilo o el metanol. Es inestable a un pH fuertemente alcalino (pH > 10) y a altas temperaturas. Sus análogos acetilados, 3-AcDON y 15-AcDON, se denominan tricotec-9-en-8-ona,3-(acetiloxi)-12,13-epoxi-7,15-dihydroxy-(3α,7α) y tricotec-9-en-8-ona,15-(acetiloxi)-12,13-epoxi-3,7-dihydroxy-(3α,7α), respectivamente (38).

El DON fue aislado por primera vez en 1972 de una partida de cebada contaminada. En 2004, la EFSA reclamó más datos de ocurrencia debido a la presencia de DON en pienso animal. Empezó a ganar importancia en términos de seguridad alimentaria tras la publicación en el año 2013 de un dictamen científico en el que se recogían datos de 21 países miembros europeos y Noruega entre los años 2007 y 2012. Este dictamen informaba de un aumento en la detección de esta toxina en productos alimenticios, principalmente trigo, maíz, granos de avena y productos derivados. No obstante, la EFSA indica que es necesario conseguir más datos sobre los derivados del DON, para mejorar la estimación de la exposición a estas micotoxinas. Por otra parte, 3-AcDON, 15-AcDON y DON-3-Glu tuvieron una baja incidencia en los alimentos (39, 40).
El DON es conocido como vomitoxina por los efectos gastrointestinales que produce en humanos y animales tras su consumo. La IARC lo clasifica en el grupo 3, por lo tanto, los resultados hasta el momento descartan que sea cancerígeno.

USC DE COMPOSTELA

Además, dentro del grupo de los tricotecenos, la presente tesis doctoral se centra en el NIV y el DAS. El NIV tiene la siguiente nomenclatura (3α,4β, 7α)-12,13-epoxi-3,4,7,15-tetrahidroxitricotec-9-en-9-ona, con fórmula molecular $C_{15}H_{20}O_7$. Es un polvo cristalino de color blanco, con un punto de fusión de 222 ºC. Es una micotoxina soluble en disolventes orgánicos como el acetonitrilo o el metanol y poco soluble en agua. La estructura del NIV es muy similar a la del DON (ver Fig. 2), se diferencia en un grupo hidroxilo en el carbono 4. Esta micotoxina es un tricoteceno de tipo B y se ha detectado en granos de cereales, como trigo, arroz o avena, así como cereales procesados y legumbres (41). Es un compuesto citotóxico y genotóxico (42).

### 1.2.3. Zearalenona

La ZEA es una lactona del ácido β-resorcílico macrocíclico, en concreto, lactona del ácido 6-[10-hidroxi-6-oxo-trans-1-undecenil]-B-resorcilíco (ver Fig. 3). Según la IUPAC se denomina (-)-(3S,11E)-3,4,5,6,9,10-hexahidro-14,16-dihidroxi-3-metil-1H-2-benzoaciclotetradecin-1,7(8H)-diona. Es una micotoxina producida por *Fusarium graminearum, F. culmorun* y *F. cerealis* y suele encontrarse en alimentos como maíz, trigo, avena, arroz y otros cereales que crecen en regiones de clima moderado. La ZEA puede transformarse en formas modificadas a través de las rutas metabólicas del animal que la ingiera, los análogos más importantes son la zearalanona (ZOL o ZAN) formado a partir de la acción de la reductasa presente en la flora intestinal del animal, o los análogos formados a través de una reducción del grupo cetona que dan lugar a α-zearalenol (α-ZEA) y β-zearalenol (β-ZEA) y se muestran en la Fig. 3. Además, la reducción del grupo cetona y del doble enlace conducen a la transformación de otros dos compuestos denominados α-zearalanol (α-ZOL) y β-zearalanol (β-ZOL). Estos compuestos pueden aparecer en leche y productos derivados (43).

Son solubles en disolventes orgánicos como el acetonitrilo, acetona o metanol, pero no en agua (solubilidad= 0,002 g/100 mL, 25 ºC). Son térmicamente estables, por lo que es difícil su eliminación y/o descomposición (44, 45). Sin embargo, los isómeros α-ZEA y β-ZEA poseen una configuración *trans* en la posición del doble enlace olefínico, así como la micotoxina precursora, ZEA, que al ser irradiados con luz ultravioleta-visible (UV) o luz solar se convierten en los isómeros *cis* y son muy estables químicamente con puntos de fusión alrededor de 130 ºC (46).

La ZEA está considerada un xenoestrógeno, es decir, un compuesto disruptor-endocrino, ya que al poseer una estructura química similar a la de los estrógenos es capaz de enlazarse a los mismos receptores. De esta manera, existe un desequilibrio hormonal dentro del organismo y como consecuencia afecta al sistema reproductivo, pudiendo producir abortos o bajo peso al nacer, o incluso cáncer de próstata, de cérvix o de mama (47). Los animales monogástricos como el cerdo son más susceptibles a los efectos secundarios de esta toxina (48). Posee también propiedades inmunotóxicas, teratogénicas, carcinógenas, hematotóxicas y hepatotóxicas (49). Los efectos que provocan las formas modificadas de ZEA no están claros, no obstante, existe la hipótesis de que en el tracto gastrointestinal del animal se liberan en forma de ZEA (48).

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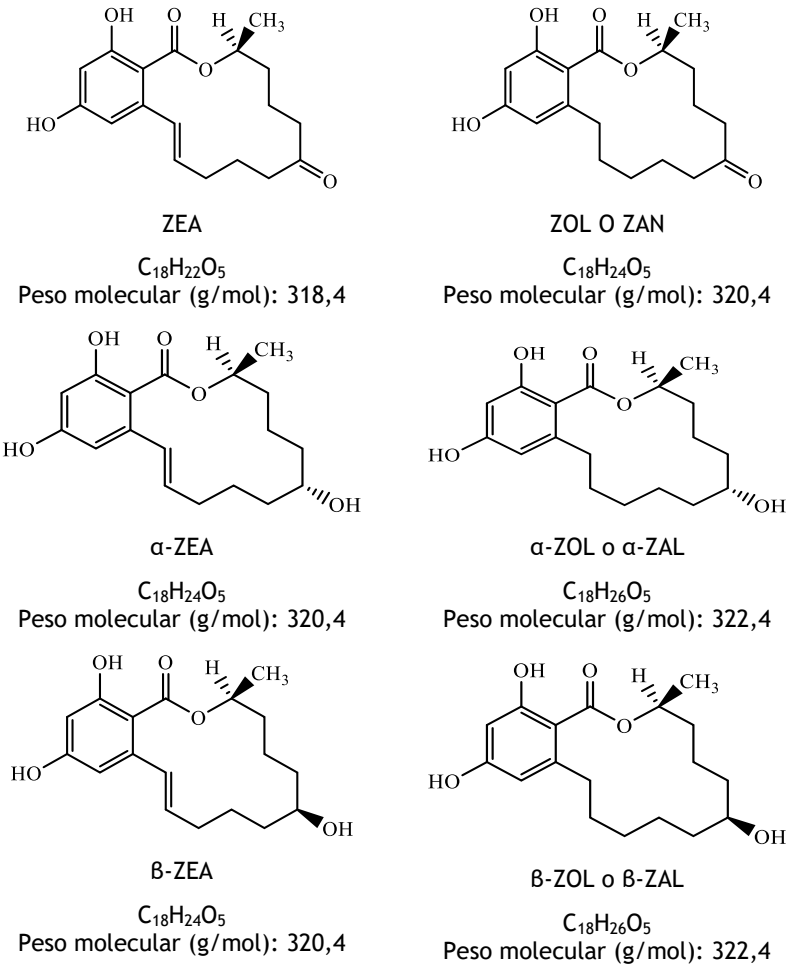



**Fig. 3**. Estructura de la ZEA y análogos

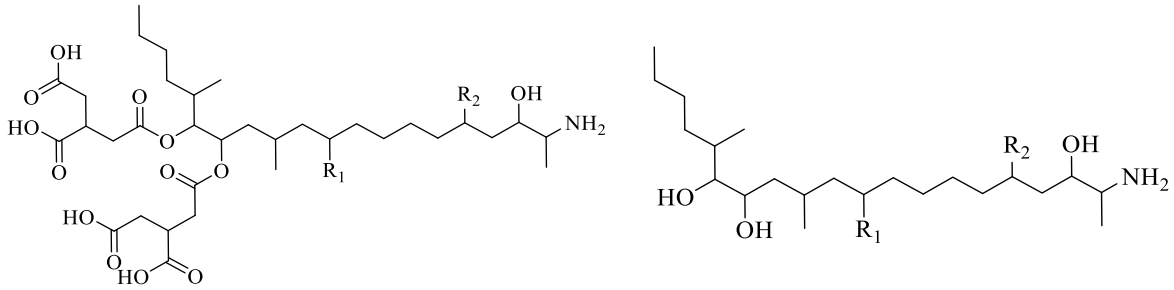
### 1.2.4. Fumonisinas

Las fumonisinas están constituidas por una estructura común de aminopoliol de cadena larga, 19 o 20 carbonos con dos grupos metilo. Poseen dos cadenas laterales de ácido propano-1,2,3-tricarboxílico unidas mediante enlace éster a los hidroxilos situados en la posición de carbono 14 y carbono 15. Las fumonisinas se clasifican en fumonisinas A, B, C y P según los grupos funcionales de su estructura. Las fumonisinas B (FBs) son las más relevantes y las que se consideran de riesgo según la EFSA, debido a su presencia en piensos y alimentos. En cambio, las otras variedades no son detectadas con frecuencia, representan menos de un 5% total de las fumonisinas. Las más conocidas son la fumonisina $B_1$ ($FB_1$), fumonisina $B_2$ ($FB_2$) y fumonisina $B_3$ ($FB_3$) por encontrarse en niveles elevados en los productos alimenticios, en especial, en maíz y productos derivados (50). Este grupo de micotoxinas está formado por más de 25 análogos producidos por especies del género *Fusarium*, especialmente por *F. verticillioides* y *F. proliferatum* (51).

Las estructuras químicas de las FBs se muestran en la Fig. 4. Son compuestos solubles en agua y en disolventes polares. Los grupos funcionales que contienen hacen que pueda reaccionar y dar lugar a formas modificadas, especialmente en procesos térmicos durante la producción de alimentos y piensos, o bien, por acción del metabolismo de plantas, hongos o animales. La $FB_1$ hidrolizada (h-$FB_1$) es una forma modificada de la $FB_1$ como consecuencia de la acción del metabolismo animal, en donde tiene lugar la hidrólisis de los grupos éster.

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También se conocen la $FB_2$ y $FB_3$ hidrolizadas (h-$FB_2$ y h-$FB_3$) y sus estructuras químicas se muestran en la Fig. 4. Estas micotoxinas y varios metabolitos como la h-$FB_1$ pueden aparecer en leche (36). La solubilidad de h-$FB_1$ es mayor en lípidos en comparación con su micotoxina precursora.



| | $R_1$ | $R_2$ | Fórmula molecular | Peso molecular (g/mol) | | $R_1$ | $R_2$ | Fórmula molecular | Peso molecular (g/mol) |
|---|---|---|---|---|---|---|---|---|---|
| $FB_1$ | OH | OH | $C_{34}H_{59}NO_{15}$ | 721,8 | h-$FB_1$ | OH | OH | $C_{22}H_{47}NO_5$ | 405,6 |
| $FB_2$ | H | OH | $C_{34}H_{59}NO_{14}$ | 705,4 | h-$FB_2$ | H | OH | $C_{22}H_{47}NO_4$ | 389,6 |
| $FB_3$ | OH | H | $C_{34}H_{59}NO_{14}$ | 705,4 | h-$FB_3$ | OH | H | $C_{22}H_{47}NO_4$ | 389,6 |

**Fig. 4.** Estructura química de las principales FBs y de las h-FBs

La toxicidad y los efectos que originan son variados y diferentes para cada especie animal. Son las responsables de la leucoencefalomalacia en caballos, esta enfermedad mortal se caracteriza por una necrosis licuefactiva de la sustancia blanca cerebral que afecta a los lóbulos frontal y parietal (52). Además, también producen edema pulmonar en cerdos y carcinomas hepatocelulares en animales de laboratorio. En humanos, se ha relacionado el consumo de maíz con elevado contenido de FBs con el cáncer de esófago (53, 54).

### 1.2.5. Ocratoxina A

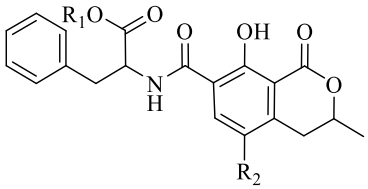
Las ocratoxinas contienen el aminoácido L-fenilalanina que está enlazado al ácido dihidroisocumárico a través de un enlace amida. Son micotoxinas producidas por distintos hongos del género Aspergillus y Penicillium. La OTA es la más importante porque aparece en los alimentos con mayor frecuencia y a niveles más altos y, además, se considera la más tóxica. En la Fig. 5, se observa la estructura de la OTA, su forma no clorada denominada como ocratoxina B (OTB) y la ocratoxina C (OTC) que es el éster de la micotoxina precursora, donde el hidrógeno del $R_1$ está sustituido por un grupo etilo ($C_2H_5$).

La OTA es un sólido de color blanco, soluble en disolventes orgánicos polares como el metanol y posee baja solubilidad en agua (0,42 mg/L). Tiene valores de pKa de 4,3 y 7,2 y en función del pH puede encontrarse en forma no iónica, monoaniónica y dianiónica. Es capaz de emitir fluorescencia al ser excitada con luz UV (55).

La OTA es producida por varias especies de Aspergillus entre las que se incluyen A. alliaceus, A. carbonarius y por especies de Penicillium, como P. verrucosum y P. nordicum. Esta micotoxina ha sido detectada en una amplia variedad de alimentos tales como cereales, granos de café verde, uvas, chocolate, legumbres, cerveza, especias, carne y leche (56, 57). Además, se han observado niveles de OTA en alimentos no legislados para esta toxina.

Tiene efectos neurotóxicos, inmunosupresores, genotóxicos y teratógenos. La IARC la ha clasificado en el grupo 2B, es decir, como posible carcinógeno humano (58). Esta toxina se acumula en los riñones provocando efectos agudos y crónicos. Se ha relacionado con nefropatías en animales mamíferos, siendo el perro y el cerdo las especies más vulnerables.

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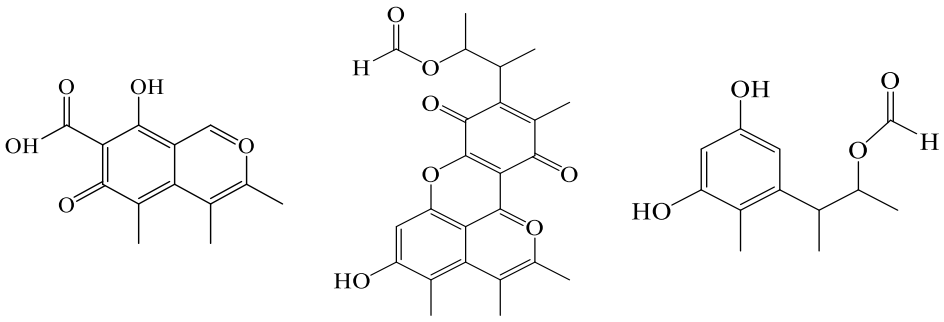


| Ocratoxina | $R_1$ | $R_2$ | Fórmula molecular | Peso molecular (g/mol) |
|---|---|---|---|---|
| OTA | H | Cl | $C_{20}H_{18}ClNO_6$ | 403,8 |
| OTB | H | H | $C_{20}H_{19}NO_6$ | 369,4 |
| OTC | $C_2H_5$ | Cl | $C_{22}H_{22}ClNO_6$ | 431,9 |

**Fig. 5**. Estructura de la OTA, OTB y OTC

### 1.2.6. Citrinina

La CTN es un policétido nombrado según la IUPAC como ácido (3R, 4S)-4,6-dihidro-8-hidroxi-3,4,5-trimetil-6-oxo-3H-2-benzopiran-7-carboxílico. En estado puro es sólido cristalino de color amarillo que se descompone a 175 ºC, aunque en presencia de agua se descompone a partir de 100 ºC. Los dos productos de descomposición se conocen como citrinina $H_1$ y citrinina $H_2$ (Fig. 6), el primer subproducto presenta mayor potencial tóxico comparado con la citrinina y el segundo no muestra toxicidad. Es insoluble en agua fría, soluble en disolución acuosa de hidróxido de sodio, carbonato de sodio o acetato de sodio. Además, es soluble en disolventes orgánicos como el metanol o acetonitrilo.



| Micotoxina | CTN | Citrinina $H_1$ | Citrinina $H_2$ |
|---|---|---|---|
| Fórmula molecular | $C_{13}H_{12}O_5$ | $C_{24}H_{23}O_7$ | $C_{12}H_{16}O_4$ |
| Peso molecular (g/mol) | 248,2 | 423,4 | 224,3 |

**Fig. 6.** Estructura de la CTN y análogos

La CTN está producida por diferentes especies de los géneros *Aspergillus, Penicillium* y *Monascus.* En el año 1932 se aisló por primera vez a partir de *P. citrinum* e inicialmente se le atribuyeron propiedades antibióticas y antifúngicas, sin embargo, se demostró su potencial tóxico que provoca daños en el riñón tras su ingesta (59). La CTN puede estar presente en variedad de alimentos como los cereales, especialmente arroz, en frutas o especias (60).

### 1.2.7. Otras toxinas reguladas

#### 1.2.7.1. Patulina

La PAT es una γ-lactona α,β-insaturada (Fig. 7) nombrada como 4-hidroxi-4H-furo [3,2c] piran-2[6H]-ona (61). Es una micotoxina soluble en agua y está producida por varias especies del género *Penicillium, Aspergillus* y *Byssochlamys* (62). La primera vez que se aisló

se consideró un compuesto con actividad antibacteriana. Sin embargo, se descubrió que la ingesta de esta micotoxina provoca inflamación del sistema gastrointestinal. Una exposición prologada puede provocar efectos que van desde la alteración de la microbiota hasta efectos neurotóxicos e inmunosupresores (63). El principal productor de esta micotoxina se conoce como *P. expansum*, y es el causante de la aparición de moho azul o manchas marrones en la fruta. La PAT suele encontrarse en frutas, como la pera o la manzana, y en productos derivados a base de frutas, como el zumo. Incluso puede aparecer en cereales y quesos (64).

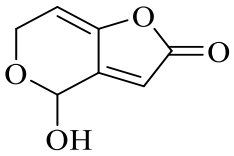



**Fig. 7.** Estructura de la PAT

#### 1.2.7.2. Alcaloides y esclerocios de cornezuelo

Los alcaloides de cornezuelo, conocidos como alcaloides ergóticos (AEs) son sólidos cristalinos, solubles en disolventes orgánicos y algunos son solubles en agua. Su estructura de ergolina consiste en un sistema de anillos tetracíclicos (Fig. 8), que están producidos principalmente por *Claviceps purpurea,* un hongo parásito que suele crecer en granos de centeno, cebada, trigo, maíz y avena. La infección por este hongo se puede detectar a simple vista, ya que se observa como brotan unas especies de puntas largas y negras que crecen hacia arriba, dentro y al lado de las espigas de los cereales (65). Los antecedentes históricos de estos compuestos se remontan a la Edad Media, donde los efectos eran bien conocidos y la enfermedad se denominaba "El fuego de San Antonio". Esta intoxicación era producida por la ingesta de alimentos contaminados con este hongo que producía convulsiones, delirios e incluso gangrena en las extremidades, llegando a provocar la muerte. Actualmente, esta enfermedad se conoce como ergotismo (66).

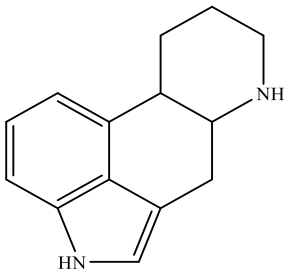



**Fig. 8.** Estructura química de AEs

## 1.3. MICOTOXINAS EMERGENTES

Las micotoxinas emergentes abarcan un amplio número de metabolitos producidos por diferentes especies que no están reguladas y no se incluyen en métodos de monitoreo rutinario, empleados en el control de alimentos y piensos. Actualmente existe una falta de datos acerca de la incidencia y toxicidad de estos compuestos en alimentos, pero hay un creciente interés por ahondar en su búsqueda para evaluar el riesgo que pueda suponer para la salud humana y animal. Se ha observado que la incidencia de algunas micotoxinas emergentes es muy alta, sobre todo en cultivos. También se ha demostrado que suelen estar presentes de forma

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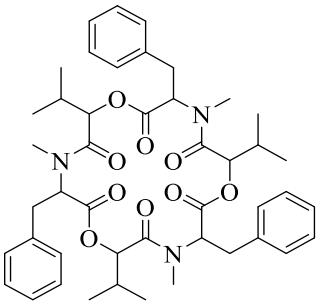
simultánea con micotoxinas reguladas, ya que los hongos productores son de la misma especie (25).

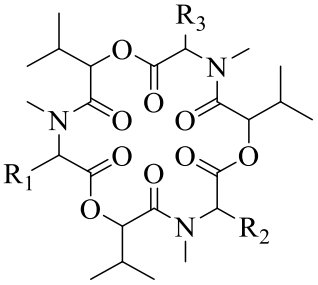
Las toxinas emergentes más conocidas por su frecuencia de aparición dentro de la cadena alimentaria son BEA, ENNs, STC, MPA, AME, ROQC, GLIO, NIV, DAS y ácido fusárico (FA) (67, 68).

### 1.3.1. Beauvericina y eniatinas

La BEA es un hexadepsipéptido cíclico que consiste en unidades alternadas de D-hidroxi-isovaleril-(ácido 2-hidroxi-3-metilbutanoico) y N-metilfenilalanina como se muestra en la Fig. 9. Está producida por hongos como *Baveria bassiana* o *Fusarium spp.* Por otro lado, las ENNs tienen una estructura muy similar, pero difieren en que en las ENNs A y B poseen los aminoácidos N-metil-valina o N-metil-isoleucina alifáticas o mezclas de estas dos. Las estructuras se muestran en la Fig. 9.

La BEA y ENNs se consideran compuestos lipofílicos porque las cadenas laterales apolares están situadas en la cara exterior de la molécula. Muestran baja solubilidad en agua y no presentan inestabilidad en condiciones de pH ácido o básico (69). Pueden actuar como nucleófilos debido a su par de electrones libres situados en el oxígeno del grupo carbonilo. El mecanismo de acción todavía no está bien definido, sin embargo, se ha demostrado que producen efectos en los canales de calcio en células de neuroblastoma humano (70). En cuanto a la incidencia en alimentos, suelen encontrarse con frecuencia en cereales y productos derivados, frutos secos, cerveza, huevos y leche (71, 72).





| | BEA | ENNA | ENNA$_1$ | ENNB | ENNB$_1$ |
|---|---|---|---|---|---|
| **Fórmula molecular** | $C_{45}H_{57}N_3O_9$ | $C_{36}H_{63}N_3O_9$ | $C_{35}H_{61}N_3O_9$ | $C_{33}H_{57}N_3O_9$ | $C_{34}H_{59}N_3O_9$ |
| **Peso molecular (g/mol)** | 783,95 | 681,5 | 667,4 | 639,4 | 653,4 |
| **$R_1$** | - | $CH(CH_3)CH_2CH_3$ sec-butyl | $CH(CH_3)CH_2CH_3$ sec-butyl | $CH(CH_3)_2$ iso-propyl | $CH(CH_3)_2$ iso-propyl |
| **$R_2$** | - | $CH(CH_3)CH_2CH_3$ sec-butyl | $CH(CH_3)CH_2CH_3$ sec-butyl | $CH(CH_3)_2$ iso-propyl | $CH(CH_3)_2$ iso-propyl |
| **$R_3$** | - | $CH(CH_3)CH_2CH_3$ sec-butyl | $CH(CH_3)_2$ iso-propyl | $CH(CH_3)_2$ iso-propyl | $CH(CH_3)CH_2CH_3$ sec-propyl |

**Fig. 9.** Estructura química de la BEA y de las ENNs

### 1.3.2. Esterigmatocistina

La STC es la abreviatura de ((3aR, 12cS)-3a,12c-dihidro-8-hidroxi-6-metoxi-7H-furo[3',2':4,5]furo[2,3-c]xanten-7-ona. Su fórmula molecular es $C_{18}H_{12}O_6$ y el peso molecular de 324,3 g/mol. En la Fig. 10 se muestra su estructura química. Es un policétido producido por más de 50 especies *Aspergillus*, principalmente *A. versicolor*. Esta micotoxina cristaliza en agujas de color amarillo pálido, y su punto de fusión está en torno a 245-246 ºC. Es soluble en

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disolventes orgánicos como el cloroformo, metanol o acetonitrilo y muestra baja solubilidad en disoluciones acuosas. Esta micotoxina, que comparte su vía biosintética con las AFs y que está relacionada con la estructura de la $AFB_1$, afecta a cereales y productos a base de cereales, además, se ha aislado de diferentes variedades de queso (73, 74).

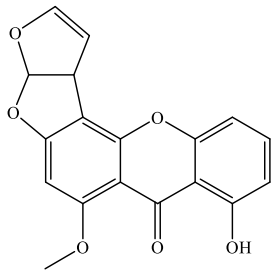



**Fig. 10.** Estructura de la STC

La STC es hepatotóxica, causa necrosis hepatocelular y hemorragias. También es capaz de causar lesiones premalignas y malignas como carcinomas hepatocelulares (75). En este sentido, la EFSA considera que se precisan métodos analíticos más sensibles y más datos de toxicidad para averiguar el posible riesgo que supone su presencia en alimentos y piensos (74).

### 1.3.3. Ácido micofenólico

El MPA se conoce como ácido 6-[4-hidroxi-6-metoxi-7-metil-3-oxo-5-ftalanil]-4-metil-4-hexenoico (Fig. 11). Su fórmula molecular es $C_{17}H_{20}O_6$ y su peso molecular de 320,3 g/mol. Es un policétido producido principalmente por especies de *Penicillium* siendo el principal productor *P. roqueforti*, y otras especies como *P. brevicompactum*, y *P. carneum* (76).
Esta micotoxina se puede encontrar en silos, jengibre, vino y queso (25). Es un agente inmunosupresor comúnmente empleado en la profilaxis del rechazo agudo en trasplantes. Los niveles en los que se encuentra en los alimentos son muy inferiores a las dosis terapéuticas en humanos, sin embargo, podría causar un cierto nivel de inmunosupresión en animales expuestos a grandes cantidades (25, 77).

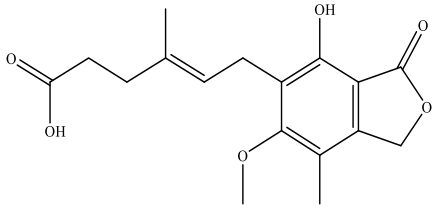



**Fig. 11.** Estructura del MPA

### 1.3.4. Roquefortina C

La ROQC (Fig. 12) es una micotoxina producida por el género *Penicillium,* en especial *P. roqueforti*. Su fórmula molecular es $C_{22}H_{23}N_5O_2$ y su peso molecular es de 389,5 g/mol. Es totalmente soluble en etanol, y la solubilidad en metanol es de 10 mg/mL. El punto de fusión es de 303,1 ºC. El hongo productor suele añadirse de manera intencionada en la producción de ciertos quesos durante la etapa de maduración. Como consecuencia, en estos productos se suelen encontrar cantidades elevadas de ROQC, pero el consumo de quesos no considera un peligro importante para la salud ya que, hasta el momento, no se han reportado intoxicaciones humanas por consumo de estos productos lácteos (64).

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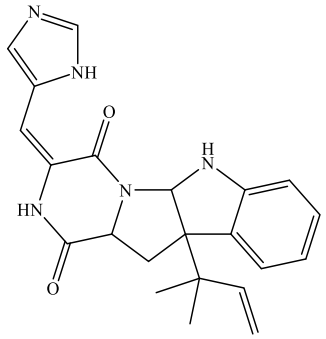


**Fig. 12.** Estructura de la ROQC

### 1.3.5. Gliotoxina

La GLIO pertenece a la clase de compuestos epipolitiodioxopiperacina, caracterizada por un enlace disulfuro. Su fórmula molecular es $C_{13}H_{14}N_2O_4S_2$ y su peso molecular es 326,1 g/mol (Fig. 13). Fue aislada originalmente de *Trichoderma virens,* como antibiótico para el control de hongos patógenos de plantas (78). Es producida por varias especies como *Aspergillus fumigatus, Eurotium chevalieri*, *Gliocladium fimbriatum,* y *Dichotomyces cejpii* y también algunas especies de *Penicillium* (79, 80). Se encuentra con frecuencia en los silos y es inmunosupresora ya que causa la apoptosis de linfocitos y macrófagos (81).

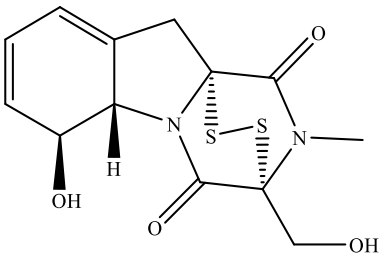
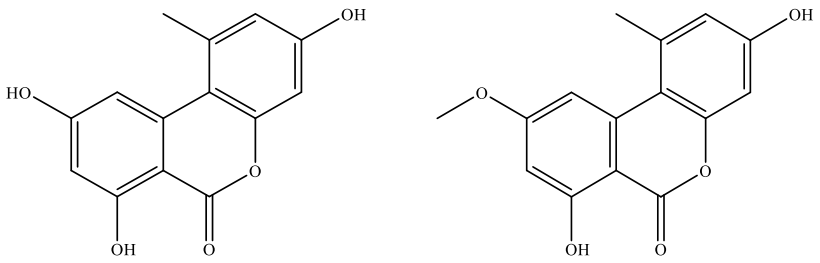


**Fig. 13.** Estructura de la GLIO

### 1.3.6. Toxinas de *Alternaria*

Dentro de las toxinas de *Alternaria*, se incluyen las dibenzo-α-pironas AOH ($C_{14}H_{10}O_5$, 258,2 g/mol) y AME ($C_{15}H_{12}O_5$, 272,3 g/mol) (Fig. 14). Estas micotoxinas son solubles en disolventes orgánicos, como el metanol o etanol, suelen encontrarse en cereales, productos derivados, tomate y productos a base de tomate, semillas de girasol y aceite de girasol, frutas, productos elaborados a base de frutas, cerveza y vino (82). Recientemente se ha detectado la presencia de AME en leche humana (83). Aunque existen pocos datos acerca de su toxicidad, ensayos *in vitro* los clasifican como citotóxicos y genotóxicos (84).



(a) (b)

**Fig. 14.** Estructura de AOH (a) y AME (b)

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### 1.3.7. Diacetoxiscirpenol

El DAS conocido también como (3α,4β)-3hidroxi-12,13-epoxi-tricotec-9-eno-3,4,15-triol-4,15-diacetato es un compuesto cristalino de color blanco, con un punto de fusión entre 12 ºC y 164 ºC. Pertenece al grupo de tricotecenos de tipo A y lo producen distintas especies *Fusarium* (36). La estructura química se diferencia de la T-2 en el $R_5$, ya que no posee el grupo isovaleril (Fig. 2) (85).

Es un compuesto soluble en disolventes orgánicos como acetato de etilo, acetona o cloroformo. Sin embargo, es menos polar que los tricotecenos de tipo B. Se mantiene estable en condiciones de pH neutro y ácido pero en disoluciones alcalinas de hidróxido sódico o hidróxido de amonio se hidroliza a un análogo denominado scirpentriol (86). Generalmente suele detectarse en cereales y productos derivados, así como también en piensos y silos de maíz. Sus efectos tóxicos son similares a los mencionados de los anteriores tricotecenos de tipo A (87).

### 1.3.8. Ácido ciclopiazónico

El CPA también conocido como ácido α-ciclopiazónico, es una toxina del ácido indol-tetrámico, con fórmula molecular $C_{20}H_{20}N_2O_3$ y peso molecular de 336,4 g/mol. Es cristalino, incoloro y soluble en disolventes orgánicos como metanol, acetonitrilo o cloroformo e insoluble en agua. Su estructura química se muestra en la Fig. 15.

Se aisló por primera vez de una cepa de *Penicillium griseofulvum.* El CPA es producido por varias especies de *Penicillium,* como *P. camemberti* y por especies de *Aspergillus,* como *A. flavus*, *A. fumigatus* y *A. oryzae.* Debido a que estas especies son productoras de AFs, el CPA se puede llegar a detectar en los mismos alimentos, como cereales y productos derivados, e incluso en leche (88, 89). El CPA se ha detectado en queso azul, probablemente porque la especie *P. camemberti* se emplea en la etapa de producción para la maduración del queso (90).

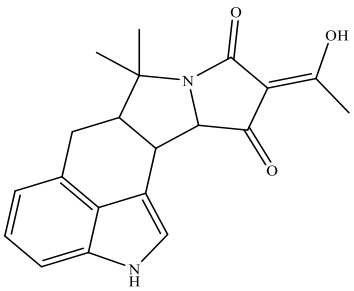



**Fig. 15.** Estructura química del CPA

## 1.4. EXTRACCIÓN DE MICOTOXINAS DE MATRICES ALIMENTARIAS

El método de extracción permite separar el analito de la matriz alimentaria. La técnica de extracción va a depender de la naturaleza de ambos y todas tienen un objetivo común que es la obtención de un extracto libre de interferentes. Existen diversos métodos para extraer compuestos de matrices alimentarias entre los que se incluyen extracción líquido-líquido, sólido-líquido, extracción en fase sólida o SPE (Solid Phase Extraction) o microextracción en fase sólida o SPME (Solid Phase Micro Extraction).

La técnica de extracción líquido-líquido es de la más empleadas cuando la matriz es líquida, permite recuperar los analitos de interés utilizando un disolvente inmiscible con la matriz. De esta forma, el analito se reparte entre las dos fases según el coeficiente de distribución. Generalmente se emplea un disolvente de naturaleza orgánica cuando la matriz es acuosa. La

extracción SPE es otra técnica aplicable a matrices liquidas. En este caso, la matriz se introduce en un cartucho que contiene una fase sólida que actúa a modo de adsorbente, y en ella se retiene el analito de interés. A continuación, este compuesto se recupera empleando un disolvente de elución que permite su recuperación selectiva, minimizando el arrastre de otras moléculas. Por ello, esta técnica también puede utilizarse como un método de limpieza en muestras complejas, después de una extracción sólido-líquido. Los procedimientos de SPE tradicionales utilizan como adsorbentes materiales de fase reversa como C8 o C18, intercambiadores catiónicos o aniónicos o materiales poliméricos. Además, también se emplean columnas de inmunoafinidad, que utilizan anticuerpos específicos contra el analito de interés, lo que permite una adsorción específica, aunque son menos usados debido a su alto coste (91). La extracción sólido-líquido consiste en extraer el analito de la matriz sólida con el uso de un disolvente adecuado y a través de distintos procedimientos. El disolvente tiene que poseer ciertas características que lo hagan apropiado, es decir, la solubilidad de las sustancias que se desean extraer deben ser afines al disolvente de extracción, las propiedades físicas como una baja viscosidad y tensión interfacial para poder penetrar a través de los poros de la matriz sólida deben ser adecuadas. Además, hay que tener en cuenta el coste y la toxicidad del disolvente (92).

Existen procedimientos de extracción más comunes como son la maceración, en los que el analito está unido débilmente a la matriz, o el soxhlet, para extraer analitos orgánicos unidos fuertemente a la matriz empleando un disolvente orgánico volátil. Por otro lado, existen técnicas de extracción que demandan menos uso de disolvente orgánico y son más respetuosas con el medio ambiente. Por ejemplo, la SPME utiliza una fase extractante a base de fibras, hechas de sílice fundida y recubierta de un adsorbente que suele ser polimérico. En este tipo de técnicas, la ratio de extractante/muestra es mucho menor (93).

Existen otros métodos de extracción y purificación como las columnas de inmunoafinidad o IAC (InmunoAffinity Column), la extracción con fluidos supercríticos o SFE (Supercritical Fluid Extraction), la extracción con microondas o MWE (Micro-Wave Assisted Extraction) o la extracción acelerada con disolventes o ASE (Accelered Solvent Extraction) (91).

En los últimos años, se popularizó la extracción basada en la metodología QuEChERS (Quick, Easy, Cheap, Effective, Ruuged y Safe). Esta se dio a conocer en el año 2003 a través de la publicación científica de Anastassiades et. al (94) donde se mencionó por primera vez este acrónimo. El método original se desarrolló para el análisis de pesticidas en frutas y verduras. La ventaja de este método frente a los convencionales fue que se basaba en los principios de la química analítica verde. El método original se basa en dos etapas. La primera consiste en una extracción sólido-líquido seguido de una partición con sales inorgánicas. En esta primera etapa se lleva a cabo la extracción de los contaminantes con la adición de 10 mL de acetonitrilo en 10 g de muestra homogenizada. Posteriormente, se añaden las sales inorgánicas sulfato de magnesio ($MgSO_4$) y cloruro sódico (NaCl) para inducir la separación de fases mediante agitación y centrifugado. La segunda etapa del método consiste en una extracción en fase sólida dispersiva (d-SPE) como etapa limpieza del extracto. En este paso se reducen o eliminan los compuestos secundarios presentes en la matriz que pueden interferir en el análisis. Para esta etapa se emplea $MgSO_4$ y PSA (amina primaria/secundaria), y mediante agitación y centrifugado se consigue un sobrenadante limpio que constituye el extracto de análisis.

A partir del método original se han realizado múltiples modificaciones con el fin de mejorar la eficacia de extracción en función de la matriz y los analitos objeto de estudio. Concretamente, el método AOAC 2007.01 validado por Lehotay (95) para analizar pesticidas en alimentos. Este método emplea 15 mL de acetonitrilo mezclados con una solución buffer de ácido acético y acetato de sodio para conseguir un pH de 4,5 que se añaden a 15 g de muestra hidratada y homogenizada (95). A continuación, se usan 6 g de $MgSO_4$ y 1,5 g de acetato de sodio, y tras

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aplicar agitación y centrifugación se consigue separar en dos fases. Posteriormente, 1 mL del sobrenadante obtenido se somete a una etapa de limpieza con $MgSO_4$ y PSA. El extracto se conserva en ácido fórmico 6,7 mM para su posterior análisis por LC-MS/MS.

Otra variante del método original es la que realizó Anastassiades (96) validada como el método estándar EN 15662 por el Comité Europeo de Estandarización (CEN, European Committe for standardizartion). En este caso se emplea la combinación de $MgSO_4$:NaCl en un ratio de 4:1 y se añade citrato tribásico de sodio dihidratado y citrato de sodio dibásico sesquihidrato para facilitar la separación de la fase acuosa y de la fase orgánica. Para llevar a cabo la d-SPE se emplean los sorbentes PSA, C18 y la sal inorgánica, $MgSO_4$ anhidro. El extracto final se conserva en ácido fórmico al 5 % en acetonitrilo. Posteriormente, se realizaron múltiples modificaciones y se extendió su uso al análisis de otros contaminantes como micotoxinas, toxinas marinas o residuos de fármacos (97).

El disolvente de extracción más empleado es el acetonitrilo porque permite extraer en un único paso una amplia gama de analitos de polaridades intermedias. Si la muestra contiene un porcentaje bajo de agua es necesario humedecer la muestra mediante la adición de agua o agua acidificada, porque facilita la entrada del disolvente dentro de la matriz y permite que la unión analito-matriz se rompa con mayor facilidad. En segundo lugar, la ratio muestra/disolvente depende de la naturaleza de la matriz y del disolvente, generalmente se aplica una proporción 1/1 porque resulta adecuada para la extracción. Las sales empleadas para inducir la separación de fases suelen ser $MgSO_4$ y NaCl en un ratio de $MgSO_4$:NaCl 1:4. Estas sales favorecen la separación de la fase orgánica y de la fase acuosa mejorando la recuperación del analito en la fase orgánica. Además, la combinación de las dos permite controlar la polaridad del medio y mejorar la forma de los picos cromatográficos (98).

## 1.5. DETECCIÓN DE MICOTOXINAS

La legislación europea establece métodos de referencia para el control oficial de ciertos contaminantes en alimentos, como las toxinas marinas. Sin embargo, en el caso de las micotoxinas, no se han establecido métodos específicos para su detección; en su lugar se permite el uso de métodos validados siempre que cumplan los criterios de funcionamiento establecidos en el Reglamento de Ejecución (UE) 2023/2782 (99). Por ello, este campo se presta a desarrollar nuevos métodos que permitan la determinación de micotoxinas en alimentos y piensos. En primer lugar, existen los métodos de cribado que proporcionan información sobre la presencia o ausencia de los contaminantes en las muestras de estudio. Se emplean con la finalidad de analizar un gran número de muestras en poco tiempo. En general, solo permite la detección cualitativa de la/s micotoxina/s en la muestra problema y sirven como sistemas de alerta (100).

Los métodos de cribado engloban varios ensayos o técnicas que poseen ciertas ventajas, entre ellas, la rapidez de respuesta y la sencillez en el tratamiento de muestra. El ensayo por inmunoadsorción ligado a enzimas, abreviado como ELISA (enzyme-linked immunosorbent assay), consiste en un método de detección basado en el enlace de la toxina a un anticuerpo específico. Se ofrece en formato kit y se puede aplicar para realizar un cribado de varias micotoxinas simultáneamente. Las ventajas que ofrece esta técnica son que se requiere poco volumen de muestra y su preparación es sencilla (101).

Sobre la misma base se han desarrollado las pruebas de flujo lateral. El método más empleado para la determinación de micotoxinas es el competitivo indirecto, donde el anticuerpo está marcado y el antígeno (toxina) está fijado a la membrana. De esta manera, el anticuerpo es suspendido en una muestra líquida que se deja fluir a través de la membrana. En ausencia de

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micotoxinas en la muestra, los anticuerpos se unen al antígeno, dando lugar a una línea T (línea test) y a la línea C (línea control). En presencia de micotoxinas por encima del límite de detección, sólo aparece la línea C, ya que los anticuerpos específicos están saturados por la unión con las micotoxinas presentes en la muestra, lo que impide la unión con el antígeno fijado en la membrana (102). Del mismo modo que en el ELISA, existen múltiples test disponibles comercialmente (102, 103). Otros métodos propuestos como técnicas de cribado incluyen los inmunosensores, el inmunoensayo por polarización de fluorescencia, los aptasensores o biochip (104-106).

Por otra parte, las técnicas analíticas de confirmación emplean una etapa de separación de los componentes de la muestra mediante técnicas como la cromatografía líquida (LC) seguida de técnicas de detección como fluorescencia (FLD), UV o espectrometría de masas (MS) (107, 108). La cromatografía de gases rara vez se emplea en la determinación de micotoxinas, debido a que son compuestos polares y no volátiles. En el caso de utilizarse, es necesario una etapa de derivatización para convertirlas en análogos o derivados volátiles (109).

Un cromatógrafo de líquidos incluye los siguientes elementos: reservorios o botellas para las fases móviles, sistema de bombeo, un inyector o dispositivo para introducir la muestra y una columna cromatográfica, normalmente dentro de un horno termostatizado. La columna está formada por un material granular formado por pequeñas partículas sólidas muy compactas denominado fase estacionaria. La muestra se inyecta en el sistema y es arrastrada por la fase móvil a través de la columna a una presión elevada, por ello se habla de cromatografía líquida de alta eficacia (HPLC). Cuando se emplean columnas empaquetadas con partículas de un tamaño inferior a 2 μm, la técnica se denomina cromatografía líquida de ultra alto rendimiento (UHPLC), este tipo de columnas aumentan el rendimiento de separación y permiten un análisis más rápido en comparación con las columnas de HPLC, con tamaños de partículas más grandes. En cuanto a fases móviles se refiere, hay dos formas de trabajar: elución isocrática, la composición de la fase móvil es constante, y elución en gradiente, en la que la composición de la fase móvil varía con el tiempo (107).

La cromatografía puede clasificarse según la polaridad de la fase estacionaria en dos tipos principales: cromatografía de fase normal y cromatografía de fase reversa. En la cromatografía de fase normal, la fase estacionaria es polar y la fase móvil es no polar. Por otro lado, en la cromatografía de fase reversa, la fase estacionaria es no polar y la fase móvil es polar. En HPLC o UHPLC, en el caso de columnas en fase normal, se emplean rellenos polares como la sílice, amida, ciano o resinas de cromatografía líquida de interacciones hidrofílicas (HILIC) para separar compuestos polares. En cuanto a columnas en fase reversa, se trabaja con resinas de sílice unidas a hidrocarburos alifáticos como C6, C8 o C18, o hidrocarburos aromáticos, como el fenil. Suelen emplearse para la separación de analitos polares, no polares, iónicos o ionizables. En general, suele emplearse la cromatografía de fase reversa para la separación de micotoxinas. El mecanismo general se basa en interacciones hidrofóbicas entre el analito y la fase estacionaria, en concreto con los hidrocarburos enlazados a la resina. El tiempo de retención es mayor para los analitos que tienen una naturaleza no polar, debido a que se quedan retenidos en la fase estacionaria. Por el contrario, los analitos polares eluyen más rápido. Este parámetro va a variar dependiendo de la interacción entre los analitos, la fase estacionaria y la fase móvil. Factores como el pH, el tipo de columna, la temperatura y la composición de la fase móvil van a influir en el tiempo de retención (110).

Las fases móviles más utilizadas en este tipo de cromatografía son agua, metanol y acetonitrilo. En ocasiones, se añaden soluciones tampón, ácidos o bases. Es conveniente que el pH de la fase móvil sea estable y las soluciones *buffer* o tampón sean adecuadas para ello, las más empleadas son el tampón de fosfato, de acetato, de trietilamina o dietilamina. A medida

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que la concentración de tampón en la fase móvil aumenta, el tiempo de retención de los compuestos polares es menor. Por otro lado, el pH de la fase móvil juega un papel importante en la sensibilidad y la selectividad de los analitos (111).

Una vez se consigue la separación de los analitos de interés del resto de componentes de la muestra, deben ser detectados en base a sus propiedades fisicoquímicas. Los detectores principales que se emplean en el análisis de micotoxinas son la FLD, la UV, la matriz de fotodiodos (PDA) y la espectrometría de masas (MS) y MS en tándem (MS/MS). Debido a la presencia de cromóforos en las micotoxinas, es posible su detección por FLD o UV. El DON, T-2 y HT-2 se detectan frecuentemente por UV, mientras que las AFs o CTN poseen propiedades fluorescentes y pueden ser medidas directamente por LC-FLD. Sin embargo, otras toxinas como las FBs carecen de cromóforos y por lo tanto se necesita una etapa de derivatización, bien sea pre- o post- columna (112).

El detector más empleado en la actualidad es el espectrómetro de masas. Este detector emplea la relación masa-carga (*m/z)* de iones positivos o negativos para la identificación de los compuestos. Por ello, las moléculas presentes en la muestra deben ser ionizadas empleando distintos sistemas entre los que destaca la ionización por electrospray (ESI) y la ionización química a presión atmosférica (APCI). En el caso de ESI, la muestra en solución pasa a través de un capilar al que se aplica un potencial eléctrico, normalmente de 2 a 6 kV, entre el capilar de entrada de la muestra y el contraelectrodo. A la salida del capilar, la muestra sale dispersa en forma de aerosol formado por pequeñas gotas cargadas que se evaporaran debido a un fenómeno de desorción del campo eléctrico o por evaporación del disolvente. A medida que las gotas se evaporan, las moléculas de la muestra se aproximan y se repelen, hasta que llega un momento en el que son atraídas hacia el contraelectrodo de entrada del espectrómetro por capilaridad dieléctrica.

Una vez ionizada la muestra, los iones pasan a una región de alto vacío y son guiados al analizador de masas que se encarga de separarlos en función de su *m/z*. Existen varios tipos de analizadores como el tiempo de vuelo (TOF), trampa de iones (IT), resonancia ciclotrónica por transformada de Fourier (FT-ICR) o el cuadrupolo (Q) (108) .

En la actualidad, los equipos más empleados en el análisis de las micotoxinas son instrumentos híbridos en los que se combinan dos analizadores. Por ejemplo, los equipos de triple cuadrupolo (QqQ) combinan dos cuadrupolos, esto les permite operar en diferentes modos de trabajo incluyendo scan de iones producto (product ion scan), scan de iones precursores (parent ion scan), scan de pérdida neutra (constant neutral Ioss scan), scan de iones seleccionados (SIM, selected ion monitoring) o monitorizando una reacción múltiple (MRM, multiple reaction monitoring). De ellos, el MRM es el modo de trabajo que permite la detección simultánea de múltiples compuestos y por ello es el más empleado en la determinación de micotoxinas (113). En el primer cuadrupolo se lleva a cabo una preselección, diferenciando los iones del analito o de los analitos de interés de otros compuestos. Luego, los iones seleccionados se dirigen hacia la celda de colisión, donde los iones precursores se disocian y forman iones producto, los cuales son monitorizados en el tercer cuadrupolo. De este modo, se obtienen al menos dos transiciones por cada analito: una transición para identificación y otra para cuantificación. Estos analizadores permiten una alta selectividad y sensibilidad gracias a una ionización eficiente y la detección específica de iones por los cuadrupolos. Además, son capaces de minimizar las interferencias y mejorar la relación señal/ruido (114).

Por otro lado, los instrumentos TOF ofrecen exactitud de masa a nivel sub-ppm y permiten la medición en un amplio rango de masas. A diferencia del analizador QqQ, los iones se van a separar en función de la velocidad alcanzada en el tubo de vuelo, la cual está inversamente relacionada con la raíz cuadrada de *m/z*. Los analizadores TOF pueden acoplarse a otros

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analizadores como la IT. El sistema IT-TOF permite la elucidación tentativa de moléculas de interés aplicando el proceso $MS^n$. Su funcionamiento consiste en que los iones generados en la fuente se transportan a través de una guía de iones (DQarray) y se acumulan en un octopolo durante un tiempo de acumulación definido por el usuario. Desde allí, los iones se impulsan hacia la IT, donde pueden acumularse, permitiendo varias etapas de MS/MS. El ión precursor puede disociarse mediante inducción por colisión con moléculas neutras como el argón y luego ser separado según su tiempo de vuelo (115).

## 1.6. VALIDACIÓN DEL MÉTODO ANALÍTICO

La validación de un método se define, según la ISO/IEC 17025, como la *"confirmación, a través del examen y aportación de evidencias objetivas, de que se cumplen los requisitos particulares para un uso específico previsto*". La validación de un método analítico es la única forma de demostrar que el método empleado es adecuado para el fin previsto, obteniendo un resultado fiable. Según la Guía Eurachem sobre la *Adecuación al uso de métodos analíticos* para validar un método es necesario evaluar las características de desempeño, así como valorar la idoneidad del método (116). Las características de desempeño son las siguientes: selectividad, límite de detección (LOD) y cuantificación (LOQ), rango o intervalo de trabajo, veracidad, precisión y robustez.

Selectividad: Se relaciona con la capacidad del método para identificar y/o cuantificar los analitos en presencia de compuestos secundarios, en una muestra de la matriz en las condiciones establecidas por el método.

LOD: Se define como la mínima cantidad de analito en una muestra que puede ser detectada. Existen varias formas de calcularlo basándose en la señal/ruido (S/N), o en la desviación estándar (SD) de la respuesta analítica del análisis de blancos y la pendiente (m) de la curva de calibrado

LOQ: Se define como la mínima cantidad de analito en una muestra que puede ser determinada con precisión y veracidad. Existen varias opciones para calcular este parámetro, entre ellas, se suele aplicar una de estas dos ecuaciones:

$$\mathrm{LOQ} = \frac{10}{\mathrm{m}}$$

$$\mathrm{LOQ} = 3.3 \cdot \mathrm{LOD}$$

Intervalo de trabajo: La capacidad de un método analítico para generar resultados directamente proporcionales a la concentración del analito o valor del parámetro de la muestra, dentro de ese rango de trabajo.

Veracidad o exactitud: Grado de concordancia entre el valor medio obtenido de una serie de resultados y un valor de referencia aceptado.

Precisión: Es el grado de concordancia entre los resultados independientes de una medición obtenidos en condiciones de repetibilidad o de reproducibilidad.

Repetibilidad: Se refiere a la variabilidad de los resultados cuando una medición se repite varias veces en condiciones idénticas, utilizando el mismo equipo y operario.

Reproducibilidad: Es la variabilidad observada cuando las mediciones se realizan en diferentes laboratorios, con equipos distintos y por distintos operarios.

Precisión intermedia: Es la medida de precisión cuando se realizan mediciones en el mismo laboratorio, pero bajo condiciones variables, como diferentes días, diferentes equipos, o diferentes operadores.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Robustez: Susceptibilidad de un método analítico a los cambios de las condiciones experimentales.

La UE establece procedimientos a seguir para la validación de los métodos analíticos y la interpretación de los resultados a través de la Decisión de la Comisión 2002/657/EC, cuando no existen regulaciones específicas para resolver un caso concreto. No obstante, en el año 2006 se publica el Reglamento (CE) Nº 401/2006, que se establece los métodos de muestreo y análisis para el control oficial del contenido de micotoxinas en productos alimenticios. Este reglamento estableció los requisitos generales y específicos que debían cumplir los métodos empleados para el análisis de las micotoxinas. Los requisitos generales hacen referencia a los parámetros que deben ser evaluados durante la validación: exactitud, aplicabilidad, LOD, LOQ, precisión, repetibilidad, reproducibilidad, recuperación ($R_E$), selectividad, sensibilidad, linealidad e incertidumbre de medición. Los requisitos específicos son los criterios de funcionamiento que tienen que cumplir los métodos para el análisis de las micotoxinas en productos alimenticios. Estos criterios incluyen el intervalo de concentración, la desviación estándar relativa ($RSD_R$ y $RSD_r$) y la $R_E$ para cada micotoxina (117). El Reglamento (CE) Nº 401/2006 ha sido recientemente derogado por el Reglamento de Ejecución (UE) 2023/2782, que establece nuevos requisitos específicos para el análisis de micotoxinas en productos alimenticios. Este nuevo reglamento se centra en criterios actualizados, basados en la $R_E$, precisión y LOQ (99). Debido a que los cambios introducidos son sustanciales, se establece un periodo de adaptación hasta el año 2029, durante el cual los requisitos específicos del Reglamento (CE) 401/2006 seguirán aplicándose a los métodos que hayan sido validados antes de la entrada en vigor del nuevo Reglamento (99). En la Tabla 2 se muestran las principales características y requisitos de funcionamiento de los dos reglamentos. En concreto, se especifican para cada nivel de concentración (µg/kg) la $RSD_r$ %, la $RSD_R$ % y la $R_E$. Además, en el nuevo reglamento se incluye un nuevo parámetro adicional: el LOQ.

Por lo tanto, la legislación comunitaria no establece ningún método de análisis específico, pero sí exige que los métodos empleados sean validados de acuerdo con los criterios generales y específicos establecidos en el Reglamento (UE) 2023/2782. De esta manera, la validación de un método analítico garantiza su fiabilidad (116).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

| Contenido (µg/kg) | | Reglamento (CE) Nº 401/2006 | | | Reglamento de Ejecución (UE) 2023/2782 | | | |
|---|---|---|---|---|---|---|---|---|
| | | $RSD_r$ % | $RSD_R$ % | $R_E$ % | $RSD_r$ % | $RSD_R$ % | $R_E$ % | LOQ (µg/kg) |
| $AFM_1$ | 0,01-0,05 | Derivada de la ecuación de Horwitz[a] | | 60-120 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,5*Límite máximo |
| | > 0,05 | | | 70-110 | | | | |
| AFs ($B_1$, $B_2$, $G_1$, $G_2$) | < 1 | Derivada de la ecuación de Horwitz[a] | | 50-120 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,1 ($AFB_1$) ≤ 1 |
| | 1-10 | | | 70-110 | | | | |
| | > 10 | | | 80-110 | | | | |
| OTA | < 1 | ≤ 40 | ≤ 60 | 50-120 | ≤ 20 | ≤ 25 | 70-120 | ≤ 3 |
| | 1-10 | ≤ 20 | ≤ 30 | 70-110 | ≤ 20 | ≤ 25 | 70-120 | ≤ 10 |
| PAT | < 20 | ≤ 30 | ≤ 40 | 50-120 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,5*Límite máximo |
| | 20-50 | ≤ 20 | ≤ 30 | 70-105 | ≤ 20 | ≤ 25 | 70-120 | |
| | > 50 | ≤ 15 | ≤ 25 | 75-105 | ≤ 20 | ≤ 25 | 70-120 | |
| DON | >100-≤ 500 | ≤ 20 | ≤ 40 | 60-110 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,5*Límite máximo |
| | > 500 | ≤ 20 | ≤ 20 | 70-120 | ≤ 20 | ≤ 25 | 70-120 | |
| ZEA | ≤ 50 | ≤ 40 | ≤ 50 | 60-120 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,5*Límite máximo |
| | > 50 | ≤ 25 | ≤ 40 | 70-120 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,5*Límite máximo |
| $FB_1$, $FB_2$ | ≤ 500 | ≤ 30 | ≤ 60 | 60-120 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,5*Límite máximo |
| | > 500 | ≤ 20 | ≤ 30 | 70-110 | ≤ 20 | ≤ 25 | 70-120 | |
| T-2, HT-2 | 50-250 | ≤ 40 | ≤ 60 | 60-130 | ≤ 20 | ≤ 25 | 70-120 | ≤ 0,5*Límite máximo |
| | > 200 | ≤ 30 | ≤ 50 | 60-130 | ≤ 20 | ≤ 25 | 70-120 | |
| | 100-200 | ≤ 40 | ≤ 60 | 60-130 | ≤ 20 | ≤ 25 | 70-120 | |
| | > 200 | ≤ 30 | ≤ 50 | 60-130 | ≤ 20 | ≤ 25 | 70-120 | |

**Tabla 2.** Características y requisitos de funcionamiento para cada micotoxina por el Reglamento (CE) Nº 401/2006 y Reglamento de Ejecución (UE) 2023/2782. [a]Los valores de precisión se calculan a partir de la ecuación de Horwitz, $RSD_R = 2^{(1-0,5\log C)}$; siendo $RSD_R$ la desviación estándar relativa calculada a partir de los resultados obtenidos en condiciones de reproducibilidad y C el cociente de concentración (117).

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# HIPÓTESIS Y OBJETIVOS

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# 2. HIPÓTESIS Y OBJETIVOS

Las micotoxinas son metabolitos secundarios producidos, en su mayoría, por hongos filamentosos de los géneros *Aspergillus*, *Penicillium* y *Fusarium* que, en condiciones óptimas de humedad y temperatura, proliferan sobre distintos alimentos destinados al consumo humano y animal. El crecimiento de los hongos y la consiguiente contaminación con micotoxinas puede darse en el campo o durante las etapas de transporte, procesado y almacenamiento de los alimentos. Además, las micotoxinas presentes en los cereales pueden sufrir arrastre hacia productos elaborados, o hacia productos de origen animal, como la leche y derivados. La legislación establece niveles máximos permitidos en productos alimenticios para los grupos de micotoxinas más tóxicas y que se detectan con mayor frecuencia. Estos grupos de toxinas son monitorizadas en programas de control mediante métodos analíticos que deben cumplir criterios de funcionamiento establecidos. Sin embargo, el riesgo de contaminación e intoxicación no está totalmente controlado porque, además de las micotoxinas reguladas, se han descrito nuevas toxinas denominadas micotoxinas emergentes y se ha observado que la mayoría de estos compuestos pueden conjugarse con otras moléculas dando lugar a formas modificadas. Todos estos compuestos no están contemplados en la legislación y no se analizan de forma rutinaria. En este sentido, es necesario disponer de métodos analíticos y estudiar su presencia en materias primas y productos derivados para determinar su incidencia y evaluar el riesgo de exposición en la cadena alimentaria.

En este contexto los objetivos de la presente tesis doctoral son:

1. Desarrollar y validar métodos analíticos para el análisis simultáneo de micotoxinas reguladas, emergentes y modificadas en matrices vegetales y animales.
2. Estudiar la presencia de micotoxinas en materias primas y productos elaborados para determinar su arrastre a lo largo de la cadena alimentaria.

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# MATERIAL Y MÉTODOS

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# 3. MATERIAL Y MÉTODOS

Esta sección contiene los materiales, reactivos, muestras comerciales, métodos analíticos y preparación de las muestras que se han empleado o llevado a cabo durante la presente tesis doctoral.

## 3.1. REACTIVOS Y MATERIALES

Metanol, acetonitrilo, ácido acético glacial, sulfato de magnesio anhidro y cloruro de sodio fueron adquiridos de Panreac Química S.A. (Barcelona, España). El ácido fórmico fue suministrado por Merck (Madrid, España), el formiato de amonio y el acetato de amonio por Fluka (Buchs, Suiza). La C18 Polygoprep$^{TM}$ 60-50 fue adquirida a través de Macherey-Nagel (Düren, Alemania). Los filtros centrífugos de membrana ultrafree-MC durapore de tamaño de poro de 0,22 µm fueron suministrados por Millipore (Billerica, EEUU). El agua se obtuvo del sistema Milli-Q Plus de Millipore (Millipore, EEUU).

Los estándares analíticos se adquirieron a través de las siguientes casas comerciales:

- Sigma (Madrid, España): $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, ENNA, ENNB, $ENNA_1$, $ENNB_1$, GLIO, ROQC, ZEA
- Romer Labs (Tulln, Austria): 3-AcDON, 15-AcDON, $AFM_1$, AOH, AME, CPA, CTN, DAS, $FB_1$, $FB_2$, h-$FB_1$, HT-2, MPA, NEO, STC, T-2, T-2 triol y ZOL
- Santa Cruz biotechnology (Santa Cruz, CA): CTA
- CIFGA S.A. (Lugo, España): OTA
- ENZO (Barcelona, España): BEA

El estándar analítico de la adenosina se adquirió en Merck (Madrid, España).

Quesos: Se adquirieron en supermercados locales. Se evaluaron 38 muestras de queso de distintos lotes, en concreto, 12 muestras de queso emmental, 10 de queso azul, 9 de queso brie y 7 de queso camembert.

Bebidas vegetales: Se adquirieron en supermercados locales. Se evaluaron 16 muestras de bebida de avena (con porcentajes de avena entre 11 % y 15 %), 6 muestras de bebida de arroz (entre 15% y 17% de arroz), 7 muestras de bebida de almendra (entre 2 % y 14 % de almendra) y 9 muestras de bebida de soja (8 % y 15 % de soja). Las muestras contienen ingredientes secundarios como estabilizadores, aromas, sal, azúcares y vitaminas.

Yogures: Se adquirieron en supermercados locales. Se evaluaron 27 muestras de yogur natural y 16 muestras de yogur de avena.

Muestras de TMR: Se evaluaron muestras procedentes de granjas situadas en Vila do Conde (Norte de Portugal). En el año 2019 se colectaron 33 muestras durante el otoño. En 2020, se colectaron 3 muestras en otoño y 15 en primavera. En 2022, se colectaron 2 muestras en otoño, 5 en invierno, y 4 en primavera.

Las cepas de *Gnomoniopsis smithogilvyi* (*Gnomoniaceae*, *Diaporthales*) fueron aisladas de castañas procedentes de bosques afectados por un brote de este hongo en el sur de Galicia. De todos los aislados, 5 se seleccionaron para realizar el estudio de la producción de metabolitos.

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## 3.2. INSTRUMENTACIÓN

### 3.2.1 Análisis UHPLC-MS/MS

Para el análisis dirigido se ha empleado un sistema UHPLC Agilent 1290 Infinity acoplado a un espectrómetro de masas triple cuadrupolo (Agilent 6460) equipado con una fuente de ionización electrospray (Agilent Technologies, Waldbronn, Germany). La adquisición de datos se ha llevado a cabo con el software Agilent MassHunter LCMS Acquisition Console y el procesado de datos con el software QQQ Quantitative Analysis.

Para separar los analitos, se ha empleado una columna cromatográfica Waters Acquity UPLC HSS T3 100 mm x 2.1 mm, 1.8 µm. Se ha empleado a una temperatura de 40 ºC. Las fases móviles y el gradiente de elución se optimizaron en función de las necesidades de cada método, siendo las más habituales:

Fase móvil A: agua con ácido fórmico (0,1 %) y formiato de amonio (5 mM) o agua con ácido fórmico (1 %) y formiato de amonio (5mM).

Fase móvil B: metanol o metanol/agua/ácido fórmico 97/2/1 (v/v/v).

El flujo de las fases móviles fue de 0,3 mL/min y se aplicó una elución en gradiente de 13 minutos de duración según la Tabla 3.

| Tiempo (min) | % B |
|---|---|
| 0,0 | 0 |
| 0,5 | 14 |
| 2,0 | 14 |
| 3,0 | 60 |
| 3,5 | 60 |
| 8,0 | 100 |
| 10,0 | 100 |
| 10,5 | 0 |
| 13,0 | 0 |

**Tabla 3.** Gradiente empleado para el análisis

Los parámetros del espectrómetro de masas fueron los siguientes: temperatura del gas de nebulización ($N_2$) 400 ºC; flujo del gas de nebulización, 12 L/min; temperatura del gas de secado, 350 ºC; flujo del gas de secado, 8 L/min; nebulizador, 45 psi; voltaje de capilaridad, 4000 V; voltaje de la boquilla, 0 V. El análisis fue llevado a cabo en modo ESI negativo y positivo a través de un método dinámico de MRM, es decir, se siguieron dos transiciones *m/z* para cada micotoxina como se muestran en la Tabla 4.

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| Micotoxina | Precursor *m/z* y modo | Producto *m/z* (CE) | | Polaridad | FV | CAV | Tiempo de retención (min) |
|---|---|---|---|---|---|---|---|
| | | Identificación | Cuantificación | | | | |
| **$AFB_1$** | 313 $[M+H]^+$ | 285 (24) | 241 (44) | Positiva | 144 | 2 | 6,54 |
| **$AFB_2$** | 315 $[M+H]^+$ | 287 (28) | 259 (32) | Positiva | 147 | 1 | 6,25 |
| **$AFG_1$** | 329 $[M+H]^+$ | 243 (28) | 200 (48) | Positiva | 132 | 2 | 6,15 |
| **$AFG_2$** | 331 $[M+H]^+$ | 245 (32) | 217 (40) | Positiva | 146 | 2 | 5,95 |
| **$AFM_1$** | 329 $[M+H]^+$ | 273 (25) | 259 (29) | Positiva | 120 | 2 | 6,00 |
| **AOH** | 257 $[M+H]^-$ | 213 (20) | 215 (24) | Negativa | 155 | 2 | 7,34 |
| **AME** | 271 $[M+H]^-$ | 256 (20) | 228 (28) | Negativa | 125 | 6 | 8,40 |
| **BEA** | 801 $[M+NH_4]^+$ | 134 (70) | 784 (14) | Positiva | 90 | 2 | 10,15 |
| **CPA** | 335 $[M+H]^-$ | 154 (28) | 140 (24) | Negativa | 255 | 2 | 8,80 |
| **CTA** | 394 $[M+H]^+$ | 379 (24) | 350 (32) | Positiva | 83 | 2 | 7,35 |
| **CTN** | 251 $[M+H]^+$ | 205 (20) | 233 (9) | Positiva | 110 | 2 | 6,70 |
| **DAS** | 384 $[M+NH_4]^+$ | 307 (4) | 105 (36) | Positiva | 90 | 2 | 6,65 |
| **DON** | 297 $[M+H]^+$ | 249 (8) | 203 (12) | Positiva | 74 | 2 | 5,00 |
| **DOM-1** | 281 $[M+H]^+$ | 109 (16) | 79 (48) | Positiva | 90 | 6 | 5,50 |
| **15 Ac-DON** | 339 $[M+H]^+$ | 261 (8) | 137 (8) | Positiva | 75 | 2 | 5,72 |
| **3 Ac-DON** | 339 $[M+H]^+$ | 261 (8) | 213 (8) | Positiva | 75 | 2 | 5,72 |
| **ENNA** | 699 $[M+NH_4]^+$ | 100 (60) | 228 (28) | Positiva | 170 | 2 | 10,50 |
| **$ENNA_1$** | 685 $[M+NH_4]^+$ | 210 (28) | 668 (12) | Positiva | 145 | 2 | 10,35 |
| **ENNB** | 657 $[M+NH_4]^+$ | 196 (28) | 640 (12) | Positiva | 90 | 2 | 10,10 |
| **$ENNB_1$** | 671 $[M+NH_4]^+$ | 196 (30) | 654 (14) | Positiva | 130 | 2 | 10,25 |
| **$FB_1$** | 722 $[M+H]^+$ | 352 (40) | 334 (40) | Positiva | 170 | 1 | 7,20 |
| **$FB_2$** | 706 $[M+H]^+$ | 336 (40) | 318 (48) | Positiva | 165 | 1 | 8,10 |
| **FA** | 180 $[M+H]^+$ | 162 (8) | 134 (16) | Positiva | 88 | 2 | 6,10 |
| **FX** | 355 $[M+H]^+$ | 175 (8) | 71 (20) | Positiva | 90 | 4 | 5,40 |
| **GLIO** | 327 $[M+H]^+$ | 263 (10) | 245 (14) | Positiva | 86 | 2 | 6,50 |
| **h-$FB_1$** | 406 $[M+H]^+$ | 388 (9) | 370 (17) | Positiva | 105 | 2 | 7,05 |
| **HT-2** | 447 $[M+Na]^+$ | 345 (16) | 285 (20) | Positiva | 108 | 2 | 7,20 |
| **MPA** | 321 $[M+H]^+$ | 207 (16) | 303 (0) | Positiva | 80 | 2 | 7,60 |
| **NEO** | 400 $[M+H]^+$ | 215 (16) | 169 (28) | Positiva | 74 | 2 | 5,45 |
| **OTA** | 404 $[M+H]^+$ | 239 (22) | 102 (75) | Positiva | 84 | 2 | 8,15 |
| **ROQC** | 390 $[M+H]^+$ | 193 (40) | 322 (16) | Positiva | 150 | 2 | 7,15 |
| **STC** | 325 $[M+H]^+$ | 281 (40) | 310 (24) | Positiva | 130 | 1 | 8,40 |
| **T-2 TOXIN** | 484 $[M+NH_4]^+$ | 215 (16) | 197 (16) | Positiva | 84 | 2 | 7,80 |
| **T-2 TRIOL** | 405 $[M+Na]^+$ | 303 (16) | 125 (12) | Positiva | 110 | 2 | 6,70 |
| **ZEN** | 317 $[M+H]^-$ | 273 (20) | 131 (28) | Negativa | 123 | 2 | 8,25 |
| **ZOL** | 319 $[M+H]^-$ | 275 (24) | 107 (28) | Negativa | 120 | 2 | 8,10 |

**Tabla 4.** Parámetros de detección UHPLC-MS/MS. a ion precursor (m/z) y modo de ionización, ion producto (m/z) empleado para la identificación, ion producto (m/z) empleado para la cuantificación, energía de colisión (CE), voltaje de fragmentación (FV), voltaje de la celda de aceleración (CAV) y tiempo de retención (min) de cada micotoxina.

### 3.2.2 Análisis UHPLC-MS-IT-TOF

Para el análisis no dirigido se ha empleado un sistema Shimadzu (Kyoto, Japón). El sistema UHPLC consiste en dos bombas (LC-30AD), un desgasificador (DGU-20), un autoinyector (SIL-10AC) con un rack de muestras refrigerado y un horno de columna (CTO-10AS). La columna empleada fue Waters ACQUITY HSS T3, 100 mm x 2,1 mm, 1,8 µm (Waters, Milford, MA), que se mantuvo a una temperatura de 40 ºC. Las fases móviles empleadas consistieron en (A) agua con 0,1 % de ácido fórmico y (B) metanol. El flujo de la fase móvil fue de 0,3 mL/min y se empleó una elución en gradiente que se detalla a continuación: el eluyente B se mantuvo en 0 % durante 0,5 min, a continuación, se incrementó hasta 50 % de B en 0,5 min y se mantuvo durante un tiempo de 2,5 min. Posteriormente, el gradiente se incrementó hasta 100 % de B en 3 min y se mantuvo durante 3,5 minutos. Finalmente, el gradiente descendió hasta el 0 % B en 0,5 min y las condiciones iniciales se mantuvieron durante 3,5 min para re-equilibrar la columna cromatográfica. El volumen de muestra inyectado en el UHPLC fueron 5 µL.

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El espectrómetro IT-TOF de Shimadzu (Kyoto, Japón), equipado con una fuente ESI, mantuvo las siguientes condiciones de trabajo: voltaje del detector, 1,65 kV; temperatura del bloque térmico, 200 ºC; flujo del gas de nebulización, 1,5 L/min; presión del gas de secado, 105 kPa. La presión en la trampa iónica fue de 1,8 x $10^{-2}$ Pa, mientras que la región del TOF estuvo en 1,4 x $10^{-4}$ Pa. El método MS operó en modo positivo y negativo full scan MS entre un rango de masas de 150 y 1000 Dalton (Da). El tiempo de acumulación de iones se configuró en 20 ms, con un tiempo de evento de 300 ms con tres repeticiones. Para los experimentos de disociación inducida por colisión (CID, del inglés collision-induced-dissociation), los iones se aislaron en $MS^1$ con un rango de tolerancia de 1 Da. El gas de colisión empleado fue el argón, con un parámetro de energía de colisión configurado al 50 % y el parámetro de frecuencia a 45 kHz. A continuación, los rangos de masas del método en $MS^2$ se ajustaron para cada metabolito, donde el tiempo de acumulación de iones se estableció en 30 ms.

## 3.3. PREPARACIÓN DE LA MUESTRA

### 3.3.1. Extracción basada en QuEChERS

El método de extracción se basó en la metodología QuEChERS, los protocolos concretos fueron optimizados para cada tipo de matriz analizada. De manera general, se detalla a continuación el procedimiento a seguir: se pesan la muestra homogenizada en un tubo Falcon y se añade un volumen de disolución acuosa acidificada. Durante 5 minutos se homogeniza por agitación mediante vórtex. A continuación, se añade el volumen correspondiente de acetonitrilo y se vuelve a homogenizar durante 5 minutos. Más tarde, se añaden las sales $MgSO_4$ y NaCl y se agitan durante 30 s y se centrifuga la mezcla a 3134 *g,* durante 10 min. Como se muestra en la Fig. 16, este paso favorece la separación de la fase acuosa (parte inferior) y de la fase orgánica (parte superior).

Después de esta primera etapa, se lleva a cabo una etapa de limpieza a través de una extracción de dispersión en fase sólida (d-SPE). A un volumen concreto de extracto orgánico se le añade una cantidad de $MgSO_4$ anhidro y, en menor proporción, C18. Esta mezcla se agita durante 30 s y se centrifuga (3134 *g*, 10 min). El sobrenadante se trasvasa a un vial silanizado y se evapora hasta sequedad. Finalmente, se redisuelve en un volumen de una mezcla de acetonitrilo/agua/ácido acético (49/50/1 v/v/v), se agita durante 30 s y se sonica. El extracto final se filtra a través de 0,22 µm (16000 *g,* 5 min) y se analiza por UHPLC-MS/MS.

En algunos casos es necesario omitir la etapa de limpieza, en estes casos el extracto orgánico se evapora hasta sequedad y se redisuelve en la mezcla de disolventes según lo mencionado anteriormente.

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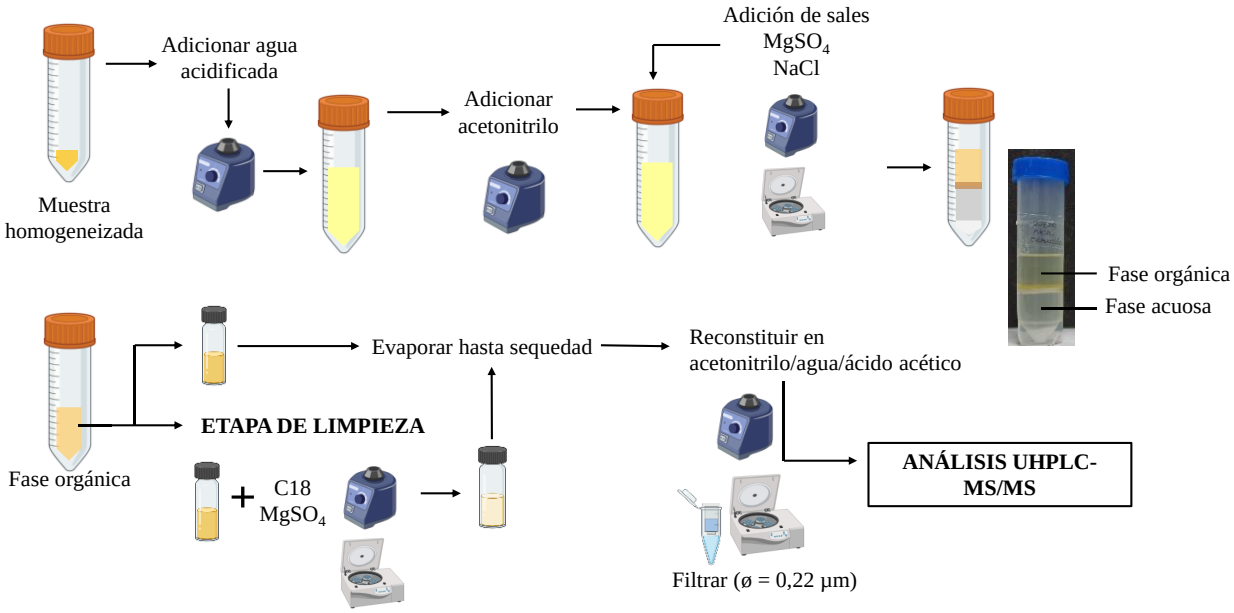



**Fig. 16.** Esquema del método de extracción basado en la metodología QuEChERS

### 3.3.2. Extracción de hongos *Gnomoniopsis smithogilvyi* (*Gnomoniaceae, Diaporthales*)

Para establecer el perfil de metabolitos producido por la especie *G. smithogilvyi,* se seleccionaron 5 aislados cultivados en PDA (del inglés, potato dextrose agar) a 25 ºC en oscuridad durante una semana y paralelamente se prepararon controles, es decir, PDA sin hongo. Una vez transcurridos los siete días, de cada cultivo se cortaron 6 discos de agar de 6 mm de diámetro y también de los controles. A continuación, se adicionaron 0,5 mL de una mezcla de acetonitrilo/agua/ácido acético (49/50/1 v/v/v) a los discos de agar y se agitaron durante 3 min. El extracto resultante se filtró a través de 0,22 µm y se conservó a -20 ºC.

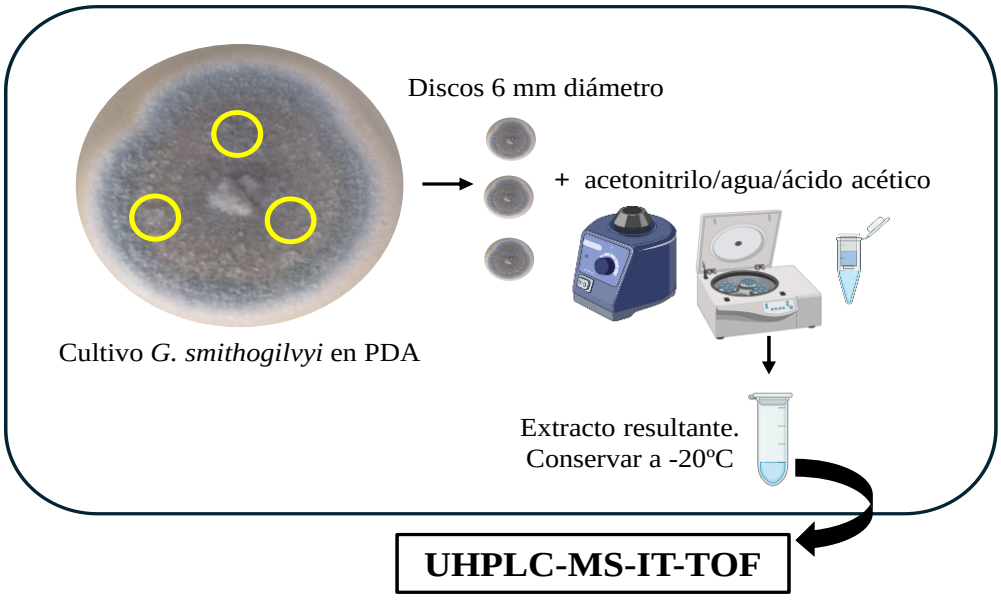



**Fig. 17.** Extracción de cultivo de *G. smithogilvyi* en PDA para su posterior análisis UHPLC-MS-IT-TOF

### 3.3.3. Caracterización del perfil metabólico de *G. smithogilvyi*

En primer lugar, se emplearon estándares analíticos de micotoxinas para verificar el rendimiento del método. En segundo lugar, se analizaron los extractos con el objetivo de realizar un cribado para detectar posibles metabolitos. Los iones fueron seleccionados para CID. En

función de la masa exacta del ion precursor y del ion producto, se predijo la fórmula molecular del compuesto mediante un software predictor ad hoc (Formula Predictor Software, Shimadzu Corp., Kyoto, Japón). Se emplearon varios filtros para incrementar la precisión de la predicción, aplicando la regla del nitrógeno y la regla del grado de insaturación.

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# RESULTADOS Y PUBLICACIONES

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# 4. RESULTADOS Y PUBLICACIONES

En esta sección se recopilan los resultados obtenidos durante el desarrollo de la presente tesis doctoral. El conjunto de datos se encuentra recogido en los siguientes artículos publicados en revistas internacionales incluidas en el JCR. Los resultados se agrupan en las siguientes secciones:

**SECCIÓN 1: MICOTOXINAS EN MATERIAS PRIMAS**

1. Occurrence of mycotoxins in total mixed ration of dairy farms in Portugal and carry-over to milk
2. First description of adenosine production by *Gnomoniopsis smithogilvyi*, causal agent of chesnut brown rot

**SECCIÓN 2: MICOTOXINAS EN PRODUCTOS ELABORADOS**

3. Detection of mycotoxins in cheese using an optimized analytical method based on a QuEChERS extraction and UHPLC-MS/MS quantification
4. Identification of mycotoxins in yogurt samples using an optimized QuEChERS extraction and UHPLC-MS/MS detection
5. Application of a multi-toxin detect method to analyze mycotoxins occurrence in plant-based beverages

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## SECCIÓN 1: MICOTOXINAS EN MATERIAS PRIMAS

### 1. Occurrence of mycotoxins in total mixed ration of dairy farms in Portugal and carry-over to milk

**Resumen:**
Este estudio tiene como objetivo explorar la presencia de micotoxinas en raciones totalmente mezcladas (TMR, por sus siglas en inglés) empleadas para la alimentación de ganado lechero y su posible transferencia a la leche. Se recolectaron un total de 87 muestras de TMR en granjas del norte de Portugal entre 2019 y 2022. Para el análisis de las muestras se empleó un método basado en una extracción QuEChERS seguida de detección por UHPLC-MS/MS. El método fue validado internamente en términos de efecto matriz, linealidad, sensibilidad, exactitud y precisión. Las toxinas reguladas detectadas con mayor frecuencia fueron las fumonisinas, con una tasa de positividad del 74 %, mientras que el deoxinivalenol y la zearalenona se encontraron en aproximadamente el 35 % de las muestras. Entre las toxinas emergentes, la beauvericina y las eniatinas mostraron las tasas de detección más altas. Además, se recolectaron muestras de leche de 21 granjas, proporcionando información sobre la transferencia de estas toxinas y la roquefortina C a la leche, con una tasa estimada que oscila entre el 2 % y el 10 %.

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**Información revista:**

**Occurrence of mycotoxins in total mixed ration of dairy farms in Portugal and carry-over to milk**

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# Occurrence of mycotoxins in total mixed ration of dairy farms in Portugal and carry-over to milk

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ABSTRACT

This study aims to explore the presence of mycotoxins in total mixed rations (TMR) employed for feeding dairy cattle and their potential carry-over to milk. A total of 87 TRM samples were collected in farms from the north of Portugal from 2019 to 2022. A method based on a QuEChERS extraction followed by UHPLC-MS/MS detection was employed for sample analysis. The method was in-house validated in terms of matrix effect, linearity, sensitivity, accuracy and precision. The most frequently detected regulated toxins were fumonisins, with a positivity rate of 74%, while deoxynivalenol and zearalenone were found in approximately 35% of the samples. Among the emerging toxins, beauvericin and enniatins exhibited the highest detection rates. In addition, milk samples were collected from 21 farms, providing insights into the carry-over of these toxins and roquefortine to milk, with an estimated rate ranging between 2% and 10%.

## 1. Introduction

In the European Union (EU), dairy production is the second-biggest agricultural industry. Over the past 20 years, there has been a progressive change in the dairy production landscape, with a remarkable 50% rise in the milk yield per dairy cow (Karlsson et al., 2023). This achievement was accompanied by a shift towards intensified feeding systems, involving the partial or complete substitution of grazing. This was possible through the implementation of balanced rations, commonly known as Total Mixed Rations (TMR). TMR are produced by blending different proportions of forages, byproducts, concentrates, minerals, vitamins, and additives. This comprehensive mixture is formulated to provide a balanced and nutritionally complete diet for livestock, meeting their specific needs for maintenance and production (Schingoethe, 2017).

One of the main risks associated with TMR is that most of their constituents are susceptible to mycotoxin contamination (Garcia et al., 2023). Mycotoxins are fungal metabolites that evoke a toxic response when introduced in low concentration to higher vertebrates and other animals by a natural route (Bennett, 1987). The most significant groups of mycotoxins, such as aflatoxins, ochratoxin A (OTA), deoxynivalenol (DON), fumonisins, or zearalenone (ZEN), are regulated in many nations throughout the world (Sainz et al., 2018). However, there are many other fungal metabolites, not currently subject to legislative regulations despite their known toxicity, which are known as emerging mycotoxins. According to their frequency of occurrence and concentrations, the most significant compounds in this group include, among others, alternariol (AOH), enniatins, beauvericin (BEA), sterigmatocystin (STG), mycophenolic acid (MPA), and alternariol monomethyl ether (AME) (Gruber-Dorninger et al., 2017; Pérez-Fuentes et al., 2022).

While the composition of TMR may differ significantly among farms, in Portugal, maize silage is usually their main constituent (Garcia et al., 2023). In a recent study, it was discovered that more than 82% of maize silage samples exhibited the simultaneous presence of DON and BEA. Additionally, there were *Penicillium* and *Aspergillus* toxins, such as STG or roquefortine C (RC), which were found in up to 5% and 12% of samples, respectively (González-Jartín et al., 2022). The use of contaminated silage implies the transfer of mycotoxins to TMR. In this

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context, the presence of mycotoxins in the diet of dairy cows is of particular concern. Although ruminants are generally considered to have lower susceptibility to mycotoxins due to the capacity of the rumen microbiota to degrade these substances, the economic implications remain substantial. In this sense, mycotoxins are associated with decreased livestock production and elevated veterinary care costs. Furthermore, there is a potential transfer of mycotoxins to milk, posing a possible risk for human consumption. Although aflatoxin $M_1$ ($AFM_1$) is still a major concern in this food, new observations have shown that milk consistently has low levels of enniatins and beauvericin (BEA) (González-Jartín, Rodríguez-Cañás et al., 2021; Zain, 2011).

Currently, there is limited data on the presence of mycotoxins in TMR. Although various analytical methods have been employed in different regions to assess the presence of these contaminants in TMR, most of them have been developed to analyze one or a few toxins. In China, a high-performance liquid chromatography with fluorescence detection (HPLC-FLD) method was utilized for the analysis of aflatoxin $B_1$ ($AFB_1$), revealing the presence of this mycotoxin in all samples (Xu et al., 2021). A similar approach was used in Mexico, where aflatoxins were also detected in all samples, with an average concentration of 26.0 g/kg in the forage incorporated into TMR (Álvarez-Días et al., 2022). The same technique was applied in Lithuania to analyze $AFB_1$, ZEN, and T-2 toxin, being detected in 49%, 52%, and 55% of the 119 analyzed samples, respectively (Vaičiulienė et al., 2021). In this country, another small survey was conducted using lateral flow tests to analyze ZEN and DON in 12 samples, being detected in 11 and 7, respectively (Falkauskas et al., 2019). ELISA kits were employed in Jordan to analyze aflatoxins, T-2 toxin, DON, ZEN, and fumonisins, highlighting a high occurrence of T-2 toxin (Bani et al., 2020). Another study in Iran, using an ELISA method, found $AFB_1$ in all analyzed samples (Shad et al., 2019). The most comprehensive approach utilized so far has been a liquid chromatography coupled to mass spectrometry (LC-MS) method to detect mycotoxins, phytoestrogens, and other metabolites. In this way, ZEN, fumonisin $B_1$ ($FB_1$), and DON were detected in more than 84% of samples, while $AFB_1$ and OTA, previously reported in Mexico, were not detected (Penagos-Tabares, Sulyok, et al., 2023).

The presence of mycotoxins in animal feed poses a significant public health concern, as these contaminants enter the food chain, inducing toxicity in animals. Additionally, they have the potential to lead to undesirable residues in animal-derived food products, including milk, meat, or eggs (González-Jartín et al., 2023). Nowadays, dairy cows are housed in intensive farming systems, where they are provided with TMR as their primary feed (Schingoethe, 2017). However, not much is known about the presence of mycotoxins in this matrix. In this context, the objective of this study was to validate a method capable of analyzing both regulated and emerging mycotoxins in TMR. The secondary goal was to investigate the prevalence of these mycotoxins in dairy farms located in Portugal from 2019 to 2022. Furthermore, the study aimed to establish any potential transfer of toxins from TMR to milk.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Chemical reagents, including methanol, acetonitrile (ACN), glacial acetic acid (100%), anhydrous magnesium sulfate ($MgSO_4$), and sodium chloride (NaCl), were supplied by Panreac Quimica S.A. (Barcelona, Spain). Formic acid and ammonium formate were procured from Merck (Madrid, Spain) and Fluka (Buchs, Switzerland), respectively. Water was purified in a Millipore Milli-Q Plus system (Millipore, Bedford, MA). Millipore also provided the Ultrafree-MC Durapore membrane centrifugal filters with a 0.22 μm pore size.

Sigma (Madrid, Spain) provided the solid standards, including DON, ZEN, $FB_1$, $AFB_1$, aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$), aflatoxin $G_2$ ($AFG_2$), RC, gliotoxin (GLIO), fusaric acid (FA), enniatin A (ENNA), enniatin $A_1$ ($ENNA_1$), enniatin B (ENNB), enniatin $B_1$ ($ENNB_1$) and BEA. OTA standard was from Laboratorios CIFGA S.A. Romer Labs (Tulln, Austria) supplied the analytical standards, including T-2 toxin, HT-2 toxin, neosolaniol (NEO), fumonisin $B_2$ ($FB_2$), citrinin (CTN), alternariol (AOH), alternariol methyl ether (AME), deepoxy-deoxynivalenol (DOM-1), diacetoxyscirpenol (DAS), T-2 triol, hydrolyzed fumonisin $B_1$ (h-$FB_1$), mycophenolic acid (MPA), and STG.

### 2.2. Sampling

Between November 2019 and April 2022, 87 TMR samples were taken from intensive dairy farms in Vila do Conde, North Portugal. The sampling distribution was as follows: in 2019, 33 samples were collected during the Autumn season. In 2020, 3 samples were collected in Autumn, and 16 in Winter. In 2021, 9 samples were collected in Winter, and 15 in Spring. In 2022, 2 samples were collected in Autumn, 5 in Winter, and 4 in Spring. Each sample, weighing 2 kg, was manually gathered during discharge from at least 10 points of fence line feeders. After that, the samples were packed in plastic bags and kept at 4 °C until they were delivered to the laboratory. Twenty-four hours after feeding with the studied TMR, milk samples were taken from the milk cooling tank and stored at −20 °C until analysis.

### 2.3. LC-MS detection

A 1290 infinity ultra high-performance liquid chromatography (UHPLC) system interfaced to a 6460 triple quadrupole mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used to measure the amount of mycotoxins in TMR samples. The detection conditions had been previously optimized for the analysis of feed and milk (González-Jartín, Alfonso et al., 2021; González-Jartín, Rodríguez-Cañás et al., 2021). Briefly, the separation process employed a Waters ACQUITY HSS T3 column with dimensions of 100 mm × 2.1 mm (inside diameter) and a particle size of 1.8 μm (Waters, Milford, MA), maintaining a temperature of 40 °C. The mobile phases consisted of water containing 0.1% formic acid and 5 mM ammonium formate (mobile phase A), and methanol (mobile phase B). The elution gradient was as follows: an initial hold time of 0.5 min at 0% B was followed by an increase to 14 % B within 0.5 min, and maintained for 1.5 min. Subsequently, the eluent B percentage was elevated to 60% within 1 min and held for 0.5 min. The gradient then progressed to 100% B within 4.5 min, and this composition was kept for 2 min. Finally, a shift to 0% B occurred within 0.5 min, maintaining this percentage for 2.5 min. The flow rate was set at 0.3 mL/min, and a 5 μL injection volume was employed.

The Agilent 6460 triple quadrupole mass spectrometer was configured with an electrospray ionization source (ESI) employing Agilent Jet Stream Technology. The ion source parameters were set as follows: capillary voltage at 4000 V, positive nozzle voltage at 1500 V, negative nozzle voltage at 0 V, nebulizer pressure at 45 psi, sheath gas at 12 L/min and 400 °C, and nebulizer gas at 8 L/min and 350 °C. For each mycotoxin, the fragmentor voltage (FV), cell accelerator voltage (CAV), collision energy (CE), and mass transitions were individually optimized using MassHunter Optimizer software (Table S1).

### 2.4. Mycotoxin extraction in TMR

The samples underwent a comprehensive homogenization process, following which a 2.5 g portion was accurately measured and placed into a 50 mL Falcon tube and submitted to an extraction procedure previously optimized for silage (González-Jartín et al., 2022). In this sense, samples were subjected to extraction with 10 mL of acetic acid (1%) through vigorous shaking for 5 min using a vortex mixer (Nahita Blue, Auxilab, Spain) at 2500 rpm. Next, 20 mL of acetonitrile (ACN) were introduced, and the stirring process was repeated. Following this, 8 g of $MgSO_4$ and 2 g of NaCl were incorporated, and the mixture was stirred for 1 min. Then, samples were centrifuged at 3134×*g* for 10 min,



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and the upper portion of the extract was transferred to a new tube. Finally, 100 μL of the extract were evaporated to dryness and subsequently reconstituted with 400 μL of the sample solvent, consisting of ACN/water/acetic acid [49:50:1 (v/v/v)]. Before UHPLC-MS/MS analysis, aliquots were filtered by using 0.22 μm centrifugal filters.

### 2.5. In-house validation of the analytical procedure

The validation of the method encompassed key parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and precision. To initiate the validation process, calibration curves were constructed at nine different points using both solvent and TMR extracts. The concentration ranges were from 1.5 to 192 μg/kg for aflatoxins and 50–12800 μg/kg for most of other toxins. These curves were used to calculate the matrix effect and linearity, which were measured by the correlation coefficient (R). Signal suppression/enhancement (SSE) was calculated to express the matrix effect in accordance with the SANTE/11312/2021 guidelines (SANTE, 2021). This was done by comparing the slopes of curves created in both solvent and blank extracts of TMR. The SSE was determined using the following formula: $SSE(\%) = 100 \times \frac{\text{slope of spiked extracts curve}}{\text{slope of standards curve in solvent}}$. The coefficient of variance (CV) was calculated by measuring three consecutive batches of calibration curves constructed in TMR extracts.

In compliance with EU-RL guidelines (Wenzl et al., 2016), blank extracts were analyzed and the following formulas were used to determine the LOD and LOQ: LOQ = 3.3 × LOD, and LOD = 3.9 × Sb/m. In this case, m denotes the slope of the calibration curve made in the sample solvent, and Sb stands for the standard deviation of the noises seen in 10 blank samples.

Accuracy and intra-day precision were determined based on the Eurachem and SANTE guidelines and previous validation procedures (Eurachem, 2014; SANTE, 2021; Sulyok et al., 2006). However, the number of required samples by the guidelines was reduced, and the recovery was evaluated from three replicate blank samples (n = 3) spiked at a single contamination level: 4.8 μg/kg for aflatoxins, 640 μg/kg for CTN, and DOM-1, and at 320 μg/kg for other compounds. The calculation of the amount of each analyte in the extracts relied on solvent-based calibration curves. This approach facilitated the assessment of apparent recoveries ($R_A$) and relative standard deviation (RSD). $R_A$ was determined by comparing the measured concentration in the spiked samples with the expected concentration according to the following equation $R_A(\%) = 100 \times \frac{\text{area spiked sample}}{\text{area standard}}$. The RSD provided insight into the precision of the method by indicating the variability within the replicate samples. Furthermore, the recovery of the extraction ($R_E$) was assessed by applying the SSE to the $R_A$ as follows: $R_E(\%) = 100 \times \frac{R_A}{SSE}$, offering an overall measure of the efficiency of the extraction process.

## 3. Results

### 3.1. Method validation for TMR analysis

The study of linearity involved the creation of calibration curves at nine concentration levels. R values were higher than 0.995 within the investigated concentration range. Subsequently, LODs and LOQs were calculated, as indicated in Table 1. LOQs were found to range from 0.9 μg/kg for $AFB_1$ to 287.6 μg/kg for DOM-1, and the repeatability within-batch, expressed as the CV%, showed a variation lower than 5% for most toxins.

The matrix effect was evaluated through the SSE (Table 1), which varied from 37% for HT-2 to 147% for NEO. In general, the matrix caused signal variations lower than 25%, with most of the toxins showing values in the range of 78–125%. Type A trichothecenes, namely DON and DOM-1, showed factors of 53.1% and 65.1%, respectively; whereas fumonisins showed no matrix effect. Similarly, variations lower than 13% were observed for ENNs and BEA. Next, the recovery ($R_E$) was studied, and the obtained data are shown in Table 1. Recoveries from TMR ranged from 61% for RC, STG and NEO to 95% for GLIO; however, the $R_E$ for CTN was 40%. The precision was high for most mycotoxins, with RSD values lower than 5%. Exceptions were $AFG_2$, $FB_1$, HT-2 toxin, and h-$FB_1$, for which RSD values were higher but always below 17%.

**Table 1**
Performance characteristics of the analysis method.

| Toxin | LOD (μg/kg) | LOQ (μg/kg) | Coefficient of variance (CV, %) | Matrix effect (SSE, %) | Apparent recovery ($R_A$, %) | Precision (RSD, %) | Accuracy ($R_E$, %) |
|---|---|---|---|---|---|---|---|
| $AFB_1$ | 0.27 | 0.9 | 4.7 | 82.8 | 62.6 | 5.9 | 75.6 |
| $AFB_2$ | 0.30 | 1.0 | 3.4 | 79.1 | 59.2 | 3.7 | 74.8 |
| $AFG_1$ | 0.36 | 1.2 | 3.3 | 72.8 | 52.3 | 6.9 | 71.8 |
| $AFG_2$ | 0.39 | 1.3 | 2.3 | 71.4 | 52.1 | 11.9 | 72.9 |
| DON | 21.0 | 69.9 | 5.3 | 53.1 | 36.7 | 0.5 | 69.2 |
| $FB_1$ | 20.4 | 67.9 | 3.5 | 101.8 | 83.8 | 11.3 | 82.4 |
| $FB_2$ | 25.4 | 84.6 | 4.9 | 103.1 | 67.3 | 4.3 | 65.2 |
| CTN | 78.3 | 260.6 | 0.5 | 124.3 | 49.8 | 4.7 | 40.0 |
| OTA | 10.3 | 34.2 | 4.8 | 95.6 | 63.8 | 3.2 | 66.7 |
| T-2 toxin | 9.6 | 31.9 | 4.7 | 95.2 | 62.4 | 5.6 | 65.5 |
| HT-2 toxin | 9.9 | 33.0 | 2.2 | 37.3 | 29.9 | 10.0 | 80.4 |
| ZEN | 7.1 | 23.5 | 1.6 | 98.4 | 63.9 | 2.8 | 64.9 |
| AOH | 29.2 | 97.3 | 2.3 | 74.9 | 47.3 | 5.4 | 63.1 |
| AME | 6.5 | 21.6 | 2.1 | 85.0 | 55.2 | 2.9 | 64.9 |
| BEA | 2.2 | 7.2 | 4.4 | 92.6 | 60.7 | 2.4 | 65.4 |
| ENNA | 0.5 | 1.6 | 5.7 | 87.3 | 67.9 | 4.4 | 77.9 |
| $ENNA_1$ | 0.6 | 2.0 | 3.9 | 88.1 | 55.0 | 2.0 | 62.5 |
| ENNB | 1.5 | 5.0 | 3.5 | 97.1 | 59.9 | 5.2 | 61.7 |
| $ENNB_1$ | 1.1 | 3.7 | 5.0 | 91.7 | 58.6 | 2.7 | 63.9 |
| FA | 39.2 | 130.5 | 10.9 | 59.4 | 39.8 | 2.3 | 67.0 |
| DOM-1 | 86.4 | 287.6 | 6.4 | 65.1 | 51.5 | 5.5 | 79.1 |
| DAS | 12.6 | 42.1 | 2.3 | 116.7 | 84.5 | 8.0 | 72.4 |
| T2 triol | 22.2 | 73.9 | 6.4 | 46.2 | 33.5 | 7.2 | 72.4 |
| NEO | 21.1 | 70.3 | 4.1 | 147.5 | 90.6 | 6.1 | 61.5 |
| h-$FB_1$ | 24.3 | 80.8 | 12.7 | 117.4 | 90.2 | 17.4 | 76.8 |
| MPA | 17.9 | 59.6 | 2.2 | 108.8 | 82.0 | 3.2 | 75.4 |
| RC | 0.5 | 1.6 | 5.6 | 40.1 | 24.6 | 3.6 | 61.4 |
| STG | 0.6 | 2.0 | 3.0 | 79.2 | 48.7 | 1.9 | 61.4 |
| GLIO | 14.8 | 49.4 | 2.0 | 55.6 | 52.8 | 5.9 | 94.9 |



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### 3.2. TMR analysis

Complete analysis results are shown in Table S2. A total of 16 mycotoxins were detected in the samples collected over the 4 years of the study. As shown in Table 2, the occurrence varied significantly. $AFB_1$ was identified in 2% of samples, followed by OTA, detected in 9%. DON and ZEN were found in approximately one-third of the samples, while fumonisins were more frequently detected, occurring in 74% of cases. Regarding emerging compounds produced by *Fusarium* species, such as BEA and enniatins, they were present in up to 88% of the samples. Compounds produce by *Aspergillus* or *Penicillium*, such as MPA, STG, and RC, were detected in 9%, 18%, and 31% of samples, respectively. The only *Alternaria* toxin detected was AME, present in 14% of the samples.

As shown in Fig. 1 and Table 3, 2019 exhibited the lowest prevalence for the most common toxins over the years, with exceptions for ZEN observed in 2020 and DON in 2021. Compared with previous years, 2022 showed a high prevalence of DON (81%), RC (81%) and ZEN (100%), with double the average of 2019–2021. However, the most frequently detected mycotoxins were $FB_1$, enniatins, and BEA, as they were present in more than 80% of the samples since 2020. As shown in Fig. 2 and Table 3, over the four years, the frequency of ZEN in winter (26%) was lower than in autumn (37%) and spring (63%). Fumonisins, enniatins, and BEA exhibited a similar pattern, with low positivity in autumn (below 50%, except for BEA at 72%), increasing to over 90% in winter, and slightly decreasing in spring. On the other hand, DON and RC displayed unique patterns: nearly 50% of autumn samples were positive for DON, decreasing to 40% in winter and 25% in spring, while RC showed the opposite pattern.

Toxin concentrations, expressed as μg/kg, are shown in Tables 2 and 3. $AFB_1$ was found with a maximum concentration of 2.9 μg/kg. The median concentrations of DON and $FB_1$ were similar, around 220 μg/kg, though $FB_1$ showed higher variability with a maximum concentration of 3541 μg/kg. ZEN and OTA were present at lower amounts, with median concentrations of 68 μg/kg and 36 μg/kg, respectively. However, in some cases, the concentration of ZEN was high, reaching up to 817 μg/kg. Concerning the most prevalent emerging toxins, such as BEA and enniatins, their mean concentrations were lower, at 38 μg/kg and 96 μg/kg, respectively. Less frequent toxins such as RC and MPA appeared in some samples in very high amounts, particularly RC, with one sample containing 15,144 μg/kg.

### 3.3. Carry-over to milk

Regulated mycotoxins, such as DON, ZEN, OTA, and fumonisins were detected in several TMR samples, however, none were identified in the corresponding milk samples (Table S3). Moreover, no transfer was observed for AME, STG, or MPA. On the contrary, a carry-over was established for RC, BEA and enniatins. In the case of RC, it was identified individually in TMR, with an average concentration of 609 μg/kg, and in milk, average 5.83 μg/kg. However, on three farms, this toxin was detected simultaneously in both TMR and milk. The average consumption was 17.38 mg of RC, with an excretion of 0.49 mg, resulting in a transfer rate of 2.8%. Regarding BEA, the transfer rate was 1.06%, while for ENNA, $ENNA_1$, ENNB, and $ENNB_1$ were 4.85%, 6.66%, 10.10%, and 5.54%, respectively.

## 4. Discussion

### 4.1. Method validation for TMR analysis

A method of extraction that was originally created for the analysis of mycotoxins in maize and grass silage was now validated for TMR analysis (González-Jartín et al., 2022). The method demonstrated satisfactory linearity, $R > 0.995$, and high repeatability within-batch. The obtained LOQs allow the quantification of regulated mycotoxins below legal limits or recommendations (European Commission, 2006, 2011).

**Table 2**
Contamination levels of the studied samples.

| (μg/kg) | $AFB_1$ | DON | ZEN | $FB_1$ | $FB_2$ | OTA | ENNA | $ENNA_1$ | ENNB | $ENNB_1$ | BEA | FA | AME | RC | STG | MPA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Median | 2.8 | 220.1 | 68.2 | 218.2 | 129.0 | 36.0 | 4.6 | 5.5 | 15.7 | 7.0 | 51.3 | 131.1 | 19.8 | 107.6 | 13.3 | 98.9 |
| Mean | 2.8 | 293.2 | 108.1 | 328.9 | 141.2 | 49.6 | 6.1 | 6.7 | 37.9 | 15.9 | 96.4 | 140.3 | 19.2 | 1752.0 | 159.5 | 375.8 |
| 25% Percentile | 2.8 | 146.4 | 58.7 | 152.2 | 83.0 | 20.7 | 1.8 | 3.4 | 8.7 | 5.6 | 26.5 | 91.3 | 8.8 | 51.3 | 2.4 | 54.1 |
| 75% Percentile | 2.9 | 452.8 | 106.9 | 307.7 | 173.2 | 87.9 | 9.3 | 9.7 | 64.7 | 9.6 | 143.6 | 172.0 | 26.5 | 1010.0 | 23.9 | 735.5 |
| Maximum | 2.9 | 735.5 | 817.8 | 3541.0 | 416.3 | 99.3 | 22.2 | 32.7 | 186.2 | 135.2 | 1485.0 | 275.9 | 42.6 | 15144.0 | 1916.0 | 1579.0 |
| Detection rate (%) | 2.3% | 42.5% | 39.1% | 74.7% | 59.8% | 9.2% | 27.6% | 40.2% | 71.3% | 58.6% | 88.5% | 19.5% | 13.8% | 31.0% | 18.4% | 9.2% |

Concentration levels are expressed as μg/kg, and the detection rate is the percentage of samples above the LOQ shown in Table 1.



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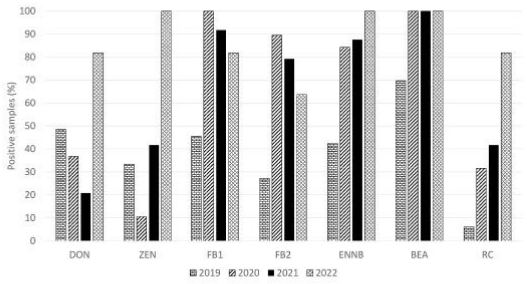



**Fig. 1.** Percentage of contamination across various sampling years.

Specifically, the only mycotoxin with established maximum limit is $AFB_1$, with a maximum tolerable level of 20 μg/kg in feed materials, which is approximately 20 times the LOQ of the method (European Commission, 2011). While other methods with lower LOQs have been previously developed (Table 4), they only allow the detection of a specific group of toxins, mainly aflatoxins (Álvarez-Días et al., 2022; Gallo et al., 2020; Xu et al., 2021), whereas the proposed method provides a comprehensive assessment of both regulated and emerging toxins.

The matrix effect arises from co-eluting compounds with the target analyte, potentially leading to the suppression or enhancement of the analytical signal. Several approaches can be employed to correct the matrix effect; in this case, the SSE factor was determined by comparing the slope of calibration curves constructed in the solvent with those in the matrix (Fabregat-Cabello et al., 2016). In general, the matrix caused signal variations lower than 25%; however, hydrophilic trichothecenes, specifically type A trichothecenes (DON and DOM-1), constitute the group of compounds most significantly affected. These toxins usually present a high SSE factor since polar compounds are generally more prone to high suppression (Lago et al., 2021). Consequently, the extraction involving the analysis of a diluted sample, while leading to relatively high detection limits, facilitated minimal matrix effect. This is particularly significant in the analysis of TMR, given the variations in composition observed from one farm to another, which can lead to important variation in the SSE caused by the matrix. Therefore, the employed approach guarantees accurate and robust results, addressing potential challenges linked to the matrix effect.

Lastly, an assessment of the accuracy was conducted. To do this, a solvent-based calibration curve was used to calculate the apparent recovery ($R_A$), which was then corrected with the SSE factor to obtain the $R_E$, which served as an accuracy measure. Simultaneously, the RSD of the recovery was used to evaluate precision. Apart from CTN, the recoveries ranged from 61% to 94%, with precision consistently below 17% (Table 1). Consequently, the method aligns with European regulations for most compounds since it meets performance criteria as outlined in the Commission Implementing Regulation (EC) 2023/2782 (European Commission, 2023). However, the method was only in-house validated, using a single concentration of toxin established at a level lower than the maximum amount allowed or recommended in feed. If the method was to be used for official analysis, it would be necessary to establish the performance characteristics with a higher number of samples and using different concentrations of toxin (European Commission, 2023). In this sense, the latest SANTE guidances considers the LOQ of the method as the lowest level that has been validated by applying the complete analytical method (SANTE, 2021).

### 4.2. TMR analysis

The analysis method was validated for 29 mycotoxins, and 16 of them were detected in the samples collected over the 4 years of the study (Table 3). However, the occurrence varied significantly, as it was also

**Table 3**
Contamination levels of the studied samples by year and season.

| | Year | N | $AFB_1$ | DON | ZEN | $FB_1$ | $FB_2$ | OTA | ENNA | $ENNA_1$ | ENNB | $ENNB_1$ | BEA | FA | AME | RC | STG | MPA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Autumn** | **2019** | **33** | 2.79 (1) | 239 (16) | 61 (11) | 229 (15) | 146 (9) | <LOD | <LOQ | <LOQ | 108.8 (14) | 133.3 (3) | 220 (23) | 139 (15) | <LOD | 57 (2) | 21 (1) | <LOD |
| | **2020** | **3** | <LOD | 610 (2) | 104 (1) | 194 (3) | 109 (2) | <LOD | 7.4 (1) | 11.5 (1) | 12.9 (2) | 10.8 (2) | 38 (3) | <LOD | <LOD | <LOD | <LOD | <LOD |
| | **2022** | **2** | <LOD | 547 (2) | 91 (2) | <LOD | <LOD | <LOD | <LOQ | 3.7 (2) | 35.7 (2) | 9.8 (2) | 35 (2) | <LOD | <LOD | 46 (1) | 79 (1) | <LOD |
| | **Total** | **38** | 2.79 (1) | 307 (20) | 69 (14) | 223 (18) | 139 (11) | <LOD | 7.4 (1) | 6.3 (3) | 90.0 (18) | 63.0 (7) | 188 (28) | 139 (15) | <LOD | 54 (3) | 50 (2) | <LOD |
| **Spring** | **2021** | **15** | <LOD | 353 (3) | 53 (8) | 655 (13) | 182 (10) | 58 (2) | 3.4 (5) | 4.3 (5) | 16.7 (12) | 5.2 (12) | 39 (15) | <LOD | 22 (4) | 4525 (10) | 479 (5) | 569 (5) |
| | **2022** | **4** | <LOD | 133 (2) | 198 (4) | 167 (4) | 52 (3) | 87 (2) | 0.5 (4) | 2.0 (4) | 31.3 (4) | 11.1 (4) | 7 (4) | <LOD | 7 (3) | 6 (4) | 9 (3) | 33 (1) |
| | **Total** | **19** | <LOD | 265 (5) | 101 (12) | 540 (17) | 152 (13) | 73 (4) | 2.1 (9) | 3.3 (9) | 20.4 (16) | 6.6 (16) | 46 (19) | <LOD | 15 (7) | 3234 (14) | 303 (8) | 480 (6) |
| **Winter** | **2020** | **16** | <LOD | 443 (5) | 112 (1) | 214 (16) | 109 (15) | 36 (2) | 13.1 (7) | 13.6 (9) | 19.6 (14) | 11.9 (14) | 41 (16) | 153 (2) | 33 (3) | 96 (6) | 1 (4) | <LOD |
| | **2021** | **9** | 2.86 (1) | 372 (2) | 85 (2) | 464 (9) | 223 (9) | <LOD | 6.9 (2) | 5.7 (9) | 11.4 (9) | 8.4 (9) | 65 (9) | <LOD | <LOD | <LOD | <LOD | <LOD |
| | **2022** | **5** | <LOD | 86 (5) | 244 (5) | 116 (5) | 48 (4) | 17 (2) | 3.1 (5) | 2.7 (5) | 5.0 (5) | 4.1 (5) | 12 (5) | <LOD | 13 (2) | 326 (4) | 12 (2) | 64 (2) |
| | **Total** | **30** | 2.86 (1) | 283 (12) | 187 (8) | 273 (30) | 137 (28) | 27 (4) | 8.6 (14) | 8.1 (23) | 14.3 (28) | 9.4 (28) | 43 (30) | 153 (2) | 25 (5) | 188 (10) | 4 (6) | 64 (2) |

The average concentration is expressed as μg/kg, and the number of positive samples (above LOQ) is shown in brackets. Total number of samples (N).



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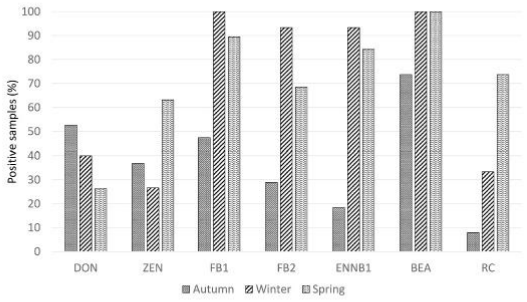



**Fig. 2.** Percentage of contamination based on the sampling season.

observed in previous studies (Table 4). Similar results regarding the occurrence of aflatoxins (2%) and ZEN (40%) were found in maize silage used for TMR preparation from the same geographical area. However, the occurrence of DON was higher, reaching 80%, while that of fumonisins was lower, standing at 40% (González-Jartín et al., 2022). Other study conducted in the South of Portugal detected $AFB_1$ in 10% of raw materials used in TMR production, while the presence of ZEN and DON was significantly higher, albeit with a limited number of samples evaluated (Viegas et al., 2020). A prior investigation assessing mycotoxins in Spanish TMR reported a prevalence of 15% for $AFB_1$, 17% for DON, 16% for ZEN and 34% for fumonisins. The elevated occurrence of aflatoxins may be explained by the high sensitivity exhibited by the UHPLC-FLD method and the fact that the sampling was done in farms that had presented problems related to aflatoxins in previous years (Rodríguez--Blanco, Marín, et al., 2020; Rodríguez-Blanco, Ramos, et al., 2020). Therefore, the results obtained are in the range of other studies carried out in the same geographical area while it is lower than in other countries such as Mexico or China (Álvarez-Días et al., 2022; Xu et al., 2021).

Regarding emerging compounds, the prevalence of toxins produced by *Fusarium* species, such as BEA and enniatins, is remarkable, being present in up to 88% of the samples. On the contrary, compounds produce by *Aspergillus* or *Penicillium* were less frequently found, with MPA detected in 9%, STG in 18%, and RC in 31% of samples. The only *Alternaria* toxin detected was AME, present in 14% of the samples. No prior information regarding emerging toxins in TMR from areas close to the current study location has been identified. A previous study, carried out in Mexico, detected enniatins in a similar proportion as that observed in Portugal, while AME and MPA were more frequently detected in up to 42% of TMR samples (Penagos-Tabares , Sulyok et al., 2023). Similarly, a study conducted in Pakistan detected, among others, BEA in all samples, STG in 37% and AME in 90% (Penagos-Tabares , Mahmood et al., 2023).

The seasonal disparities in sample collection may have contributed to the observed variations in toxin prevalence (Table 3). Specifically, the low prevalence of fumonisins, ENNB, and BEA in 2019 could be attributed to the fact that all 33 samples collected during that year were obtained in the autumn. Regarding DON, a notable reduction was observed in 2021, where 15 of the 24 samples were obtained during the spring. Additionally, the lowest occurrence of ZEN was noted in 2020, with 16 out of the 19 samples collected during winter. However, samples were taken most consistently during this season with other 9 samples in 2021 and 5 in 2020. Across the 4 years, the frequency of ZEN in winter was lower than in autumn and spring. On the other hand, during spring, a notable increase in samples contaminated with ZEN and RC is observed. Although the variation in toxin occurrence and concentration depending on the time of year has been previously demonstrated (González-Jartín et al., 2022), in the case of the TMR it is very difficult to predict since the origin of the raw materials used for their production can also vary.

The only toxin regulated for animal feeding is $AFB_1$. It was found in very few samples, with a maximum concentration of 2.9 μg/kg (Table 2), below the 5 μg/kg permitted in feed for dairy cattle (European Commission, 2011). Except for one sample that had 817 μg/kg of ZEN, exceeding the 500 μg/kg set as maximum recommended amount for dairy cattle, the observed levels for regulated mycotoxins comply with

**Table 4**
Summary of reports of mycotoxins in TMR and related products.

| Data | | Current study | González-Jartín et al. (2022) | Viegas et al. (2020) | Rodríguez-Blanco, Marín, et al. (2020), Rodríguez-Blanco, Ramos, et al. (2020) | Penagos-Tabares, Mahmood et al. (2023) | Penagos-Tabares, Sulyo et al. (2023) | Á lvarez-Días et al. (2022) | Xu et al. (2021) |
|---|---|---|---|---|---|---|---|---|---|
| **Extraction and analysis, number of toxins included in the method** | | QuEChERS UHPLC-MS/MS, 29 mycotoxins | QuEChERS UHPLC-MS/MS, 39 mycotoxins | Solid-liquid using a SPE cartridge UHPLC-MS/MS, 16 mycotoxins | Solid-liquid, UHPLC-FLD and UHPLC-MS/MS, 9 + 5 mycotoxins | Solid-liquid, UHPLC-MS/MS, >500 mycotoxins | Solid-liquid, UHPLC-MS/MS, >800 mycotoxins and other metabolites | Solid-liquid LC-FLD, 4 toxins | Solid-liquid LC-FLD, 1 toxin |
| **Matrix and number of samples** | | TMR, 87 | Maize silage, 45 | TMR raw materials, 9 | TMR, 193 | TMR, 30 | TMR, 30 | TMR, 99 | TMR, 22 |
| **Geographical area, and season** | | North of Portugal, all | North of Portugal, all | South of Portugal, unknown | North of Spain, all | Pakistan, unknown | Mexico, summer | Mexico, unknown | China Autumn -Winter |
| **Occurrence % and LOD of the detection method** | **$AFB_1$** | 2.3% (0.27 μg/kg) | 2% (0.71 μg/kg) | 10% (0.06 μg/kg) | 15% (0.1 μg/kg) | 40% (Unknown) | 0% (Unknown) | 100% (2.5 μg/kg) | 100% (0.03 μg/kg) |
| | **DON** | 42.5% (21 μg/kg) | 82% (28.94 μg/kg) | 80% (1 μg/kg) | 17% (0.75 μg/kg) | 0% (Unknown) | 53% (3.6 μg/kg) | – | – |
| | **ZEN** | 39.1% (7.1 μg/kg) | 39% (8.34 μg/kg) | 100% (0.07 μg/kg) | 16% (0.25 μg/kg) | 43% (Unknown) | 68% (2.8 μg/kg) | – | – |
| | **$FB_1$** | 74.7% (21 μg/kg) | 41% (18.7 μg/kg) | 30% (0.5 μg/kg) | 34% (30 μg/kg) | 93% (Unknown) | 47% (16 μg/kg) | – | – |
| | **OTA** | 9.2% (10.3 μg/kg) | 0% (11.8 μg/kg) | 50% (0.13 μg/kg) | – | 7% (Unknown) | 0% (Unknown) | – | – |
| | **ENNB** | 71.3% (1.5 μg/kg) | 50% (0.93 μg/kg) | – | – | 20% (Unknown) | 68% (0.9 μg/kg) | – | – |
| | **BEA** | 88.5% (2.2 μg/kg) | 94% (0.43 μg/kg) | – | – | 100% (Unknown) | 100% (0.3 μg/kg) | – | – |
| | **RC** | 31% (0.5 μg/kg) | 18% (7.15 μg/kg) | – | – | – | – | – | – |



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the recommendations (European Commission, 2006).

### 4.3. Carry-over to milk

To assess whether the presence of toxins in the TMR poses a direct risk for their occurrence in milk, several farms were chosen. Milk samples were collected from the milk tank, which contained the combined production of several cows, 24 h after the animals were fed with the TMR. These samples were then analyzed using a method that had been previously validated for the detection of 40 mycotoxins in this specific matrix (González-Jartín, Rodríguez-Cañás et al., 2021). No transfer was observed for toxins commonly found in TMR such as DON, ZEN and $FB_1$. It was previously reported that rumen microorganisms are capable of degrading these contaminants in a high proportion, while reports on $FB_1$ degradation are inconsistent although it seems to be barely metabolized (Loh et al., 2020). In orally exposed ruminants, fumonisins exhibit very limited bioavailability, undergoing extensive biotransformation and rapid excretion primarily through the fecal route, which may explain their absence in milk (EFSA, 2018). Moreover, no transference was neither observed for MPA, STG, or AME. MPA is an immunosuppressive agent whose pharmacokinetics has been extensively studied in humans. This small molecule exhibits high protein binding, which limits the amount of free compound available for transfer into breast milk. It can only be detected in breast milk after the administration of high doses, with minimal excretion through this route (<0.02%) (Krutsch et al., 2023). Regarding STG, the limited available information suggests that its absorption is minimal, with excretion occurring via bile and urine (EFSA, 2013). Similarly, a low absorption has been observed for AME (Islam et al., 2023).

On the contrary, a carry-over was established for RC, BEA and enniatins. The calculation method, described by Guo et al. (Guo et al., 2021) was employed, using an average intake of 43 kg of TMR. This TMR includes 30–35 kg of silage, 1.5 kg of straw, and 8–10 kg of mixture. Additionally, a daily milk production rate of 40 L per cow was considered in the analysis. In the case of RC, a transfer rate of 2.8% was obtained while for BEA, ENNA, $ENNA_1$, ENNB, and $ENNB_1$ were 1.06%, 4.85%, 6.66%, 10.10%, and 5.54%, respectively. At the moment, most research efforts are focused on studying the carry-over of aflatoxins, with reported values ranging from 1% to 6.2%. In addition, it has also been reported the transference of T-2 toxin and $FB_1$ with rates lower than 2 and 0.05%, respectively (Fink-Gremmels, 2008; Guo et al., 2021).

RC and enniatins are only partially degraded by rumen microbiota, making systemic absorption feasible (Debevere et al., 2020). Although enniatins and BEA have been previously found in human and animal milk, the transfer ratio has not been studied (González-Jartín, Rodríguez-Cañás et al., 2021; Pietruszka et al., 2023; Rubert et al., 2014). These compounds are *in vitro* substrates of the ABCG2 transporter, which facilitates the excretion of both endogenous and exogenous substances from the bloodstream into milk (García-Lino et al., 2019). Consequently, substrates of this transporter can appear in high concentrations in milk (Fink-Gremmels, 2008). The specific carry-over for BEA and enniatins, which are within the range of the observed one for aflatoxins, was determined for the first time in this study.

## 5. Conclusion

The proposed analytical method, based on a QuEChERS extraction followed by UHPLC-MS/MS detection, enables the quantification of both regulated and emerging toxins, aligning with current legislation. The study revealed a high prevalence of fumonisins and BEA, while toxins such as DON, ZEN, and RC were present in approximately 35% of the samples. On the other hand, OTA and MPA exhibited a lower occurrence, each found in less than 10% of the samples. Notably, aflatoxins were detected in only 2% of the samples, highlighting a relatively low incidence. Furthermore, the study observed variable transfer rates of enniatins, BEA, and RC from TMR to milk.

## CRediT authorship contribution statement

**Jesús M. González-Jartín:** Writing – original draft, Validation, Investigation. **Inés Rodríguez-Cañás:** Validation, Investigation. **Rebeca Alvariño:** Validation, Investigation. **Amparo Alfonso:** Writing – review & editing, Project administration, Methodology. **María J. Sainz:** Methodology. **Mercedes R. Vieytes:** Methodology. **Ana Gomes:** Resources. **Isabel Ramos:** Resources. **Luis M. Botana:** Supervision, Funding acquisition.

## Declaration of competing interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2024.110682.

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**2. First description of adenosine production by *Gnomoniopsis smithogilvyi*, causal agent of chesnut brown rot**

**Resumen:**
*Gnomoniopsis smithogilvyi (Gnomoniaceae, Diaporthales*) es el principal agente que causa la pudrición parda de la castaña en todo el mundo. La pudrición de los frutos provoca alteraciones en las cualidades organolépticas y una disminución en la producción de frutos, lo que resulta en pérdidas económicas significativas. En 2021, hubo un importante brote de pudrición de castañas en el sur de Galicia (noroeste de España). Se estudió el perfil de metabolitos secundarios de *G. smithogilvyi*, especialmente para determinar su capacidad de producir micotoxinas, como ocurre con otros hongos causantes de pudriciones, debido a las posibles consecuencias sobre la seguridad del consumo de castañas. Los metabolitos secundarios producidos por aislados de *G. smithogilvyi* que crecieron en medio de agar dextrosa de patata (PDA) fueron identificados utilizando cromatografía líquida acoplada a espectrometría de masas de alta resolución. Se identificaron tres metabolitos con propiedades farmacológicas y fito-toxicológicas interesantes basadas en su masa exacta y patrones de fragmentación, a saber, adenosina, oxasetina y fitosfingosina. Se evaluó la capacidad de *G. smithogilvyi* para producir adenosina en cultivos de PDA, encontrándose concentraciones que oscilan entre 176 y 834 µg/kg. De manera similar, se descartó la producción de micotoxinas, lo que indica que el consumo de castañas con lesiones necróticas no representa un riesgo para la salud del consumidor en términos de micotoxinas.

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**First description of adenosine production by *Gnomoniopsis smithogilvyi*, causal agent of chestnut brown rot**

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RESEARCH

# First description of adenosine production by *Gnomoniopsis smithogilvyi*, causal agent of chestnut brown rot

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**Abstract**
*Gnomoniopsis smithogilvyi* (*Gnomoniaceae*, *Diaporthales*) is the main causal agent of chestnut brown rot on sweet chestnut worldwide. The rotting of nuts leads to alterations in the organoleptic qualities and decreased fruit production, resulting in significant economic losses. In 2021, there was an important outbreak of chestnut rot in southern Galicia (Spanish northwest). The profile of secondary metabolites from *G. smithogilvyi* was studied, especially to determine its capability for producing mycotoxins, as happens with other rotting fungi, due to the possible consequences on the safety of chestnut consumption. Secondary metabolites produced by isolates of *G. smithogilvyi* growing in potato dextrose agar (PDA) medium were identified using liquid chromatography coupled with high-resolution mass spectrometry. Three metabolites with interesting pharmacological and phyto-toxicological properties were identified based on their exact mass and fragmentation patterns, namely adenosine, oxasetin, and phytosphingosine. The capacity of *G. smithogilvyi* to produce adenosine in PDA cultures was assessed, finding concentrations ranging from 176 to 834 µg/kg. Similarly, the production of mycotoxins was ruled out, indicating that the consumption of chestnuts with necrotic lesions does not pose a health risk to the consumer in terms of mycotoxins.

**Keywords** *Gnomoniopsis castaneae* · Mass spectrometry · Oxasetin · Phytosphingosine · UHPLC-MS-IT-TOF

## Introduction

Natural products are small molecules, primary and secondary metabolites, produced by living organisms. Plants, animals, or microorganisms serve as sources of bioactive metabolites with interesting pharmacological properties. These compounds are regularly used in the pharmaceutical and biotechnology industries, as many drugs are either based on naturally occurring molecules or are derivatives thereof (Mathur and Hoskins 2017).

Fungi constitute a large group of eukaryotic organisms with a global distribution, found in nearly all ecosystems. Metabolites derived from fungi represent a significant source of pharmaceutical compounds, such as penicillin, vancomycin, or lovastatin, some of the industrially important secondary metabolites (Devi et al. 2020). The ecological functions of many of these metabolites remain unknown; however, some can play an essential role against insect predators or competitors. Although more than 1500 compounds have been isolated from fungi, many species have not yet been

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studied, and, every year, new species of fungi are discovered (Keller 2019).

In 2012, an undescribed ascomycete was first reported independently by Shuttleworth et al. in Australia and Visentin et al. in Italy, albeit under different names, namely *Gnomoniopsis smithogilvyi* L.A. Shuttleworth, E.C.Y. Liew & D.I. Guest and *Gnomoniopsis castaneae* Tamietti, respectively (Shuttlewoth et al. 2012; Visentin et al. 2012). A comparative morphological and five-marker phylogenetic analysis revealed that both names represent the same species, *G. smithogilvyi* (*Gnomoniaceae*, *Diaporthales*) being the current name of the fungus, and *G. castaneae* its synonym (Shuttleworth and Guest 2017; Shuttleworth et al. 2016). This fungus was isolated as the causal agent of an unknown nut rot that began to affect up to 80% of sweet chestnut (*Castanea sativa* Mill.) production, depending on location and year, in Europe and Australia (Morales-Rodriguez et al. 2022). In 2018, *Gnomoniopsis smithogilvyi* was reported in the Portuguese region of Trás-os-Montes (Coelho and Gouveia 2021), which shares a border with Galicia in the Spanish northwest. However, it was not until September 2021 that the fungus was identified in sweet chestnut orchards in southern Galicia, causing an outbreak of chestnut brown rot (Aguín et al. 2023). The climate in this region is characterized by cold winters, high temperatures and extreme drought in the summer (MeteoGalicia 2010), conditions well tolerated by chestnuts (Freitas et al. 2021). Nevertheless, in 2021, the months of May, June and July (chestnut flowering period) experienced colder temperatures and higher rainfall compared to the 1981–2010 averages (MeteoGalicia 2022). Shuttleworth and Guest (2017) have linked climatological conditions, such as temperature and rainfall, to the infection of chestnut flowers by ascospores of *G. smithogilvyi*. In particular, rainfall during the flowering period could be responsible for an increase in the incidence of chestnut rot (Lione et al. 2015; Williams et al. 2007).

It is remarkable that *G. smithogilvyi* can also behave as an endophyte, living asymptomatically within healthy plant tissue. It may remain latent in healthy chestnuts and only cause rotting during post-harvest if optimal temperature and water conditions are present (Vettraino et al. 2021).

Several filamentous fungi that cause spoilage and/or live endophytically in food commodities, either pre-harvest or post-harvest, pose a global concern due to their ability to produce mycotoxins. These fungal secondary metabolites can be toxic to humans, other mammals, poultry, and fish mainly upon ingestion (Steyn 1995). The study of the secondary metabolites profile, particularly mycotoxins, in fungi affecting edible fruit is essential to guarantee food safety, especially when fungi live endophytically or cause small lesions that may go unnoticed, since humans or animals can consume these products. The main mycotoxigenic fungal species belong to the genera *Aspergillus*, *Penicillium* and *Fusarium*. However, it is worth noting that species within these genera are also known to produce metabolites with antifungal, anticancer, or antibacterial properties, which are of interest in therapeutics (Devi et al. 2020).

The wide array of secondary metabolites produced by fungi coupled with the high complexity of the crude extracts makes the discovery of natural products challenging. In this context, mass spectrometry (MS) technologies have proven to be a useful tool for characterizing the diversity of natural products (Louie et al. 2020). There are several types of mass analyzers, each with unique features and benefits. The ion trap (IT) serves as both a mass spectrometer, offering a wide mass range and variable mass resolution, and an ion storage device that can confine gaseous ions for a period of time, allowing subsequent fragmentation. In contrast, a time-of-flight (TOF) analyzer provides accurate mass measurement, making it useful for determining empirical formulae. However, it does not provide any information on the sequence of fragmentations (Li et al. 2007). Hybrid systems, such as high-resolution MS–IT–TOF, leverage the distinct properties of each analyzer to provide more complete information on the metabolites present in an extract (González-Jartín et al. 2017). In this context, the aim of this work was to decipher the unknown potential of *G. smithogilvyi* to produce secondary metabolites using the ultra-high-performance liquid chromatography coupled to mass spectrometry—ion trap—time of flight (UHPLC-MS-IT-TOF), a technique that allows the detection of both known and unknown metabolites, based on the determination of their exact mass.

## Material and methods

### Isolation and identification of *G. smithogilvyi* from sweet chestnut trees

In September 2021, 100 chestnuts with visible symptoms of chestnut brown rot were collected from eight orchards severely affected by the disease in southern Galicia (10–15 chestnuts per orchard). The collected samples were individually placed in paper bags and transported in portable coolers to the laboratory. In all chestnuts, each burr contained 2–3 nuts. Affected nuts, when dissected, showed brown lesions extending over most of the kernel; some nuts were mummified (Fig. S1). Small pieces of infected burrs, kernels and mummified nuts were surface disinfected using a 2% sodium hypochlorite solution for 5 min, followed by rinsing in distilled water and blotting on dry sterile filter paper. Using a sterile scalpel, pieces were plated onto potato dextrose agar (PDA) media (Sharlau, Barcelona, Spain) and incubated at 25 °C in the dark for seven days. Subsequently, mycelium growing from pieces was transferred to new PDA plates to obtain pure cultures.



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The identification of isolates involved an initial reliance on morphological characteristics, followed by subsequent verification through molecular methods and phylogenetic analysis (Aguín et al. 2023).

Genomic DNA was extracted directly from visible mycelium in samples of burrs and nuts, as well as from mycelium of pure cultures grown in PDA. For molecular identification, the rDNA internal transcribed spacer (ITS) and the β-tubulin (*TUB2*) and the translation elongation factor-1α (*TEF-1* α) genes were amplified and sequenced. The primers used were ITS1F (Gardes and Bruns 1993), ITS4 (White et al. 1990), T1/Bt2b (O'Donnell et al. 1998), and EF1-728F/EF1-986R (Carbone and Kohn 1999), respectively. Subsequently, multiple sequence alignment-based phylogenetic analysis was carried out using ITS, *TUB2* and *TEF-1*α sequences.

All isolates were identified as *G. smithogilvyi*, five being selected for studying metabolite production.

### Characterization of the *G. smithogilvyi* metabolites profile

In order to establish the profile of metabolites produced by *G. smithogilvyi,* the selected isolates were subcultured in PDA at 25 °C in the dark. Control plates (PDA without fungi) were also prepared. After one week of incubation, three agar plugs (6-mm diameter) were cut from each resultant monosporic culture, and from the controls. Next, 0.5 mL of an acetonitrile/water/acetic acid mixture [49:50:1 (v/v/v)] were added to the agar plugs and the mixture was stirred in a vortex mixer for 3 min. The resulting extract was then filtered using 0.22 µm centrifugal filters (Millipore Ultrafree-MC, Billerica, MA) and frozen at − 20 °C until analysis.

The analysis of these extracts was performed using a UHPLC-MS-IT-TOF instrument from Shimadzu (Kyoto, Japan). The UHPLC system consisted of two pumps (LC-30AD), a degasser (DGU-20A), an autoinjector (SIL-10AC) with a refrigerated rack, and a column oven (CTO-10AS). Compounds were separated in a 100 mm × 2.1 mm (inside diameter), 1.8 µm, Waters ACQUITY HSS T3 column (Waters, Milford, MA) at 40 °C. Mobile phases were (A) water containing 0.1% formic acid and 5 mM ammonium formate and (B) methanol. The flow rate of the mobile phase was maintained at 0.3 mL/min, and the elution gradient (14 min) was as follows: Initially, eluent B was held at 0% for 0.5 min, and then it was increased to 50% B within 0.5 min. After 2.5 min at 50% B, the gradient was further increased to 100% B within 3 min and maintained for 3.5 min. Finally, the gradient was changed to 0% B over 0.5 min, and the column was re-equilibrated for 3.5 min. The injection volume was set at 5 µL.

The mass spectrometer, an IT-TOF instrument from Shimadzu (Kyoto, Japan), was equipped with an electrospray ionization (ESI) interface. Operating conditions were as follows: detector voltage, 1.65 kV; curved desolvation line and heat block temperature, 200 °C; nebulizing gas flow, 1.5 L/min; drying gas pressure, 105 kPa. The pressure in the ion trap was $1.8 \times 10^{-2}$ Pa while the pressure in TOF region was $1.4 \times 10^{-4}$ Pa. The MS method was operated in positive and negative full scan MS mode with a mass range 150–1000 Da. The ion accumulation time was set to 20 ms, with an event time of 300 ms with three repetitions. For collision-induced dissociation (CID) experiments, ions were isolated in $MS^1$ with a tolerance range of 1 Dalton (Da), using argon as the collision gas with a collision energy parameter set at 50%, and the frequency parameter at 45 kHz. The mass ranges of the method in $MS^2$ were adjusted for each metabolite, and the ion accumulation time was set at 30 ms.

First, the performance of the method was checked using mycotoxin standards (Fig. S2). Once the usefulness of the method for the analysis of these metabolites was confirmed, extracts were analyzed in scan mode for screening purposes. Next, the detected peaks were fragmented to study their fragmentation pathway and predict their molecular formula. These were cross-referenced in different databases in order to establish if they matched known mycotoxin or other fungal metabolites.

### Quantification of adenosine production

To quantify adenosine production by the fungi, a linear calibration curve was constructed in the extraction solvent across six concentration levels: 31.25, 62.5, 125, 250, 500, and 1000 ng/mL. To assess the matrix effect, the sample with the lowest adenosine signal was utilized to create a calibration curve in the extract, conducted in triplicate at concentrations of 125, 250, 500, 1000 and 2000 ng/mL. Simultaneously, equivalent curves were generated in solvent, and their slopes were employed to calculate the signal suppression/enhancement (SSE) caused by matrix according to the following equation:

$$SSE(\%) = \frac{Slope\ of\ spiked\ extracts\ curve}{Slope\ of\ standards\ curve\ in\ solvent} \times 100.$$

## Results

### Isolation and identification of *G. smithogilvyi* from sweet chestnut trees

In the resulting fungal cultures on PDA medium, colonies developed in concentric circles, exhibiting a creamy white to gray or light brown woolly mycelium, with brownish to black conidiomata being abundantly produced. The conidiomata were globose to sub-globose, containing hyaline, oval,



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obovoid, fusoid and multi-guttulate conidia (Fig. 1). These characteristics matched those described for *G. smithogilvyi* (Shuttleworth et al. 2012). BLAST (Basic Local Alignment Search Tool) and phylogenetic analysis using sequences available at Genbank (http:/blast.ncbi.nlm.nih.gov) allowed for the conclusive identification of all isolates as *G. smithogilvyi*, with five selected for the study of metabolite production. The ITS sequences of these five isolates are deposited in the NCBI GenBank database under the accession numbers OM417081, OM417082, OR789625, OR789626, and OM417083.

## Characterization of the *G. smithogilvyi* metabolites profile

Extracts of *G. smithogilvyi* cultures were analyzed in scan mode for screening purposes. In this way, several peaks that were not present in the control sample (PDA without fungi) were identified (Fig. 2).

A high intense peak was detected at 3.4 min (Fig. 3A). The extract ion chromatogram of the peak showed the ion at *m/z* 268.1051 (Fig. 3B), which was selected for CID leading to a fragment at *m/z* 136.0601 in $MS^2$ spectrum (Fig. 3C). The molecular formula of the compound was predicted based on the accurate mass of parent and product ions using an ad hoc predictor software (Formula Predictor Software, Shimadzu Corp., Kyoto, Japan). In this sense, several settings were employed for an accurate prediction, including the double bond equivalents (Table S1) and a HC ratio ranging from 0.2 to 3.1. The nitrogen rule was applied, and all the isotopes (Fig. S3) were employed in the prediction. According to the obtained data, the most probable formula for the compound at *m/z* 268.1051 was $C_{10}H_{13}N_5O_4$, with a low mass difference (4.1 ppm) between the measured mass and the theoretical mass of the predicted formula. This formula corresponds to the $[M+H]^+$ ion of adenosine, a nucleoside that is composed of adenine and d-ribose. Furthermore, the fragment ion at *m/z* 136.0601 (− 132.0450 Da) matched this molecule since it may be formed due to the loss of a glycosidic moiety $[M+H\text{-}C_5H_4O_4]^+$. The presence of this compound was confirmed using an analytical standard (Merck, Madrid, Spain) through the coincidence of the retention time and the fragmentation pattern between the standard and the compound present in the extract.

Next, a peak eluting at 5.8 min was studied (Fig. 4A). The extract ion chromatogram showed a high intense ion at *m/z* 360.2166 (Fig. 4B), the fragmentation of this ion led to different fragments in $MS^2$ spectrum (Fig. 4C). In this sense, two main ions at *m/z* 316.1897 and *m/z* 272.1633 were observed. As shown in Fig. 4D, the subsequent fragmentation of the ion *m/z* 272.1633 led to three ions in $MS^3$ (*m/z* 228.1366, *m/z* 198.0912 and *m/z* 172.0733), which were also detectable in the $MS^2$ spectrum (Fig. 4C). Applying the formula predictor software, the most probable formula for the compound was determined to be $C_{21}H_{29}NO_4$, which may correspond to oxasetin, with a low mass difference (0.83 ppm) between the theoretical and the measured masses. Subsequently, it was studied if the detected

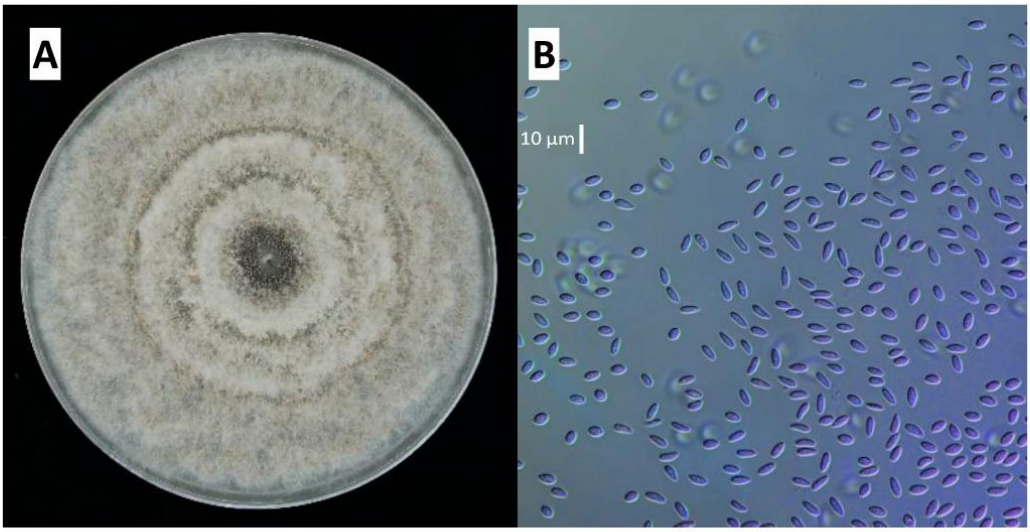



**Fig. 1** **A** Isolate of *G. smithogilvyi* on PDA. **B** Hyaline conidia of *G. smithogilvyi* (scale bar: 10 µm)



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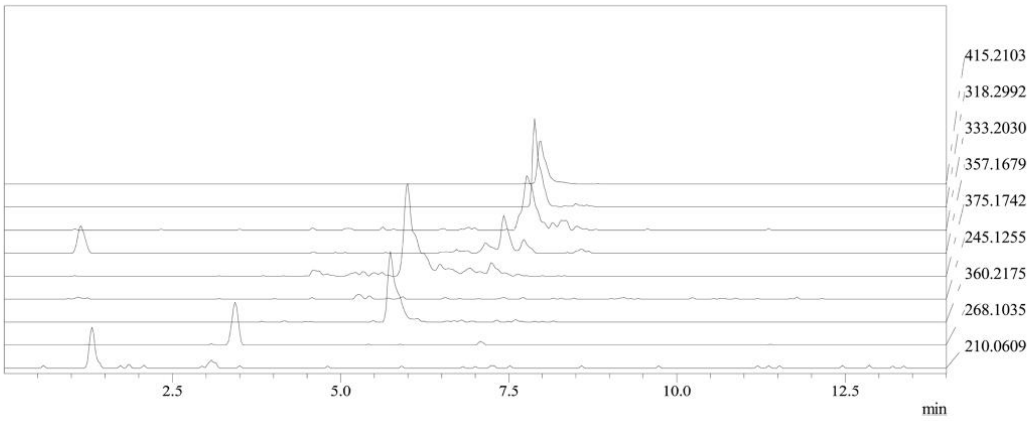



**Fig. 2** UHPLC-MS-IT-TOF chromatogram showing the main ions detected in the *G. smithogilvyi* extract

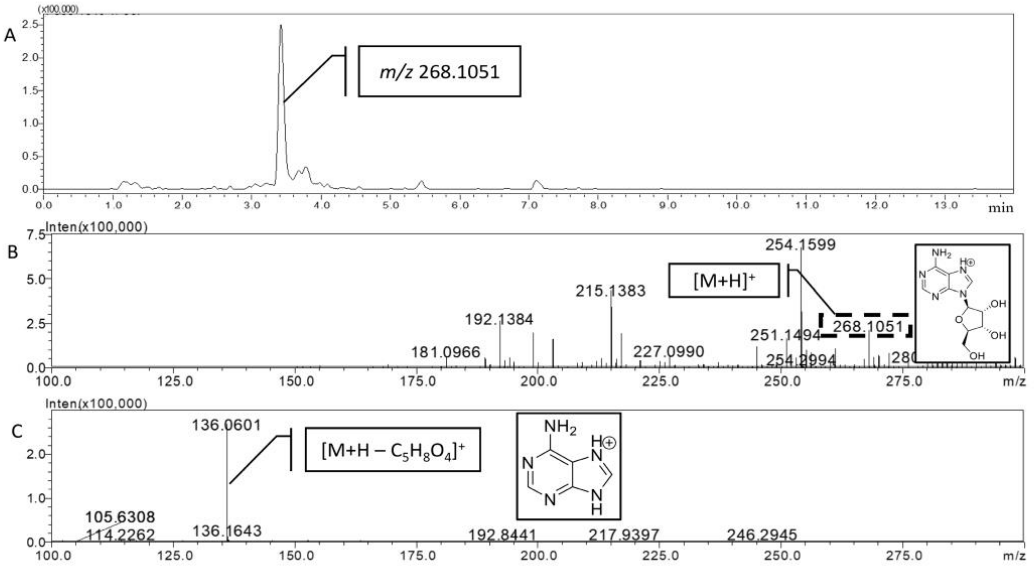



**Fig. 3** **A** UHPLC-MS-IT-TOF chromatogram of the compound eluting at 3.4 min. **B** Accurate $MS^1$ spectrum of the compound. **C** Accurate $MS^2$ spectrum of the ion at *m/z* 268.1051

fragment ions were compatible with the structure of the proposed compound. The most probable formula for the $MS^2$ ion at *m/z* 316.1897 was $C_{19}H_{25}NO_3$ ($[M+H-C_2H_4O]^+$) (Fig. 5C). Additionally, a high intense ion was also detected in $MS^2$ at *m/z* 272.1633 ($[M+H-C_4H_8O_2]^+$); the subsequent fragmentation of this ion led to an $MS^3$ compound at *m/z* 228.1366 $[M+H-C_4H_8O_2-C_2H_4O]^+$, along with the ion at *m/z* 198.0912 $[M+H-C_4H_8O_2-C_4H_{10}O]^+$ and at *m/z* 172.0733 $[M+H-C_4H_8O_2-C_6H_{12}O]^+$ (Fig. 5D and Table S1). Consequently, the exact mass and fragmentation pattern allowed the tentative identification of the compound as oxasetin.

Finally, a third high intense compound was identified with a retention time of 8.0 min (Fig. 5A). The extract ion



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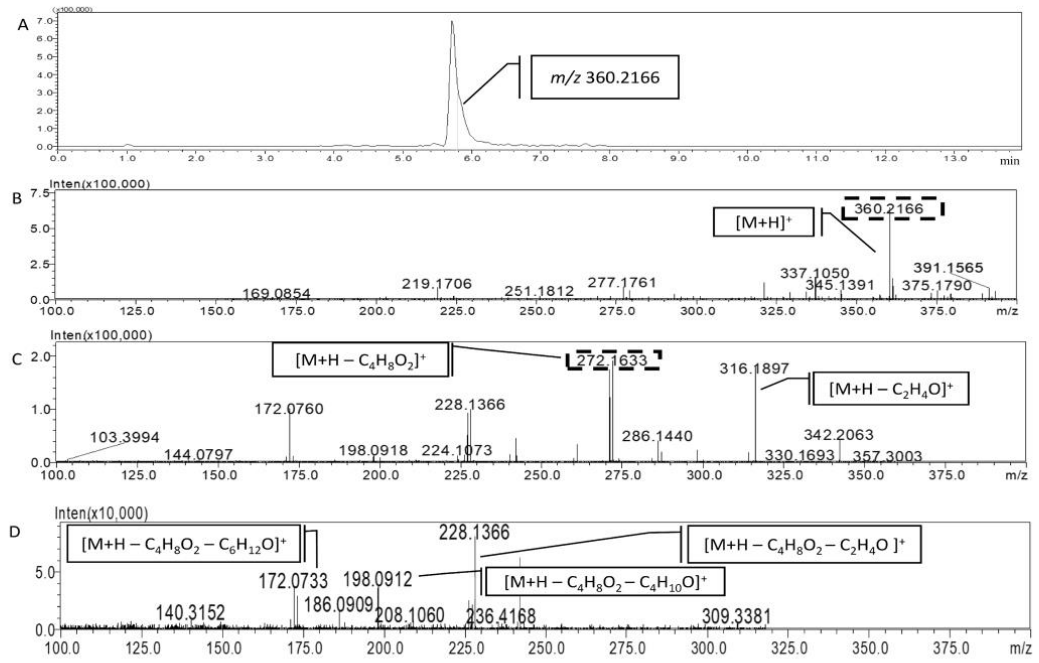



**Fig. 4** **A** UHPLC-MS-IT-TOF chromatogram of the compound eluting at 5.8 min. **B** Accurate $MS^1$ spectrum of the compound. **C** Accurate $MS^2$ spectrum of the ion at *m/z* 360.2166. **D** Accurate $MS^3$ spectrum of the $MS^2$ ion at *m/z* 272.1633

chromatogram displayed an ion at *m/z* 318.2992 (Fig. 5B), and its fragmentation in the $MS^2$ spectrum resulted in two main fragments (Fig. 5C). Employing the exact mass of parent and products ions, the most probable formula was predicted to be $C_{18}H_{39}NO_3$. This formula may correspond to phytosphingosine, with a low mass difference (3.46 ppm) between the theoretical and measured masses. To achieve accurate identification, the fragmentation process was examined. In the $MS^2$ spectrum, the first product ion at *m/z* 300.2869 was observed, which may correspond to the loss of a water molecule ($[M+H-H_2O]^+$). Next, a high intense ion at *m/z* 256.2626 ($[M+H-C_2H_6O_2]^+$) was observed and its subsequent fragmentation led to a fragment in $MS^3$ spectrum at *m/z* 102.0891 $[M+H-C_2H_6O_2-C_{11}H_{22}]^+$ (Fig. 5D), which can correspond to the aminopentenol molecule present in the compound. Therefore, the exact mass of the compound and its fragmentation pattern allowed for the tentative identification as phytosphingosine.

While additional molecules were found in the extract, their identification remains elusive based on available literature. The main detected compounds and their fragments are shown in Table S2.

## Quantification of adenosine production

The quantification of adenosine production by the fungal isolates involved the use of a linear calibration curve characterized by a high regression coefficient (R) of 0.998. The data underwent correction for matrix effect, given the observed SSE factor of $55.2 \pm 3.1$. Following this correction, adenosine was quantified in all five isolates, with concentrations determined in triplicate as follows: $176.2 \pm 28.8$; $321.8 \pm 16.6$; $351.3 \pm 40{,}1$; $421.5 \pm 177.1$ and $833.8 \pm 87.9$ µg/kg.

## Discussion

Filamentous fungi are a promising group of microorganisms that produce bioactive metabolites with diverse chemical entities and structural functions (Nagarajan et al. 2021). *G. smithogilvyi* (syn. *G. castaneae*) has emerged as a pathogen that causes chestnut brown rot on sweet chestnut worldwide (EPPO 2022) and is currently recognized as the primary agent causing nut rot in chestnut (Lione et al. 2015). Despite its impact, the secondary metabolite profile of *G.*



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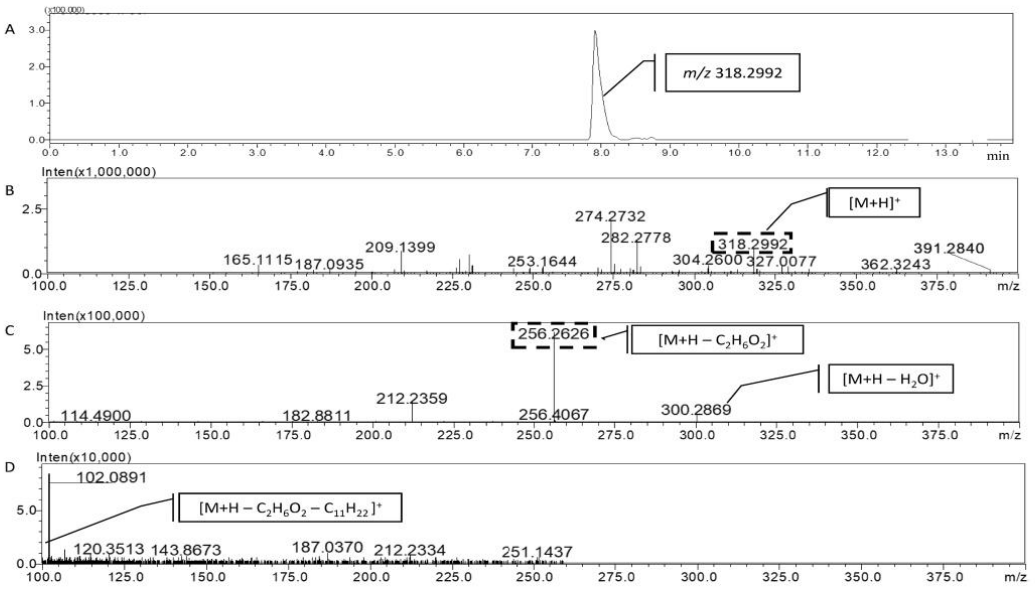



**Fig. 5** **A** UHPLC-MS-IT-TOF chromatogram of the compound eluting at 8 min. **B** Accurate $MS^1$ spectrum of the compound. **C** Accurate $MS^2$ spectrum of the ion at *m/z* 318.2992. **D** Accurate $MS^3$ spectrum of the $MS^2$ ion at *m/z* 256.2626

*smithogilvyi* remains unknown.. It is crucial to determine if this fungus is capable of producing mycotoxins, as observed in other rotting fungi, due to the potential consequences for the safety of chestnut consumption (Lione et al. 2019).

*Gnomoniopsis smithogilvyi* can live as an endophyte in healthy chestnuts, and if it produces toxic secondary metabolites in kernels, they could be inadvertently ingested. On the other hand, affected nuts may be rejected by the consumer when eaten raw (since brown lesions are visible); however, as the chestnut shell often shows no visible symptoms of disease, when kernels are eaten after being cooked or industrially processed (roasted in shell, into flour, etc.), necrotic lesions caused by the fungus might be also inadvertently ingested.

To elucidate the profile of mycotoxins and other secondary metabolites from *G. smithogilvyi*, five isolates were grown in PDA for a week. In this sense, a straightforward approach to study metabolite profiles of fungi is to analyze agar plugs from pure Petri dish cultures. In fact, this method is widely employed to establish the metabolites profile of fungi (González-Jartín et al. 2019b). Thus, three agar plugs were sampled from each *G. smithogilvyi* culture, and an acetonitrile/water/acetic acid mixture [49:50:1 (v/v/v)] was used as a extraction solvent, allowing the analysis of both lipophilic and hydrophilic compounds (González-Jartín et al. 2019a). TOF instruments facilitated the identification of molecules produced by the fungus based on their exact mass, an approach previously reported for the analysis of fungal metabolites (González-Jartín et al. 2017; Şenyuva et al. 2008).

As detailed below, the profile of secondary metabolites produced by *G. smithogilvyi* was studied for the first time.

The peak at *m/z* 268.1051, detected at 3.4 min (Fig. 3), was tentative identified as adenosine based on the most probable formula and the fragmentation pattern. This identification was further supported by the observation of the fragment ion at *m/z* 136.0601 in previous experiments (Jin et al. 2017). The presence of adenosine was confirmed using an analytical standard, where the retention time and fragmentation pattern of the standard matched those of the compound present in the extract. Adenosine was detected in all five isolates, with the concentrations ranging from 176.2 to 833.8 µg/kg. However, toxin production could be higher since it was not possible to correct the data with the recovery of the extraction as no blank samples were available for its calculation. In addition, this production was only determined in PDA, but it could be higher using other culture media or grown conditions (González-Jartín et al. 2019b).

Adenosine is a purine nucleoside that regulates central nervous, cardiovascular, peripheral, and immune systems. Indeed, this metabolite can be used in therapeutics due to its antiarrhythmic properties in supraventricular tachycardia



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(Borea et al. 2018). This compound is a major nucleoside in *Cordyceps* spp., a genus of entomopathogenic fungi in the class Ascomycetes, commonly employed in traditional Chinese medicines (Singpoonga et al. 2020; Yang et al. 2007). Adenosine has also been isolated from *Penicillium* sp., exhibiting high antioxidant activity (Yuan et al. 2014). This metabolite is the main precursor for the biosynthesis of cordycepin (Kaushik et al. 2020), the main bioactive component in *Cordyceps* (Ashraf et al. 2020); however, this metabolite was not detected in the extracts.

Next, a peak at *m/z* 360.2166 eluting at 5.8 min was studied (Fig. 4). The most probable formula corresponds to oxasetin, a polyketide initially discovered from the fungus *Vaginatispora aquatica,* and later detected in *Lophiostoma* sp. (He et al. 2002; Shushni et al. 2013). Both fungi belong to the *Lophiostomaceae* family and were isolated from decaying wood submerged in a Hong Kong River and driftwood on the coast of the Baltic Sea, respectively. There are no other studies regarding the identification of this compound by mass spectrometry. However, the observed fragment ions are compatible with the structure which supports the tentatively identification of oxasetin. This compound was reported to be active in vitro against some Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecalis*, while showing no activity against *Escherichia coli* (a Gram-negative bacterium) and the yeast *Candida albicans* (He et al. 2002).

Finally, the ion at *m/z* 318.2992, with a retention time of 8.0 min, was studied (Fig. 5). The most probable formula ($C_{18}H_{39}NO_3$) corresponds to phytosphingosine. In addition, some of the detected fragments have been reported in a previous study (Li et al. 2020). This compound is a sphingosine analogue, a structural component of sphingolipids found in fungi, plants, and some mammalian tissues (Li et al. 2006; Mota et al.). Sphingolipids are essential structural components of eukaryotic cell membranes. In filamentous fungi, glycosphingolipids are crucial for growth and pathogenesis, and, consequently, in the last few decades, they have received increasing attention for their possible role in the establishment of fungal infection (Mota et al. 2018). In particular, production of phytosphingosine seems crucial for filamentous fungal growth and viability, as has been shown for *Aspergillus nidulans* (Mota et al. 2018). On the other hand, it is well established that some sphingosine analogues inhibit sphinganine–N-acetyltransferase and ceramide synthase hampering sphingolipid biosynthesis (Möbius and Hertweck 2009). This inhibition is a common tactic employed by phytopathogenic fungi such as *Fusarium* spp., which produce fumonisins, and *Alternaria alternata,* which produces the host-specific toxin AAL-toxin (Spassieva et al. 2002; Williams et al. 2007). These phytotoxic compounds disrupt sphingolipid metabolism in susceptible tomato varieties (Abbas and Boyette 1992; Abbas and Riley 1996). Additionally, sphingolipids are important regulators in the pathogenicity of several pathogenic fungi, contributing to fungal growth in hosts under stressful conditions, maintenance of cell wall integrity, and the production of several virulence factors (Song et al. 2020). Therefore, the production of phytosphingosine by *G. smithogilvyi* may be related to its high phytotoxicity on chestnuts.

In conclusion, in this study, we first report the profile of secondary metabolites from *G. smithogilvyi*, the main causal agent of chestnut brown rot on sweet chestnut. Isolates of this pathogen were obtained from affected burrs and nuts of sweet chestnut during an outbreak of the disease in Southeastern Galicia in September 2021. Our findings demonstrate that *G. smithogilvyi* does not produce any mycotoxins. However, three secondary metabolites with interesting properties—adenosine, oxasetin and phytosphingosine—were identified based on accurate mass measurement and fragmentation patterns. The presence of adenosine was confirmed using an analytical standard. This metabolite is a well-known antiarrhythmic drug, while the production of phytosphingosine may be related to the high phytotoxicity of *G. smithogilvyi* on sweet chestnut.

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**Author contributions** Jesús M. González-Jartín: Investigation, Writing—Original Draft; Olga Aguín: Resources; Inés Rodríguez-Cañás: Investigation; Rebeca Alvariño: Investigation; María J. Sainz: Conceptualization; Mercedes R. Vieytes: Methodology; Cristina Rial: Investigation; Pilar Piñón: Investigation; Carmen Salinero: Project administration; Amparo Alfonso: Methodology, Conceptualization; Luis M. Botana: Funding acquisition, Supervision.

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**Data availability** The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.



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## Declarations

**Competing interests** The authors declare no competing interests.

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## SECCIÓN 2: MICOTOXINAS EN PRODUCTOS ELABORADOS

### 3. Detection of mycotoxins in cheese using an optimized analytical method based on a QuEChERS extraction and UHPLC-MS/MS quantification

**Resumen:**
Las micotoxinas pueden producir efectos tóxicos en los seres humanos; por lo tanto, es de gran importancia determinar su presencia en los productos alimenticios. Este trabajo presenta un método confiable para la cuantificación de 32 micotoxinas en queso. El procedimiento de análisis se optimizó basándose en un proceso de extracción QuEChERS y la detección por cromatografía líquida de ultra alta eficiencia acoplada a espectrometría de masas en tándem (UHPLC-MS/MS). El método de análisis se validó para cuatro variedades de queso (emmental, azul, brie y camembert) en términos de linealidad, sensibilidad, efecto matriz, exactitud y precisión. Se lograron valores satisfactorios de precisión y exactitud, con recuperaciones superiores al 70 % para la mayoría de las micotoxinas. El método desarrollado se aplicó al análisis de 38 muestras comerciales de queso. Se encontró una alta presencia de beauvericina y eniatinas, que varió del 31 % para eniatina A al 100 % para eniatina B. La ocratoxina A se detectó en tres muestras en concentraciones que pueden representar un riesgo para la salud humana.

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# Detection of mycotoxins in cheese using an optimized analytical method based on a QuEChERS extraction and UHPLC-MS/MS quantification

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ABSTRACT

Mycotoxins can produce toxic effects on humans; hence, it is of high importance to determine their presence in food products. This work presents a reliable method for the quantification of 32 mycotoxins in cheese. The analysis procedure was optimized based on a QuEChERS extraction process and the ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) detection. The analysis method was validated for four cheese varieties (emmental, blue, brie and camembert) in terms of linearity, sensitivity, matrix effect, accuracy and precision. Satisfactory precision and accuracy values were achieved, with recoveries above 70% for most mycotoxins. The developed method was applied to the analysis of 38 commercial cheese samples. A high occurrence of beauvericin and enniatins were found, ranging from 31% for enniatin A to 100% for enniatin B. The ochratoxin A was detected in three samples at concentrations that may pose a risk to human health.

## 1. Introduction

Cheese is one of the most widely consumed dairy products worldwide, especially in Europe (Fox, Guinee, Cogan, & McSweeney, 2017). Different types of milk and a wide range of technologies can be employed to manufacture of a great variety of cheeses, which are diverse in morphological features, texture, and flavor. These dairy products are exposed to the growth of pathogenic and spoilage microorganisms such as bacteria, yeasts and filamentous fungi (Kure & Skaar, 2019).

Certain filamentous fungi belonging to *Aspergillus, Penicillium, Alternaria*, and *Fusarium* genera are known to produce toxic metabolic substances known as mycotoxins (Sainz, González-Jartín, Aguín, Mansilla, & Botana, 2018). Although more than 400 mycotoxins are known, only a few are of major concern because of their high toxicity. In this sense, maximum limits in food and feed have been established for some mycotoxins that are a public health problem at levels at which they can appear in food. This group includes aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT), fumonisins (FBs), citrinin (CTN), zearalenone (ZEN), deoxynivalenol, T-2 toxin and HT-2 toxin (EC_1881, 2006).

The group of emerging mycotoxins comprises compounds for which there are little data about their toxicity and presence in food. This group includes, among others, enniatins (ENNs), beauvericin (BEA), sterigmatocystin (STC), mycophenolic acid (MPA) and *Alternaria* toxins (Pérez-Fuentes et al., 2021). Exposure to fungal toxins is not restricted to regulated and emerging mycotoxins since these compounds can be modified by fungi, plant or animal metabolism which leads to products with potential toxicity but not considered in the legislation. They are called modified mycotoxins (González-Jartín, Alfonso, Sainz, Vieytes, & Botana, 2018).

In some cases, the fungal contamination of cheese occurs intentionally since mould species are used for manufacturing and ripening to provide specific characteristics or prolong shelf-life (Soda, Madkor, & Tong, 2000). Fungi species employed with this purpose usually belong to the genus *Penicillium*, such as *P. roqueforti* and *P. camemberti*, which are used in the manufacture of blue and white cheeses, respectively (Hymery et al., 2014). The presence of mycotoxins in cheese depends on various factors such as water activity, temperature or the substrate (Becker-Algeri et al., 2016). For instance, *P. roqueforti* strains produce high amounts of roquefortine C (ROQC) when the ripening temperature is high. In addition, this fungus can produce PAT, penicillic acid and PR

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toxin. Similarly, *P. camemberti* produces cyclopiazonic acid (CPA) (Dobson, 2017).

Mycotoxins can also appear in cheese due to the use of contaminated milk. For example, if animals are exposed to feed contaminated with aflatoxin $B_1$ ($AFB_1$), this compound will be metabolized leading to aflatoxin $M_1$ ($AFM_1$), which will be excreted in milk (Fink-Gremmels, 2008). To avoid intoxications, the European legislation establishes a maximum level of 0.05 μg/kg for $AFM_1$ in dairy milk and milk for the manufacture of dairy products. However, other mycotoxins such as FBs, OTA, ZEN, cyclopiazonic acid (CPA), T-2 toxin, ROQC, ENNs and BEA can appear in this matrix (González-Jartín, Rodríguez-Cañás, et al., 2021). In cheese, certain mycotoxins have been detected including OTA, CTN, STC, CPA, ROQC, MPA and $AFM_1$ (Dobson, 2017). However, European legislation does not establish maximum limits for mycotoxins in this matrix. Nevertheless, the European Food Safety Authority (EFSA) has demanded more occurrence data on OTA in cheese to evaluate if its presence in this product may pose a risk to humans (EFSA, 2020).

Different methodologies have been proposed for the analysis of mycotoxins including screening techniques such as enzyme-linked immunosorbent assay (ELISA) and confirmation techniques like liquid chromatography (LC) coupled to mass spectrometry (MS), fluorescence (FD) or ultraviolet (UV) detection. Recently, Ultra-Performance Liquid Chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) has become the technique of choice for analysis of wide range of contaminants in food, including mycotoxins, since it allows the simultaneous determination and accurate quantification of several analytes at low levels in a short run time (Rodríguez et al., 2017).

The high content of cheese in fat and protein and the chemical diversity of mycotoxins that can appear in this product make developing analytical procedures challenging. In fact, few methods currently available to extract mycotoxins from cheese. Some examples include a solid-phase microextraction procedure for the determination of CPA and MPA in white and fermented blue cheeses, respectively, or a solid–liquid extraction for the determination of nine mycotoxins in mould cheeses (Kokkonen, Jestoi, & Rizzo, 2005b).

In recent years, mycotoxin extraction procedures based on QuEChERS (Quick, Easy, Cheap, Effective, Rugged & Safe) methods have been developed for the analysis of complex matrices such as fruits, juices, milk, cereal products, eggs or beer (González-Jartín et al., 2019). In this context, the aim of this work was to develop a new method for the simultaneous analysis of the main types of regulated, emerging and modified mycotoxins that may be present in cheese by the combined use of a QuEChERS-based extraction and UHPLC-MS/MS detection.

## 2. Material and methods

### 2.1. Chemicals and reagents

Pure water was obtained from a Millipore Milli-Q Plus system (Millipore, USA). Methanol, acetonitrile, acetic acid glacial 100 %, anhydrous magnesium sulphate ($MgSO_4$) and sodium chloride (NaCl) were purchased from Panreac Química S.A. (Barcelona, Spain). Formic acid was from Merck (Madrid, Spain), ammonium formate and ammonium acetate from Fluka (Buchs, Switzerland), the Polygoprep$^{TM}$ 60–50 C18 was supplied by Macherey-Nagel (Düren, Germany) and ultrafree-MC durapore membrane centrifugal filters (0.22 μm pore size) provided by Millipore (Billerica, USA).

The analytical standards $AFB_1$, aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$), aflatoxin $G_2$ ($AFG_2$), enniatin A (ENNA), enniatin $A_1$ ($ENNA_1$), enniatin B (ENNB), enniatin $B_1$ ($ENNB_1$), gliotoxin (GLIO), ROQC, and ZEN were from Sigma (Madrid, Spain). Analytics standards of 3-acetyldeoxynivalenol (3 Ac-DON), 15-acetyldeoxynivalenol (15-AcDON), $AFM_1$, alternariol (AOH), alternariol methyl ether (AME), CPA, CTN, diacetoxyscirpenol (DAS), fumonisin $B_1$ ($FB_1$), fumonisin $B_2$ ($FB_2$), hydrolyzed fumonisin $B_1$ (h-$FB_1$), HT-2 toxin, MPA, neosolaniol (NEO), STC, T-2 toxin, T-2 triol and zearalanone (ZOL) were provided by Romer Labs (Tulln, Austria). Circumdatin A (CTA) was from Santa Cruz Biotechnolgy (Santa Cruz, CA), OTA was obtained from Laboratorios CIFGA S.A. (Lugo, Spain) and BEA was supplied by Enzo (Barcelona, Spain). Cheese samples were purchased from local supermarkets.

### 2.2. Instrumentation

The UHPLC-MS/MS analysis was conducted on an Agilent 1290 Infinity UHPLC system coupled to a triple quadrupole mass spectrometer (Agilent 6460) equipped with an electrospray ionization (ESI) source (Agilent Technologies, Waldbronn, Germany). System control and data acquisition were carried out with Agilent MassHunter LCMS Acquisition Console software and data were also processed using QQQ Quantitative Analysis software.

### 2.3. Optimization of the extraction conditions

In order to optimize the extraction process, a first test was carried out in emmental cheese. In this sense, samples were artificially contaminated at 3 μg/kg of $AFM_1$, $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, and at 80 μg/kg of AME, BEA, CTA, CTN, DAS, ENNA, $ENNA_1$, ENNB, $ENNB_1$, $FB_1$, $FB_2$, MPA, OTA, STC, T-2 toxin and ZEN. The matrix effect and recoveries were evaluated, as described below, after the extraction of a 0.5 g sample with 2 mL of a 2 % formic acid solution and 2 mL of acetonitrile, a d-SPE clean-up was applied by using 1.4 mL of organic phase, 0.4 g of $MgSO_4$ and 0.1 g of C18. In addition, a further clean-up step with hexane prior to d-SPE was studied. For this purpose, 2 mL of hexane were mixed with 1.4 mL of the organic extract and shaken in a vortex for 0.5 min. Then, hexane was discarded, and the obtained extract was submitted to the same d-SPE clean-up step. Finally, 1 mL of the resultant extracts was evaporated to dryness and reconstituted with 200 μL of sample solvent.

Next, in order to optimize the percentage of acid used for the extraction, samples were artificially contaminated with 3 μg/kg of $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ and 80 μg/kg of ZEN and OTA. In this case, 0.5 g of cheese were extracted with 2 mL of aqueous solution acidified with 0.5, 1, 2 and 4 % of formic acid or acetic acid, and 2 mL of acetonitrile. After mixing for 5 min, 0.8 g of $MgSO_4$ and 0.2 g of NaCl were added to induce the separation of two phases (organic and aqueous). After centrifugation, 1 mL of the resultant organic extract was evaporated to dryness and reconstituted with 200 μL acetonitrile/water/acetic acid 49/50/1 (v/v/v). Finally, samples were filtered and analysed by UHPLC-MS/MS in order to calculate the apparent recoveries ($R_A$).

Finally, the matrix effect was evaluated at different concentration levels of the matrix extract. In this sense, emmental cheese samples were contaminated with 3 μg/kg $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$ and $AFM_1$ and with 80 μg/kg of AME, CTA, CTN, DAS, $FB_1$, $FB_2$, MPA, OTA, STC, T-2 toxin and ZEN. Samples were extracted using the method previously described. However, after the extraction process, samples were concentrated 0, 2, 5, 10 and 20 times and $R_A$ were studied.

### 2.4. Chromatography and mass spectrometric conditions

In order to separate the analytes, a Waters Acquity UPLC HSS T3 (1.8 μm, 100 mm × 2.1 mm) column was used at a temperature of 40 °C. Mobile phase A was composed of water with 1 % formic acid and 5 mM ammonium formate, and mobile phase B was composed of methanol/water/formic acid 97/2/1 (v/v/v) with 5 mM ammonium formate. The flow rate of the mobile phases was 0.3 mL/min, and a gradient elution of 13 min of duration was applied: after an initial hold time of 0.5 min at 0 %B, the gradient was increased to 14 %B within 0.5 min, and kept for 1.5 min. Later, the percentage of the eluent B was raised to 60 %B within 1 min and then maintained for 0.5 min. Next, the gradient reached the 100 %B within 4.5 min, and it was kept for 2 min. Finally, the gradient was changed to 0 % in 0.5 min and this percentage was hold for 2.5 min. The injection volume was set at 5 μL. The mass spectrometer (Agilent 6460) was operated in ESI modes using the following ionization source



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parameters: nebulization gas (nitrogen) temperature 400 °C; nebulization gas flow, 12 L/min; drying gas (nitrogen) temperature, 350 °C; drying gas flow, 8 L/min; nebulizer, 45 psi; capillary voltage, 4000 V; nozzle voltage, 0 V. Analysis was carried out in negative and positive mode using a dynamic multiple reaction monitoring (dMRM) method monitoring two mass transitions for each mycotoxin. The fragmentor voltage (FV), collision energy (CE), cell accelerator voltage (CAV) and mass transitions had previously been optimized for most of the analysed compounds and are shown in the Table S1 (González-Jartín, Rodríguez-Cañás, et al., 2021). Fig. S1 shows the chromatogram of the 32 mycotoxins included in the present work.

### 2.5. Sample preparation

Samples consisting of the whole cheese we were milled in a food processor and then a QuEChERS extraction procedure was applied as follows: a 0.5 g portion of the homogenized sample was weighed in a Falcon tube and mixed with 2 mL of a 2 % of acetic acid solution using a multi-tube vortex (Nahita Blue, Auxilab, Spain) at 2500 rpm for 5 min. Then, 2 mL of acetonitrile were added, again samples were mixed using the multi-tube vortex at 2500 rpm (Nahita Blue, Auxilab, Spain), for 5 min. Next, in order to induce phase-phase partitioning 0.8 g of $MgSO_4$ and 0.2 g of NaCl were added, and the shaking step was repeated for 30 s. Next, samples were centrifuged for 10 min (3134 × *g*) using Gyrozen 1236R (Spain). Then, an aliquot of 500 μL of supernatant was diluted with 500 μL of aqueous acetic acid solution (2 %), the resultant extract was filtered through a 0.22 μm using centrifugal filters (5 min, 16000 × *g*) and analysed. In addition, some further steps may be included in the protocol in order to obtain a concentrated extract (see below).

### 2.6. Method validation

#### 2.6.1. Calibration curves and performance characteristics

The performance characteristics of the analytical method were first evaluated in solvent. The linearity of the method was evaluated using the correlation coefficient (R) obtained with a linear regression model. In this sense, calibration curves were constructed in solvent at nine levels in a concentration range from 0.01 to 3 ng/mL for AFs, 0.19 to 50 ng/ml for BEA and ENNs, 4.69–600 ng/mL for ZOL and from 0.78 to 200 ng/mL for the rest of analysed mycotoxins. The within-batch repeatability of the analytical method was evaluated by calculating the relative standard deviation (RSD) of the slope of calibration curves for three consecutive batches, while the between-batch repeatability was measured through calibration curves constructed in three consecutive days. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated in solvent and in matrix following the guidance of the European Union Reference Laboratory (EU-RL) for each mycotoxin through the measurements of ten solvents namely an acetonitrile/water/acetic acid 49/50/1 (v/v/v) solution or ten extracts of blank samples (Wenzl, Ḥaedrich, Schaechtele, Robouch, & J., 2016). The LOD was calculated with the following equation, where *Sb* is the standard deviation of the measurements of the blank, and *m* is the slope of the calibration curve. The LOQ was calculated as 3.3 times the LOD. Blank extracts of emmental, brie, camembert, and blue cheese were employed to calculate the LOD and LOQ in matrix.

#### 2.6.2. Matrix effect

Blank extracts of cheese were employed to construct calibration curves in matrix at the same concentration levels employed in solvent. The slopes of solvent and matrix extract curves were used to calculate the matrix effect defined as the signal suppression/enhancement (SSE) caused by matrix according to the following equation:. A value under 100 % means signal suppression and above 100 % signal enhancement.

#### 2.6.3. Accuracy and precision

The accuracy and precision of the method were evaluated using the recovery from samples spiked before extraction. Blank samples were spiked in triplicate at 0.25 μg/kg of $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AFM_1$; 8.5 μg/kg of BEA, ENNA, $ENNA_1$, ENNB, $ENNB_1$; 16.5 μg/kg of 15-AcDON, 3-AcDON, AOH, AME, CTA, CTN, CPA, DAS, $FB_1$, $FB_2$, GLIO, HT-2, h-$FB_1$, MPA, NEO, OTA, ROQC, STC, T2 toxin, T2 triol, ZEN; and 50 μg/kg of ZOL. Calibration curves constructed in solvent were employed to measure the concentration of each analyte in the extracts. In this way, the apparent recovery ($R_A$) was calculated. Next, the accuracy was calculated by applying the SSE factor to the $R_A$ in order to obtain the recovery of the extraction ($R_E$), which was obtained by applying the following equation. The intra-day precision was studied through the relative standard deviation (RSD) of the $R_A$ (%) by analysing samples in triplicate on the same day. In this sense, the intra-day RSD ($RSD_r$) was calculated according to the following equation:, where *SD* is the standard deviation of the measurements and is the average. On the other hand, inter-day precision ($RSD_R$) was evaluated by analysing samples in triplicate for three consecutive days.

## 3. Results and discussion

Cheese is a product susceptible to being contaminated by mycotoxins, but currently, available methods to detect toxins in this matrix are scarce and are focus on detecting a few compounds (Table S2). Therefore, the aim of this study was to develop a new method for the simultaneous analysis of regulated, emerging and modified mycotoxins in cheese. In this regard, a new UHPLC-MS/MS method and a new extraction procedure were developed and validated for the analysis of different varieties of cheese, namely: emmental, blue, brie and camembert.

### 3.1. Optimization of UHPLC-MS/MS conditions

The UHPLC-MS/MS method was based on a recently developed procedure for milk analysis (González-Jartín, Rodríguez-Cañás, et al., 2021). However, the presence of mycotoxins not included in the original method, such as CPA and GLIO, has been reported in cheeses (Table S2). Consequently, the detection method was modified to include these compounds. Mass transitions were optimized for each metabolite using MassHunter Optimizer software. In the case of CPA, the best performance was obtained in the negative ion mode. The predominant signal (precursor ion) was detected at *m/z* 335 and the product ions at *m/z* 154 and *m/z* 140. In the case of GLIO, the precursor ion was detected at *m/z* 327 and the product ions at *m/z* 263 and *m/z* 245. Mobile phases used for milk analysis were water containing 0.1 % of formic acid and 5 mM ammonium formate (A) and methanol (B). However, these chromatographic conditions were not suitable for CPA analysis since a well-defined peak was not obtained (Fig. 1A). The inclusion of CPA in multi-mycotoxin methods have previously been discarded by some authors since they were unable to obtained linear calibration curves. The instability of CPA may be caused by its low pKa (2.94). This toxin can be produced by fungi employed for cheese ripening, and therefore the chromatographic method was optimized for its detection (Peromingo, Rodriguez, Nunez, Silva, & Rodríguez, 2018). In this sense, ten mobile phases were evaluated (Table S3). The mobile phase A consisted in water and mobile phase B methanol/water mixtures, in both cases containing ammonium formate or ammonium acetate and formic acid or acetic acid. As shown in Fig. 1, no signal corresponding to the CPA appears when the percentage of formic or acetic acid was of 0.1 % (Fig. 1 A-E). Methanol is used more frequently than acetonitrile because it gives better results in terms of mycotoxin sensitivity. Similarly, ammonium formate and formic acid usually lead to an increase in mycotoxins signals (Desmarchelier et al., 2010). For this reason, acetonitrile, ammonium acetate and acetic acid were discarded for the following steps, which first consisted in an increase of the percentage of formic acid to



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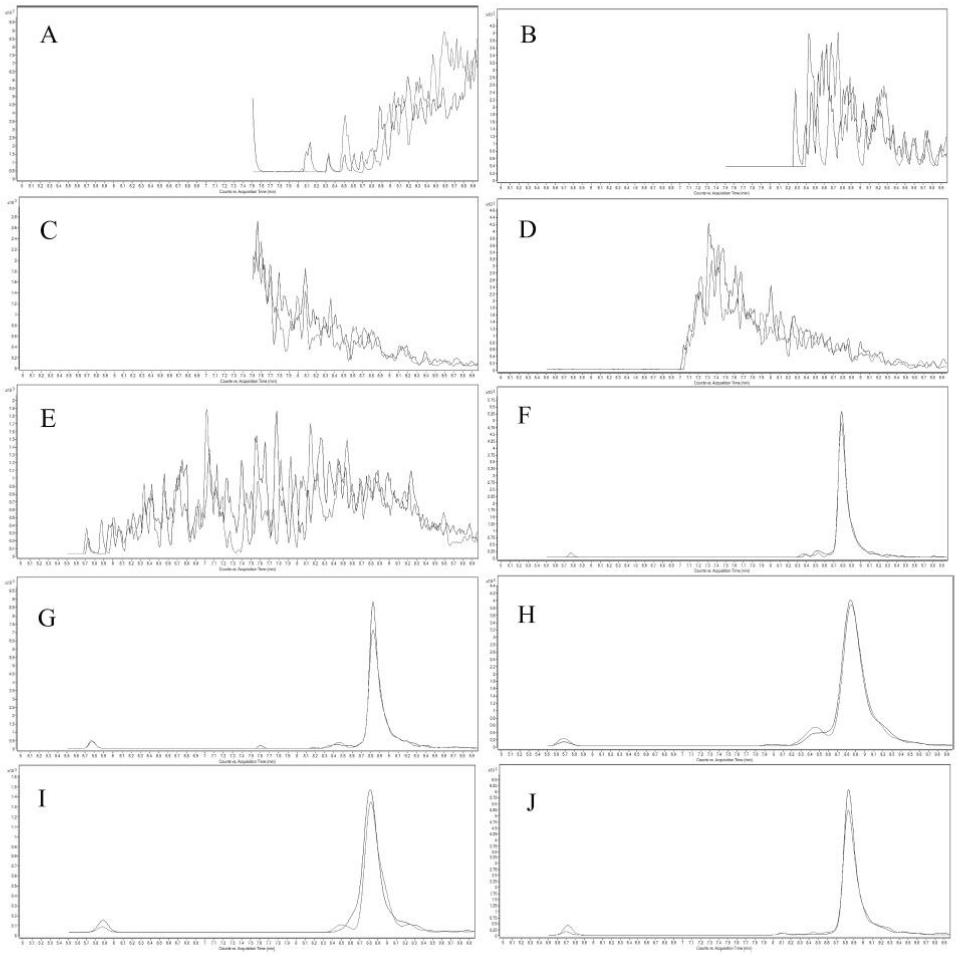



**Fig. 1.** Optimization of UHPLC conditions. Chromatograms of cyclopiazonic acid at 400 ng/mL obtained with mobile phase 1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), 8 (H), 9 (I), 10 (J).

0.5 %, in this way, a narrow peak is achieved (Fig. 1 F). Then, the percentage of formic acid was increased to 1 % leading to an increase in the intensity of the signal (Fig. 1 G). However, the inclusion of 2 % formic acid led to a broader peak (Fig. 1 H). Finally, the amount of ammonium formate was doubled (10 mM) (Fig. 1 I) and halved (2.5 mM) (Fig. 1 J). The higher intensity was observed when using the 5 mM of ammonium formate (Fig. 1 G). Therefore, a mobile phase A composed of water with 1 % formic acid and 5 mM ammonium formate, pH of 2.5, and a mobile phase B composed of methanol/water/formic acid 97/2/1 (v/v/v) with 5 mM ammonium formate, pH of 4, were chosen for analysis. With the new conditions, a well-defined CPA peak was obtained while the sensitivity for most of the analytes included in the method such as OTA or $AFM_1$ was maintained. However, the sensitivity to some compounds such as deoxynivalenol and its analogues deepoxy-deoxynivalenol and deoxynivalenol 3-glucoside was diminished, the probability of finding these toxins in cheese is very low so they were eliminated from the method (Koesukwiwat, Lehotay, Mastovska, Dorweiler, & Leepipatpiboon, 2010).

### 3.2. Optimization of extraction procedure

The original QuEChERS method was designed for pesticide analysis in fruits and vegetables. Later, it was extended to extract different compounds from several matrices (Panda, Dash, Manickam, & Boczkaj, 2021). The first step of the QuEChERS procedure is a solid–liquid or liquid–liquid extraction. The solvent most commonly employed is acetonitrile, which enables the extraction of analytes showing different polarities. This solvent is usually combined with water and different acids. In this sense, the addition of water helps the organic solvent to enter the food matrix, and the addition of acidic is useful to break the bonds between analytes and food components (Yan et al., 2020). The second step, sometimes omitted, consists of a further clean-up to remove interfering compounds, for which different sorbents such as C18, primary secondary amine (PSA) and graphitized carbon black (GCB) can be employed. In addition, other solvents, such as hexane, have been used in some protocols (Dong et al., 2019).

The different compounds present in the matrix may interference in



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the analysis, reducing or enhancing intensity of analytical signal of the target mycotoxin. This effect is of especial concern in complicate matrices such as cheese. Therefore, different clean-up steps were evaluated, all of them based on the use of C18 as a dispersive solid phase extraction (d-SPE). The procedure was initially optimized in emmental cheese with the aim of reducing the matrix effect and maximize toxins extraction. For the initial evaluation the employed method was based in a previous one developed for the analysis of milk (González-Jartín, Rodríguez-Cañás, et al., 2021). A portion of 0.5 g of sample and 2 mL of acetonitrile were chosen because the volume of the final extract was enough to perform the clean-up test. The salts used were $MgSO_4$ and NaCl in a proportion of 4:1 since is the most employed one in QuEChERS extractions (González-Curbelo et al., 2015). As shown in Fig. S3, there was not much difference in the matrix effect between samples treated with or without the application of d-SPE step with C18. However, a reduction in the $R_E$ of BEA, ENNs and CTN was observed when C18 was included for sample treatment (Fig. S3). In this sense, low recoveries of BEA and ENNs had previously been reported when using d-SPE (González-Jartín, Rodríguez-Cañás, et al., 2021). BEA and ENNs are lipophilic compounds therefore it is possible that the removal of lipids by C18 also remove some mycotoxins attached to them. The combination of C18 and hexane was proposed for the analysis of mycotoxins in egg since the hexane is highly effective in the elimination of lipids (Capriotti, Cavaliere, Piovesana, Samperi, & Laganà, 2012). However, their use was discarded since, as shown in Fig. S3, yielded low recoveries for compounds like CTN. It has been previously observed that low recoveries for CTN can be a consequence of incomplete solvent extraction, the adsorbents used in the clean-up step, as well as the evaporating process of the extract (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2014). Consequently, an extraction procedure without a clean-up step was chosen for sample analysis.

On the other hand, as the recovery for some compounds was low, the percentage of acid was optimized. As shown in Fig. 2, the increase of the percentage of formic acid causes a decrease in the $R_A$. For instance, in the case of OTA, the $R_A$ was reduced from 24 % to 9 % when the percentage of this addictive was increased from 0.5 to 4 %. In general, aqueous solution containing acetic acid resulted in higher $R_A$. Although there are low differences in the recovery obtained with the employed percentages of acetic acid, the 2 % acetic acid solution was chosen for extraction because it gave higher $R_A$ values especially for $AFG_1$ and, this percentage of addictive has previously been successfully employed for the analysis of mycotoxin in several complex matrices (González-Jartín et al., 2019; González-Jartín, Alfonso, Sainz, Vieytes, & Botana, 2021).

Finally, it was studied how the concentration of the sample modify the matrix effect. Samples were extracted using the method previously described. However, after the extraction process, samples were concentrated 0, 2, 5, 10 and 20 times and $R_A$ were studied. As shown in Fig. S4, the concentration of the sample leads to a decrease in the $R_A$ due to a progressive increase in the matrix effect. Even when the sample was not concentrated, there was a low $R_A$ for many toxins, which suggests a high matrix effect. Finally, the matrix effect (SSE factor) was calculated using calibration curves constructed at nine levels in solvent and in matrix extract diluted (1/1 v/v) with sample solvent for the four varieties of cheese studied. The SSE factor in the diluted extract was compared with a that observed when the extract was five times concentrated. For most toxins the SSE factor is highly reduced when the extract was diluted (Fig. S5). In the case of AFs, the concentration of blue and brie extracts causes an important increase in the suppression of the signal, while in emmental and camembert low differences are observed. Therefore, the diluted extract was chosen for analysis.

### 3.3. Method validation

The method was validated in terms of linearity, LODs, LOQs, matrix effect, accuracy, and precision. First, calibration curves were constructed in an acetonitrile/water/acetic acid mixture 49/50/1 (v/v/v). This solution had previously been optimized for the simultaneous analysis of several mycotoxins with different polarities (González-Jartín et al., 2019). In this solvent, the linearity of calibration curves constructed at nine levels was acceptable with a regression coefficient (R) higher than 0.995 (Table 1). In addition, repeatability of the analytical method was evaluated within-batch and between-batch. In this sense, a low deviation of the slope of calibration curves was found within- and between-batch with variations, in general, lower than 10 % and 15 %, respectively. Once the performance of the analytical method was verified, the LODs and LOQs were studied. As shown in Table 1, LODs in

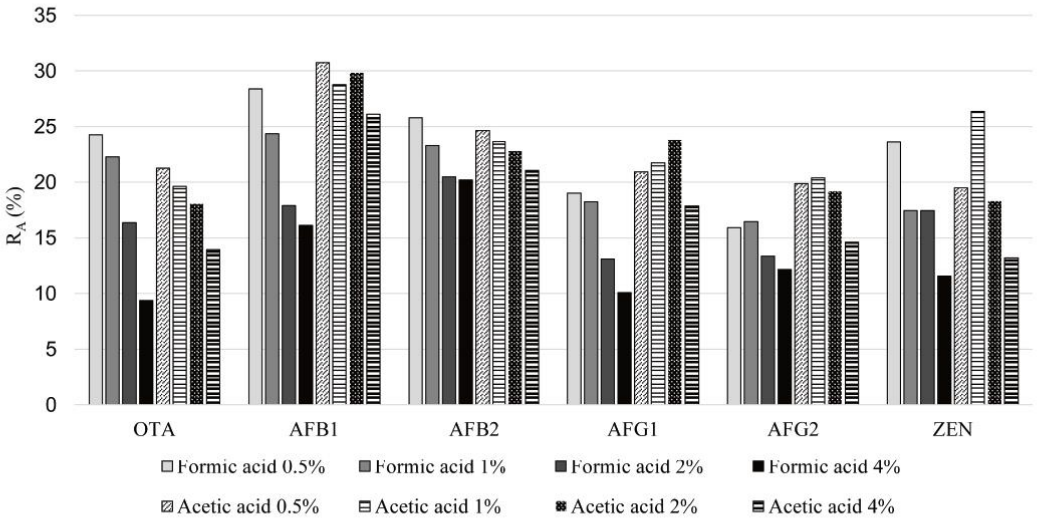



**Fig. 2.** Optimization of extraction process. $R_A$ of OTA, $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$ and ZEN using formic acid or acetic acid at 0.5 %, 1 %, 2 % and 4 % for the extraction procedure.



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**Table 1**
Performance characteristics of the analysis method in solvent. [1]Linear range (ng/mL) are usually of nine levels of concentration for each mycotoxin.

| Mycotoxin | $R^2$ | LODs (ng/ml) | LOQs (ng/ml) | Linear Range[1] ng/mL | Linear equation y = ax + b | Within batch ($RSD_r$ %) | Between-batch ($RSD_R$ %) |
|---|---|---|---|---|---|---|---|
| 15-AcDON | 0.997 | 0.490 | 1.616 | 0.78–200 | y = 3606.67x + 1293.01 | 2.71 | 11.20 |
| 3-AcDON | 0.999 | 0.480 | 1.585 | 0.78–200 | y = 2059.78x-1391.03 | 5.05 | 12.79 |
| $AFB_1$ | 0.998 | 0.004 | 0.012 | 0.01–3 | y = 162230.21x-6188.52 | 4.32 | 16.32 |
| $AFB_2$ | 0.995 | 0.002 | 0.006 | 0.01–3 | y = 119786.81x-4154.80 | 2.06 | 14.35 |
| $AFG_1$ | 0.995 | 0.006 | 0.020 | 0.01–3 | y = 150853.39x-3987 | 2.63 | 15.27 |
| $AFG_2$ | 0.995 | 0.008 | 0.027 | 0.01–3 | y = 38236.76x-1217 | 1.65 | 15.51 |
| $AFM_1$ | 0.999 | 0.007 | 0.023 | 0.01–3 | y = 16674.07x-288.05 | 6.49 | 13.90 |
| AOH | 0.996 | 0.848 | 2.798 | 1.56–200 | y = 832.30x-1204.97 | 10.85 | 18.30 |
| AME | 0.999 | 0.025 | 0.084 | 0.78–200 | y = 5853.46x-3535.69 | 3.81 | 18.54 |
| BEA | 1.000 | 0.190 | 0.628 | 0.19–50 | y = 1382.08x + 673.28 | 9.90 | 14.49 |
| CTA | 0.996 | 0.005 | 0.017 | 0.78–200 | y = 8345.99x-15044.75 | 2.38 | 16.05 |
| CTN | 0.999 | 0.428 | 1.414 | 0.78–200 | y = 1022221.84x + 81636.69 | 2.10 | 11.69 |
| CPA | 0.996 | 0.359 | 1.183 | 0.78–200 | y = 377.45x-1790.46 | 3.83 | 12.99 |
| DAS | 0.997 | 0.069 | 0.227 | 0.78–200 | y = 14229.20x-33995.12 | 3.95 | 12.77 |
| ENNA | 0.998 | 0.074 | 0.243 | 0.19–50 | y = 5664.37x-2508.23 | 7.22 | 9.50 |
| $ENNA_1$ | 0.999 | 0.006 | 0.021 | 0.19–50 | y = 12708.56x-4734.26 | 7.07 | 8.95 |
| ENNB | 1.000 | 0.277 | 0.914 | 0.39–50 | y = 2017.02x-825.68 | 7.88 | 13.00 |
| $ENNB_1$ | 0.998 | 0.033 | 0.110 | 0.19–50 | y = 5062.96x-4242.51 | 9.45 | 13.22 |
| $FB_1$ | 0.999 | 0.793 | 2.618 | 1.56–200 | y = 445.08x-1152 | 5.17 | 12.83 |
| $FB_2$ | 0.996 | 0.028 | 0.094 | 0.78–200 | y = 789.36x-1865.87 | 5.04 | 14.75 |
| GLIO | 1.000 | 2.177 | 7.185 | 3.13–200 | y = 663.67x + 304.44 | 10.83 | 13.00 |
| HT-2 TOXIN | 0.996 | 0.237 | 0.781 | 0.78–200 | y = 2857.95x-5137.20 | 4.51 | 14.65 |
| h-$FB_1$ | 0.998 | 1.108 | 3.655 | 1.56–200 | y = 1735.99–5115.82 | 6.51 | 11.27 |
| MPA | 0.995 | 0.193 | 0.638 | 0.78–200 | y = 10739.48x-24549.54 | 3.73 | 12.34 |
| NEO | 0.997 | 0.088 | 0.291 | 0.78–200 | y = 7172.70x-22088.37 | 2.49 | 13.72 |
| OTA | 1.000 | 0.139 | 0.460 | 0.78–200 | y = 3944.63x-22369.58 | 3.11 | 12.62 |
| ROQC | 0.998 | 0.024 | 0.079 | 0.78–200 | y = 131795.16x + 165756.34 | 1.69 | 13.49 |
| STC | 0.998 | 0.038 | 0.127 | 0.78–200 | y = 22338.87x + 18351.06 | 2.95 | 11.35 |
| T-2 TOXIN | 0.999 | 0.163 | 0.539 | 0.78–200 | y = 7991.51x-16428.10 | 4.79 | 14.48 |
| T2 TRIOL | 0.996 | 0.721 | 2.378 | 0.78–200 | y = 1551.23x-3858.17 | 5.27 | 19.55 |
| ZEN | 0.997 | 0.753 | 2.486 | 0.78–200 | y = 470.18x-942.92 | 8.71 | 16.81 |
| ZOL | 0.997 | 2.923 | 9.646 | 4.69–600 | y = 200.29x-520.49 | 4.65 | 18.75 |

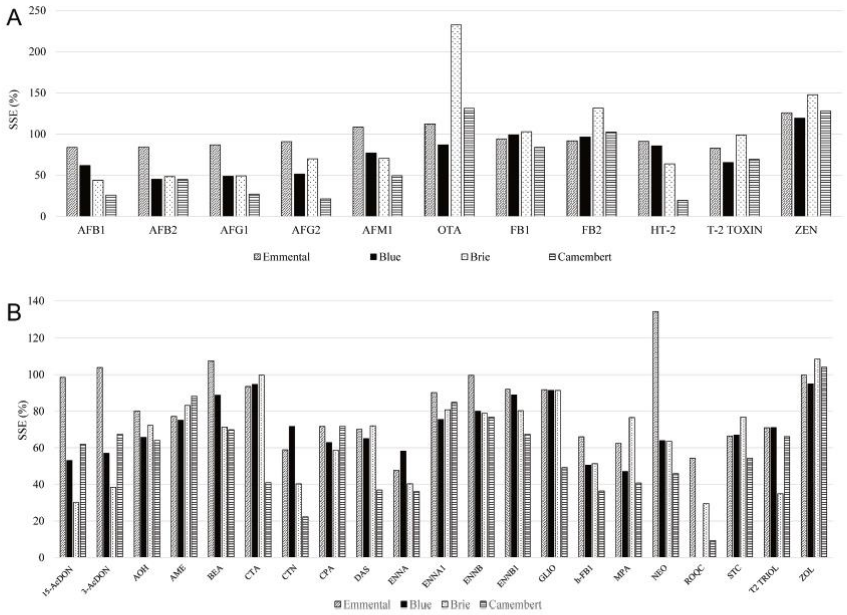



**Fig. 3.** Matrix effect of the analysis method. SSE value of emmental (diagonal stripes columns), blue (dark columns), brie (dotted columns), and camembert (horizontal stripes columns) obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



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solvent were between 0.002 and 2.923 ng/mL and LOQs in solvent were between 0.006 and 9.646 ng/mL, and the method is linear over a wide range of concentrations. After verifying that the performance method in solvent was adequate, they were calculated in matrix for each cheese variety, namely emmental (Table S4), blue (Table S5), brie (Table S6) and camembert (Table S7). The LODs obtained were between 0.020 μg/kg and 5.873 μg/kg. The LOQ, for instance, for $AFM_1$ in emmental cheese was 0.282 μg/kg, 0.244 μg/kg in blue, 0.232 μg/kg in brie and 0.224 μg/kg in camembert. Other authors had reported LOQs values of 0.6 μg/kg for AFM1 in mould cheese, 0.125 μg/kg in white cheese and 0.02 μg/kg in hard cheese (Kokkonen et al., 2005b; Škrbić, Antić, & Živančev, 2015). In the case of OTA, LOQs ranging from 0.723 to 2.272 μg/kg were obtained. Similar LOQs of OTA had been reported in grated cheese, 1 μg/kg (Biancardi, Piro, Galaverna, & Dall'Asta, 2013). However, unlike previous methods, the one proposed in this work allows the simultaneous detection of multiple toxins.

Next, the matrix effect was assessed; this phenomenon, caused by compounds that coelute with the target analyte, may lead to a suppression or enhancement of the mycotoxin signal. To correct the matrix effect, different approaches can be followed including matrix-matched calibration, standard addition or stable isotopically labelled standards (Varga et al., 2012). In this case, the SSE factor was calculated by comparing the slope of the calibration curves constructed in solvent and in matrix. Due to the variations that may exist between different batches of cheese, this factor was determined in triplicate for each cheese (Tables S4-S7). In general, there is an important matrix effect translated into a suppression of the signal for the majority of mycotoxins. As shown in Fig. 3 A, SSE values are around 60–80 % for regulated mycotoxins in emmental and blue cheeses. However, in camembert and brie cheeses there is a higher signal suppression. The mycotoxins less affected by matrix were the FBs, with SSE values from 83.96 % to 131.59 %, while the most affected compound was the HT-2 toxin, whose SSE value varied from 63.83 % in brie to 19.58 % camembert. On the other hand, the matrix barely affects the signal of OTA, although in brie there is an important enhancement of the signal (Fig. 3 A, Table S6). In the case of emerging mycotoxins, it must be highlighted and important signal suppression of ENNs in all cheeses. Similarly, the signal of ROQC is highly suppressed in camembert cheese, in blue cheese it was not possible to calculate this value because samples with low levels of ROQC were not found. In general, the emmental variety was the less affected by matrix. On the other hand, the matrix also causes a broadening of the peak shape of CPA (Fig. S6).

Finally, the precision and accuracy of the analysis method were evaluated. As shown in Fig. 4 A and Tables S4-S7, good $R_E$ values were obtained for regulated mycotoxins, all of them higher than 60 % with RSD values lower than the 15 %. For instance, the $R_E$ of OTA varied from 103 % in blue cheese to 111 % in emmental with RSD values were lower than 20 %. In the case of $AFM_1$, the obtained $R_E$ was between 79 % and 101 %. In Europe, there are no specific methods for the determination of mycotoxins in food. However, the proposed procedure must be in compliance with the legislation. In this sense, the developed method shows recoveries and RSD values which conform to European regulations. In the case of emerging toxins, accuracy values were, in general, above 60 % (Fig. 4 B). In the case of BEA, the $R_E$ was satisfactory only for emmental cheese; similarly, low $R_E$ values were obtained for ENNs in brie and camembert cheese (Fig. 4 B). On the other hand, in the case of blue cheese, it was not possible calculate the accuracy of ROQC because no blank samples were found since this toxin is produced in high concentrations by the fungi used during cheese ripening (Maragos, 2022). However, the $R_E$ of this mycotoxin in the other cheese varieties was adequate ranging from 83 % to 108 % (Fig. 4 B).

Therefore, the developed method allows the detection of 32 mycotoxins in cheese, fulfilling the minimum performance characteristic set in the legislation for regulated mycotoxins (EC_401, 2006). On the other

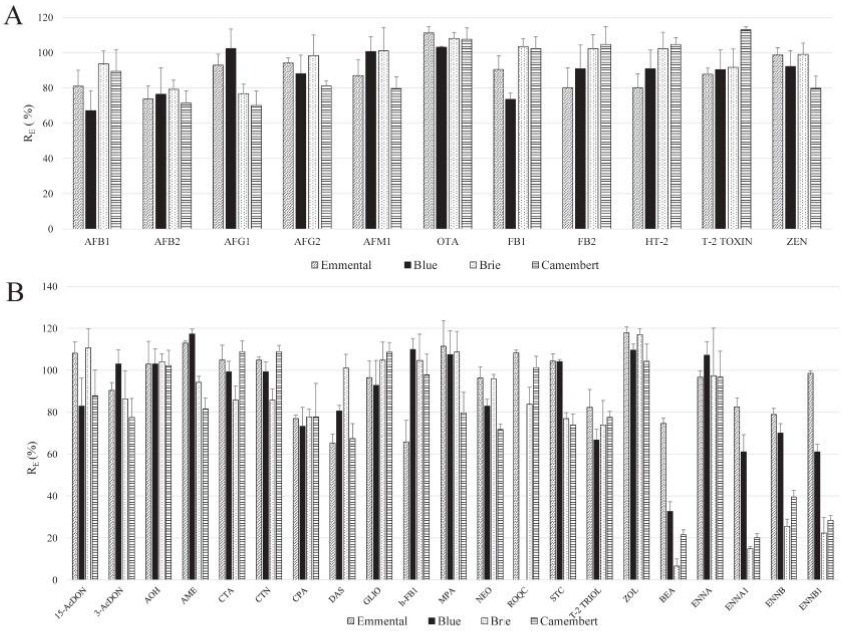



**Fig. 4.** Accuracy of the analysis method. $R_E$ value of emmental (diagonal stripes columns), blue (dark columns), brie (dotted columns), and camembert (horizontal stripes columns) obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



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hand, it represents a clear improvement over currently available methods that are summarized in table S2. An important part of these methods only allows the analysis of one or two mycotoxins at the same time, mainly $AFM_1$, MPA, CPA or CTN, using different techniques such as TLC, HPLC-UV or HPLC-FL. On the other hand, some works using mass spectrometry have been found, most of which are only optimized for the detection of OTA, STC, or CPA. Regarding the methods available for the simultaneous detection of multiple toxins there is a method that allows the quantification of AFs, OTA, MPA, penicillic acid and ROQC in blue and white cheese (Kokkonen, Jestoi, & Rizzo, 2005a). In addition, other method was identified for the analysis of 18 toxins including: MPA, ROQC, chanoclavine, ergometrinine, ergometrine, festuclavine, agroclavine, OTA, STC in cheese (Sulyok, Krska, & Schuhmacher, 2010). Therefore, among the validated methods, the proposed one detects the highest number of toxins, 32 compounds, and it was validated for some of the most important varieties of commercial cheeses. Another advantage of the optimized method is the low LOQ achieved, for instance the LOQ of $AFM_1$ varied from 0.086 μg/kg in emmental to 0.068 μg/kg in camembert, so they are about 10 times lower than the previously proposed method (Kokkonen et al., 2005b).

According to the EU Regulation, milk destined for the manufacture of dairy products, such as cheese, should not contain $AFM_1$ in a concentration higher than 0.05 μg/kg (EC_1881, 2006). However, there is no specific regulation for $AFM_1$ in cheese, although milk is concentrated during cheese manufacturing which can lead to an increase of toxin in the final product. On the other hand, Austria and Turkey stablish the limit of 0.25 μg/kg of $AFM_1$ in cheese (Becker-Algeri et al., 2016). Another mycotoxin whose presence in cheese is causing concern is the OTA (Younis, Ibrahim, Awad, & El Bardisy, 2016). At the moment, in the EU, only Slovakia has set a limit for OTA in cheese, 5 μg/kg (Duarte, Lino, & Pena, 2010). The proposed method allows the quantification of OTA at levels lower than 5 μg/kg, and $AFM_1$ can be quantified at level of 0.25 μg/kg in blue, brie and camembert cheese. However, in order to develop a method capable of detecting mycotoxins at lower levels, a further concentration step was included in the extraction protocol. In order to reduce the LOQs, mycotoxins were extracted with the developed method and analyzed in the extract 5 times concentrated. In this sense, 1 mL of the organic extract was dried using a centrifugal evaporator, then it was reconstituted with 200 μL of the acetonitrile/water/acetic acid mixture 49/50/1 (v/v/v) and filtered by using centrifugal filters of 0.22 μm. Consequently, the whole procedure was evaluated for the analysis of mycotoxins whose probability of being detected in milk is high. In this sense, $AFM_1$ is the most common aflatoxin detected in animal milk (Womack, Sparks, & Brown, 2016). Other mycotoxins have already been detected in this dairy product such as FBs, OTA, ZEN and trichothecenes like DAS, HT-2 toxin and T-2 toxin (Becker-Algeri et al., 2016). Also, AME had recently been reported for first time in animal milk (Akinyemi, Braun, Windisch, Warth, & Ezekiel, 2022). On the other hand, CTN, ROQC and MPA are frequently found in cheeses (Dobson, 2017). Consequently, the performance of the method was evaluated in the extract 5 times concentrated for the analysis of $AFM_1$, $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, AME, CTA, CTN, DAS, $FB_1$, $FB_2$, HT-2 toxin, h-$FB_1$, MPA, OTA, ROQC, STC, T-2 toxin, T-2 triol, ZEN, ZOL, 15-AcDON and 3-AcDON. Using these conditions, LODs, LOQs, SSE values were calculated as detailed before. As shown in Table S8, smaller LODs and LOQs values were achieved for certain mycotoxins in concentrated extract, as in the case of AFs. For instance, the LOQ for $AFM_1$ was reduced to 0.020 μg/kg in emmental, 0.031 μg/kg in blue cheese, 0.036 μg/kg in brie and 0.028 μg/kg in camembert. For some toxins, such as OTA in camembert cheese there are low differences in the LOQ in the concentrated (0.985 μg/kg) and diluted matrix (0.750 μg/kg), while for some compounds the sample concentration increases the LOQ as in the case of MPA in camembert cheese, which was increased from 1.183 to 5.731 μg/kg. The main effect caused by sample concentration was an important increase in the signal suppression. For instance, in the case of $AFM_1$, as shown in Table S8, when the matrix was concentrated, the SSE value decreases from 77.08 % to 43.19 % in blue cheese, this reduction in the analytical signal was more pronounced for compounds like CTN with a decrease from 71.67 % to 16.03 %. The use of a method with a higher matrix effect can lead to a greater error in the accuracy of the method. Therefore, the accuracy of contaminated cheese was studied at 3 different levels (Table S9). As shown in Fig. 5 and Table S10, the $R_E$ in the 3 levels was, in general, between 60 % and 100 %. Although, there are some exceptions, like CTN in the case of brie. On the other hand, RSD values were lower than 20 % (Table S10). Therefore, if necessary, the developed method could be used with a concentration step prior to analysis for the detection of toxins at very low levels. For example, the $AFM_1$ can be detected at 0.05 μg/kg in all cheeses, and therefore it reaches the sensitivity needed for the analysis of this compound at levels allowed in milk.

### 3.4. Application to real samples

A total of 38 cheese samples were analyzed, including 12 samples of emmental cheese, 10 of blue, 9 of brie and 7 of camembert. The results obtained are present in Table S11. BEA was found in all samples, the highest levels were detected in brie cheese, in concentrations ranging from 0.7 μg/kg to 29 μg/kg. A high incidence of ENNs was also found, but these emerging compounds were detected in low amounts. These mycotoxins had already been found in milk, and therefore a carry-over to cheese may occur (González-Jartín, Rodríguez-Cañás, et al., 2021). All the blue samples contained ROQC in concentrations higher than 250 μg/kg, although these results were not corrected for the recovery nor the matrix effect since no blank samples were found to calculate these parameters. In camembert cheese, this mycotoxin was detected in a concentration ranging from 3 μg/kg to 189 μg/kg. Although there are no reports about human health problems related to this mycotoxin, their toxicity is still not well characterized (Maragos, 2022). CTN was found in four samples, the maximum amount was detected in a blue cheese sample, with a concentration of 71 μg/kg. This mycotoxin had already been reported in cheese (EFSA, 2012). OTA was detected in brie, blue, and camembert cheeses in concentration ranging for 4.8 μg/kg to 9.6 μg/kg, respectively. Maximum levels of OTA have not been stablished in cheese or other dairy products, but the EFSA has requested more data on the presence of OTA in cheese to carry out a risk assess since high amounts of this mycotoxin were detected in traditional semi-hard cheeses (EFSA, 2020). In this sense, the concentrations found in the samples are similar to the maximum amount of OTA allowed in coffee (5 μg/kg), and much higher than those allowed for processed cereal-based foods and baby foods for infants and young children (0.5 μg/kg) (EC_1881, 2006). CPA was founded in three varieties of cheeses. The highest level (810 μg/kg) was in a brie sample, this mycotoxin was reported with a similar contamination level in taleggio cheese (Finoli, Vecchio, Galli, & Franzetti, 1999).

## 4. Conclusion

A new method was developed to quantify regulated, emerging and modified mycotoxins in cheese. The method, based on a QuEChERS extraction and UHPLC-MS/MS detection, allows the simultaneous detection of 32 mycotoxins in four varieties of cheese (emmental, blue, brie and camembert). Although the method has a large matrix effect, good accuracy and precision results have been obtained for most of the toxins in four commercial varieties of cheese. In addition, it significantly increases the number of mycotoxins that can be detected in a single analysis and improves the quantification limits of previously published methods. It was found a high occurrence of the emerging mycotoxins in samples obtained from local supermarkets; in addition, our data suggest that the presence of OTA in cheese may pose a risk to human health.



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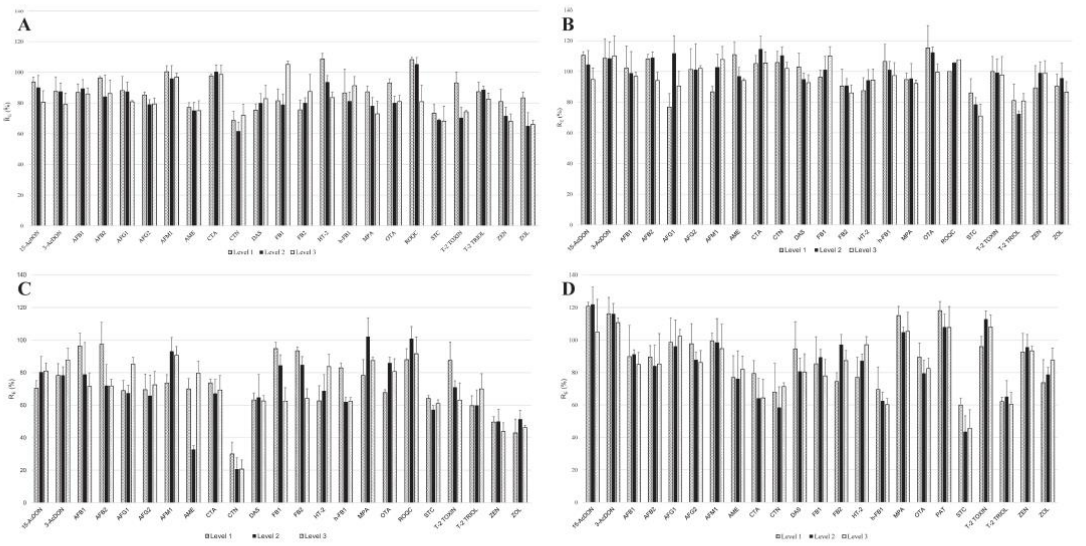



**Fig. 5.** Accuracy from the concentrated extract. $R_g$ value calculated in the five times concentrated extract for three contamination levels in emmental (A), blue, (B) brie (C) and camembert (D). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

## CRediT authorship contribution statement

**Inés Rodríguez-Cañás:** Investigation, Writing – original draft. **Jesús M. González-Jartín:** Investigation, Writing – review & editing. **Rebeca Alvariño:** Investigation. **Amparo Alfonso:** Methodology, Writing – review & editing. **Mercedes R. Vieytes:** Methodology. **Luis M. Botana:** Funding acquisition, Supervision.

## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.135182.

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**4. Identification of mycotoxins in yogurt samples using an optimized QuEChERS extraction and UHPLC-MS/MS detection**

**Resumen:**
El yogur, un producto derivado de la leche, es susceptible a la contaminación por micotoxinas. Aunque se han desarrollado varios métodos para el análisis de productos lácteos, solo unos pocos han sido específicamente validados para el yogur. Además, estos métodos se centran principalmente en la detección de aflatoxinas y zearalenona. Este estudio tuvo como objetivo realizar una investigación preliminar sobre la presencia de micotoxinas reguladas, emergentes y modificadas en yogures naturales y de avena disponibles en el mercado español. Para ello, se optimizó y validó un método de extracción basado en QuEChERS para detectar y cuantificar 32 micotoxinas utilizando cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas en tándem (UHPLC-MS/MS). El método fue validado internamente para el análisis de yogur natural y de avena en términos de linealidad, efecto matriz, sensibilidad, exactitud y precisión. Se lograron características de rendimiento satisfactorias; para la mayoría de los analitos, los LOD fueron inferiores a 2 ng/g, y las recuperaciones oscilaron entre el 60 % y el 110 %, con una precisión, expresada como la desviación estándar relativa de la recuperación, inferior al 15 %. Posteriormente, se aplicó el método validado para analizar muestras de yogur comercial, revelando una notable incidencia de beauvericina y eniatinas, con algunos análogos presentes en hasta el 100 % de las muestras. El alternariol metil éter también se encontró con frecuencia, apareciendo en el 50 % de las muestras. Además, el estudio identificó toxinas reguladas como fumonisinas, ocratoxina A y toxina HT-2. Estos resultados proporcionan nuevos datos de incidencia en el yogur, generando preocupaciones sobre los posibles riesgos para la salud de los consumidores.

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**Identification of mycotoxins in yogurt samples using an optimized QuEChERS extraction and UHPLC-MS/MS detection**

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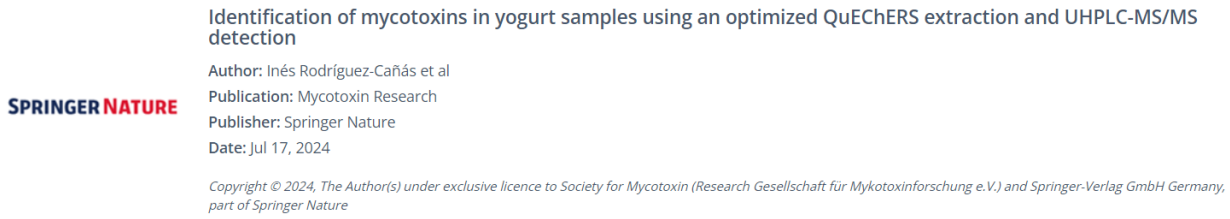
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RESEARCH

# Identification of mycotoxins in yogurt samples using an optimized QuEChERS extraction and UHPLC-MS/MS detection

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## Abstract

Yogurt, a milk-derived product, is susceptible to mycotoxin contamination. While various methods have been developed for the analysis of dairy products, only a few have been specifically validated for yogurt. In addition, these methods are primarily focus on detecting aflatoxins and zearalenone. This study aimed to conduct a preliminary investigation into the presence of regulated, emerging, and modified mycotoxins in natural and oat yogurts available in the Spanish market. For this, a QuEChERS-based extraction method was optimized and then validated to detect and quantify 32 mycotoxins using ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). The method was in-house validated for the analysis of natural and oat yogurt in terms of linearity, matrix effect, sensitivity, accuracy, and precision. Satisfactory performance characteristics were achieved; for most of the analytes, LOQs were lower than 2 ng/g, and recoveries ranged from 60 to 110% with a precision, expressed as the relative standard deviation of the recovery, lower than 15%. Subsequently, the validated method was applied to analyze commercial yogurt samples, revealing a notable incidence of beauvericin and enniatins, with some analogues found in up to 100% of the samples. Alternariol methyl ether was also frequently found, appearing in 50% of the samples. Additionally, the study identified regulated toxins such as fumonisins, ochratoxin A , and HT-2 toxin. These results provide new incidence data in yogurt, raising concerns about potential health risks for consumers.

**Keywords** Matrix effect · Mycotoxin · UHPLC-MS/MS · Yogurt · Validation

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## Introduction

Yogurt, also spelled yoghurt, is a popular food around the world since it provides many essential nutrients. Crafted through a fermentation process facilitated by lactic acid bacteria, it has been related to the prevention of gut diseases and the reduction in cholesterol levels. In addition, it is possible to fortify it with bioactive functional ingredients such as fruits, cereals, aloe vera extracts, or essential oils (Ahmad et al. 2022).

Milk is the main ingredient in yogurt; to avoid microbiological risks, raw milk undergoes a heat treatment which destroys all pathogenic microorganisms and deactivates fungal spores. However, there are contaminants such as mycotoxins that are not altered at pasteurization temperatures and therefore resist this process (Ulusoy et al. 2022; Weerathilake et al. 2014). In this sense, it has been observed that the raw milk presents some emerging mycotoxins such as beauvericin (BEA) or enniatins (ENNs) (Gonzalez-Jartin

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et al. 2021). Other toxins detected in milk include aflatoxins (AFs), zearalenone (ZEN), ochratoxin A (OTA), fumonisin $B_1$ ($FB_1$), and fumonisin $B_2$ ($FB_2$) (Flores-Flores et al. 2015; Leite et al. 2023). Furthermore, dairy products like cheese may present additional toxins coming from contamination during the manufacturing process such as roquefortine C (ROQC), citrinin (CTN), sterigmatocystin (STC), or mycophenolic acid (MPA) (Benkerroum 2016).

In the near future, an upswing in mycotoxin incidence is predictable, driven by the temperature rise associated with climate change (Chhaya et al. 2022). This shift may lead to the emergence of mycotoxins in new regions, exemplified by the expected appearance of aflatoxin $B_1$ ($AFB_1$) in Northern Europe (Battilani et al. 2016). This is especially concerning as AFs have been identified by the International Agency for Research on Cancer as human carcinogens (IARC 2002). The $AFB_1$ and other mycotoxins can cross the intestinal barrier and migrate into tissues and/or milk. After ingestion, the $AFB_1$ is hydroxylated by the animal metabolism, leading to aflatoxin $M_1$ ($AFM_1$) that is excreted in milk (Becker-Algeri et al. 2016).

To avoid toxic effects in humans, dairy products must be analyzed, and several analytical techniques can be employed. The $AFM_1$ can be detected by immunoassay such as enzyme-linked immunosorbent assay (ELISA) kits. A recent study used this technique to analyze 72 yogurts; the $AFM_1$ was detected in two. However, ELISA present some disadvantages such as low sensitivity and low specificity which can lead to inaccurate results (Cano-Sancho et al. 2010; Iqbal 2021; Matabaro et al. 2017).

The application of chromatographic techniques, combined either with fluorescence detection (FLD) or tandem mass spectrometry (MS/MS), offers remarkable selectivity and sensitivity. These methods are able to detect mycotoxins at very low levels and have become the most common way for confirmatory analysis (Shanakhat et al. 2018). For instance, a method based on high-pressure liquid chromatography (HPLC) coupled to photo-induced fluorescence detection (FLD) was developed to determine AFs in yogurt (Hamed et al. 2017). Similarly, a gas chromatography-MS/MS method was validated to detect ZEN and their modified forms in dairy products such as yogurt (D'Orazio et al. 2016). Nevertheless, the most widely employed technique is LC-MS/MS, as it enables the simultaneous determination of multiple mycotoxins in complex matrices. This makes it a highly suitable methodology, especially considering the potential coexistence of more than one mycotoxin in the same sample (González-Jartín et al. 2022).

The current study used ultra-high-performance liquid chromatography (UHPLC) coupled with an MS/MS method to explore the presence of mycotoxins in natural and oat yogurt. To detect the most relevant mycotoxins at trace levels, a swift extraction process was optimized aimed at diminishing the matrix effect and enabling the simultaneous recovery of mycotoxins from samples in a single step.

## Materials and methods

### Chemicals and reagents

Yogurt samples were purchased from local supermarkets. The natural yogurt samples have a fat content ranging between 2.5 and 3%, whereas the oat yogurt samples, comprising whole oats (1.5%) and walnuts (1%), exhibited a fat content between 3.9 and 4%. Pure water was obtained from a Millipore Milli-Q Plus system (Millipore, USA). Methanol, acetonitrile, acetic acid glacial 100%, anhydrous magnesium sulfate ($MgSO_4$), and sodium chloride (NaCl) were purchased from Panreac Química S.A. (Barcelona, Spain). Formic acid was from Merck (Madrid, Spain), while ammonium formate was from Fluka (Buchs, Switzerland). Additionally, ultrafree-MC Durapore membrane centrifugal filters with a pore size of 0.22 µm were supplied by Millipore (Billerica, USA).

The analytical standards, including $AFB_1$, aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$), aflatoxin $G_2$ ($AFG_2$), $AFM_1$, gliotoxin (GLIO), ROQC, enniatin A (ENNA), enniatin $A_1$ ($ENNA_1$), enniatin B (ENNB), enniatin $B_1$ ($ENNB_1$), and ZEN were acquired from Sigma (Madrid, Spain). OTA was from Laboratorios CIFGA S.A. (Lugo, Spain), circumdatin A (CTA) from Santa Cruz Biotechnology (Santa Cruz, CA), and beauvericin (BEA) from Enzo (Barcelona, Spain). The rest of the analytical standards used in this study were provided by Romer Labs (Tulln, Austria), including deoxynivalenol (DON), 3-acetyldeoxynivalenol (3 Ac-DON), 15-acetyldeoxynivalenol (15-AcDON), fusarenon X (FX), diacetoxyscirpenol (DAS), HT-2 toxin (HT-2), T-2, alternariol (AOH), alternariol methyl ether (AME), CPA, CTN, $FB_1$, $FB_2$, hydrolyzed fumonisin $B_1$ (h-$FB_1$), MPA, neosolaniol (NEO), STC, and zearalanone (ZAN).

### Validation process

Limits of detection (LODs) and limits of quantification (LOQs) were calculated following the guidance of the European Union Reference Laboratory (EU-RL) (Wenzl 2016). The LOD for each compound was calculated by applying the following equation: $LOD = \frac{SD}{m} \times 3.9$ where $SD$ was the standard deviation of 10 measurements of blank samples (samples with toxin levels lower than the LOD) and $m$ was the slope of the curve of calibration. The LOQ was calculated with the equation $LOQ = LOD \times 3.3$

The effect of the matrix on the analytical signal was assessed by using the signal suppression/enhancement (SSE) factor according to the following equation:



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$SSE(\%) = \frac{slope\ calibration\ curve\ in\ matrix}{slope\ calibration\ curve\ in\ solvent} \times 100$. In this sense, calibration curves were constructed in solvent and in blank matrix extracts, and their slopes were compared. The calibration levels ranged from 0.01 to 3 ng/mL in the case of AFs, from 0.78 to 200 ng/mL for $FB_1$, $FB_2$, HT-2, T-2, DON, OTA, ZEN, GLIO, MPA, AOH, AME, NEO, and STC; from 0.78 to 100 ng/mL for 15-AcDON, 3-AcDON, BEA, ENNA, $ENNA_1$, ENNB, $ENNB_1$, and h-$FB_1$; from 1.56 to 200 ng/mL for CTN; and from 1.95 to 500 for ZAN. To evaluate the linearity, the correlation coefficient ($r^2$) was determined using a linear regression model.

Accuracy and precision were assessed by calculating the average and relative standard deviation (RSD) of the recovery. To evaluate these parameters, blank samples were spiked with two different amounts of mycotoxins. The concentrations were selected based on the LOQs of each compound. For BEA and ENNs, samples were spiked at concentrations of 5 ng/g and 10 ng/g. For AFs, concentrations of 0.15 ng/g and 0.3 ng/g were utilized. ZAN was spiked at concentrations of 20 ng/g and 40 ng/g, while, for other mycotoxins, samples were contaminated at 10 ng/g and 20 ng/g.

First, the apparent recovery ($R_A$) (%) was calculated, and analytes were quantified by using solvent-based calibration curves. In this sense, the observed value of each toxin was divided by the spiked level and multiplied by 100. The RSD of the $R_A$ values obtained for each compound were determined. In this way, intra-day precision was calculated using the RSD values obtained when the recovery was calculated in triplicate ($n = 3$) for samples analyzed on the same day, while inter-day precision was determined using samples analyzed on 3 consecutive days. Finally, the SSE factor was applied to the $R_A$ obtaining the effective recovery ($R_E$%) by using the following equation: $R_E\% = \frac{R_A\%}{SSE} \times 100$.

### Sample analysis

Yogurt samples underwent homogenization, followed by the weighing of a 5 g portion into a 50-mL Falcon tube. Next, 10 mL of a 2% acetic acid solution was introduced and subjected to a 5-min mixing at 2500 rpm using a multi-vortex. Subsequently, 10 mL of acetonitrile were added, and the same mixing process was employed. A liquid-liquid partition was achieved by adding 4 g of $MgSO_4$ and 2 g of NaCl and a 30-s mixing in a vortex. Finally, samples were centrifuged for 10 min at $3134 \times g$, and a 1000 µL aliquot of the supernatant was evaporated to dryness and then reconstituted with 500 µL of acetonitrile/water/acetic acid (49/50/1 v/v/v). This reconstitution involved vortex mixing for 30 s followed by sonication for 1 min. Finally, the extract was filtered through a 0.22-µm centrifugal filter for 5 min at 10,000 rpm in preparation for analysis.

An Agilent 1290 Infinity UHPLC system coupled to a triple quadrupole mass spectrometer (Agilent 6460) equipped with an Agilent Jet Stream ESI source (Agilent Technologies, Waldbronn, Germany) was employed for UHPLC-MS/MS detection. The analysis was carried out in positive and negative modes and dynamic multiple reaction monitoring (dMRM) acquisition. Chromatographic and mass spectrometric conditions (Tables S1 and S2) have been previously optimized (Gonzalez-Jartin, et al. 2021; Rodríguez-Cañás et al. 2024).

## Results and discussion

Yogurt stands as a globally consumed dairy product, with approximately 6% of the populace including it in its diet daily (Fisberg and Machado 2015). A potential concern associated with this product is the presence of mycotoxins from milk. In addition, this product is added with ingredients that can also contain mycotoxins such as fruits or cereals (Rodríguez-Cañás et al. 2024). Nevertheless, the detection of these contaminants in yogurt remains a challenge, resulting in limited available data regarding the occurrence of mycotoxins in this dairy product. Therefore, a new method based on a QuEChERS extraction process was optimized for the recovery of mycotoxins in natural and oat yogurt. The method was validated for the detection of toxins reported in both milk, such as $AFM_1$, and oat, including trichothecenes, fumonisins, AFs, and OTA (Jia et al. 2014; Tarazona et al. 2021).

### Optimization of extraction conditions

A successful analytical method hinges on an efficient extraction procedure. Hence, it is imperative to develop methods with optimal recoveries for all targeted mycotoxins. In this context, QuEChERS protocols have proven effective in analyzing complex matrices. Nevertheless, the extraction process involves several critical steps that significantly impact the attainment of successful recoveries.

The nature of the sample and the analytes play a fundamental role in the choice of solvents. The first step consists of moistening the sample, which is why extractions are initially carried out using water, and then an organic solvent must be added to recover the lipophilic compounds (Perestrelo et al. 2019). The water employed for moistening is usually acidified, which increases the recovery of some toxins. Therefore, the first step was to evaluate the acid conditions since it was previously observed that both the nature of the acid and its concentration can affect the extraction efficiency (Rodríguez-Cañás et al. 2023). In this sense, 5 g of yogurt were extracted with 10 mL of water containing 0.5%, 1%, 2%, and 3% acetic acid or formic acid by



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mixing at 2500 rpm for 5 min (multi-tube vortex, Nahita Blue, Auxilab, Spain). The extraction protocol was followed by adding 10 mL of acetonitrile and, after vortex mixing, 4 g of $MgSO_4$ and 1 g of NaCl were added to induce phase separation. Finally, 1 mL of the supernatant was evaporated to dryness and reconstituted with 200 µL of acetonitrile/water/acetic acid (49/50/1 v/v/v). The extracts were filtered through 0.22 µm and analyzed by UHPLC-MS/MS. For each condition, a yogurt sample ($n = 1$) had been spiked before extraction with 5 ng/g of DON, OTA, T-2, ZEN; 2.5 ng/g of BEA and ENNs; and 0.075 ng/g of AFs. The comparison of the $R_A$ shows that, although there are no important differences, acetic acid yields higher recoveries (Fig. S1). Within the conditions tested using this acid, it was decided to use a 2% concentration since it shows a slight increase in the signal for ENNs, which have previously given low recoveries in dairy products such as cheese (Rodríguez-Cañás et al. 2023).

The next step was to test different volumes of water and acetonitrile. The $R_A$ (%) was evaluated ($n = 1$) using the ratios (v:v) 1:1, 2:1, 3:1, and 1:2. The utilization of higher acetonitrile-to-water ratios (10 mL of acetonitrile vs 5 mL of water) for AFs led to increased recoveries (Fig. S2). This trend was also observed for regulated toxins such as DON, OTA, T-2 toxin, and ZEN. On the contrary, for ENNs and BEA, the recovery was enhanced with higher water proportions. Due to the lipophilic nature and low solubility of these mycotoxins in water, it is plausible that the role of water was moistening the matrix, facilitating the subsequent penetration of acetonitrile. Based on the findings, extraction using 10 mL water and 10 mL acetonitrile emerges as the compromise choice for the extraction of all toxins.

Next, a separation between aqueous and organic phases must be induced. For this procedure, it is commonly employed the addition of anhydrous $MgSO_4$ and NaCl. It is known that NaCl forces polar compounds to go into the organic phase, while $MgSO_4$ induces phase partitioning (Santana-Mayor et al. 2019). Therefore, the ratio of salts may have a remarkable influence on the recovery; three tests with different proportions of salts were carried out to evaluate it. In all cases, 4 g of $MgSO_4$ was added with 1, 1.5, or 2 g of NaCl; the $R_A$ (%) was evaluated by duplicate ($n = 2$); and results are shown in Fig. 1. In the case of lipophilic compounds as BEA and ENNs, no differences were observed; however, this parameter shows an important effect in the $R_A$ (%) of hydrophilic compounds as AFs. For instance, the $R_A$% for $AFM_1$ was increased from 23 to 34%, when the amount of NaCl was doubled. Given this, the extraction was carried out by using 4 g of $MgSO_4$ and 2 g of NaCl.

The final step in the optimization process involved the study of the most suitable concentration for the extract. Analyzing a concentrated extract allows the detection of low toxin amounts, but it also carries the risk of increasing the matrix effect due to an increase in the background noise, resulting in low improvements or even a decrease in overall

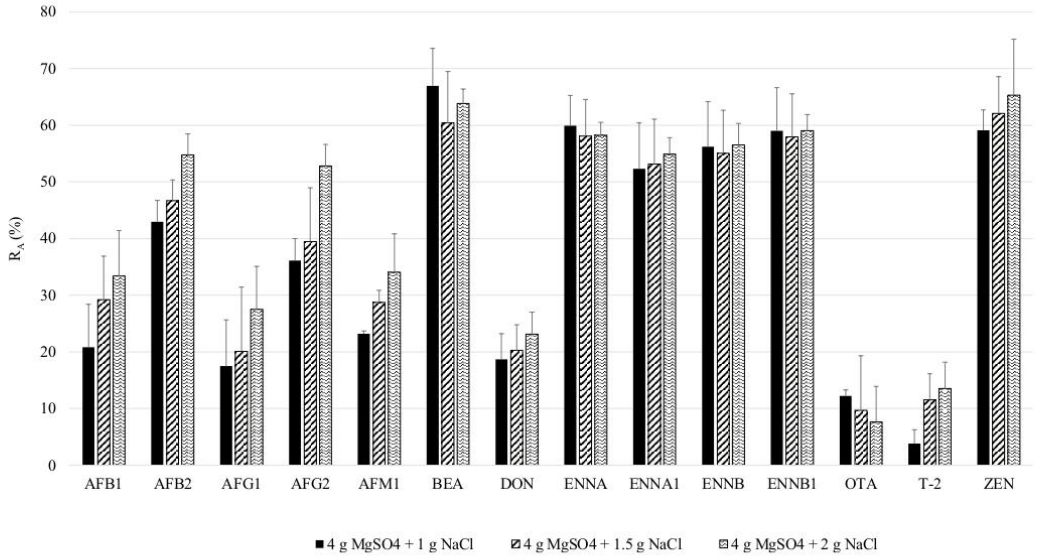



**Fig. 1** Optimization of extraction process. $R_A$% obtained by applying a QuEChERS-based extraction using 4 g of MgSO4 combined with 1, 1.5, and 2 g of NaCl



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sensitivity (Keevil 2013). One approach to maximize the sensitivity of the method is to evaluate the effect of preconcentration or dilution of the extract. The results usually vary greatly according to the mycotoxin and the matrix (Castilla-Fernández et al. 2022). Therefore, to assess this parameter, samples were spiked and extracted as it was previously explained. Finally, 1 mL of the extract was evaporated to dryness, and the residue was reconstituted with 0.5, 0.2, 0.1, or 0.05 mL of acetonitrile/water/acetic acid (49/50/1 v/v/v). Therefore, the sample was analyzed at 0, 2, 5, 10, 15, and 20 times concentrated ($n = 1$), and the $R_A$% was checked. When the extract is concentrated beyond 10 times, notably low $R_A$% was observed, particularly for AFs (Fig. S3). This signal reduction may be attributed to a substantial increase in the matrix effect. Accordingly, when the sample was not concentrated, a signal enhancement of AFs was observed, with recoveries exceeding 100%. Finally, a five-time concentrated sample was chosen for analysis as it strikes a balance between heightened sensitivity and the adverse effects of the matrix effect.

## Method validation

There are no available certified reference materials to validate the analysis of mycotoxins in yogurt. Therefore, the method was in-house validated by using artificially contaminated samples. This approach allowed us to study the performance characteristics of the method.

The LOQs ranged between 0.002 ng/g and 13.78 ng/g (Table 1). The $AFM_1$ is regulated by European Union in milk, with a maximum permitted level of 0.05 ng/g (EC_2023/915). However, there is data about its presence

**Table 1** Limits of quantification of the analysis method in natural and oat yogurt

| Analyte | Natural yogurt | | | Oat yogurt | | |
|---|---|---|---|---|---|---|
| | $r^2$ | LOD (ng/g) | LOQ (ng/g) | $r^2$ | LOD (ng/g) | LOQ (ng/g) |
| **15-AcDON** | 0.999 | 0.24 | 0.78 | 0.999 | 0.42 | 1.39 |
| **3-AcDON** | 0.996 | 0.35 | 1.16 | 0.999 | 0.52 | 1.73 |
| **$AFB_1$** | 0.998 | 0.003 | 0.01 | 0.999 | 0.003 | 0.01 |
| **$AFB_2$** | 0.997 | 0.009 | 0.03 | 0.999 | 0.024 | 0.08 |
| **$AFG_1$** | 0.997 | 0.009 | 0.03 | 0.999 | 0.024 | 0.08 |
| **$AFG_2$** | 0.998 | 0.024 | 0.08 | 0.998 | 0.009 | 0.03 |
| **$AFM_1$** | 0.999 | 0.002 | 0.01 | 0.999 | 0.002 | 0.01 |
| **AOH** | 0.999 | 2.39 | 7.89 | 0.998 | 0.74 | 2.43 |
| **AME** | 0.999 | 0.20 | 0.67 | 0.999 | 0.29 | 0.96 |
| **BEA** | 0.999 | 0.02 | 0.07 | 0.999 | 0.07 | 0.22 |
| **CTN** | 0.997 | 1.04 | 3.42 | 0.997 | 0.62 | 2.04 |
| **DAS** | 0.997 | 0.05 | 0.18 | 0.999 | 0.04 | 0.13 |
| **DON** | 0.998 | 0.66 | 2.18 | 0.999 | 0.32 | 1.07 |
| **DOM-1** | 0.999 | 2.68 | 8.86 | 0.999 | 1.65 | 5.44 |
| **ENNA** | 0.998 | 0.02 | 0.05 | 0.999 | 0.16 | 0.52 |
| **$ENNA_1$** | 0.999 | 0.01 | 0.02 | 0.999 | 0.06 | 0.21 |
| **ENNB** | 0.999 | 0.01 | 0.04 | 0.996 | 0.16 | 0.54 |
| **$ENNB_1$** | 0.999 | 0.01 | 0.04 | 0.997 | 0.09 | 0.31 |
| **$FB_1$** | 0.999 | 0.08 | 0.28 | 0.998 | 0.18 | 0.61 |
| **$FB_2$** | 0.995 | 0.36 | 1.19 | 0.997 | 0.48 | 1.58 |
| **FX** | 0.999 | 1.80 | 5.93 | 0.999 | 4.18 | 13.78 |
| **GLIO** | 0.999 | 0.42 | 1.39 | 0.998 | 0.11 | 0.36 |
| **HT-2** | 0.999 | 0.43 | 1.42 | 0.999 | 0.46 | 1.52 |
| **h-FB1** | 0.999 | 0.40 | 1.33 | 0.999 | 0.08 | 0.26 |
| **MPA** | 0.999 | 0.03 | 0.10 | 0.999 | 0.02 | 0.07 |
| **NEO** | 0.999 | 0.27 | 0.90 | 0.999 | 0.78 | 2.57 |
| **OTA** | 0.999 | 0.07 | 0.22 | 0.999 | 0.24 | 0.80 |
| **ROQC** | 0.999 | 0.04 | 0.12 | - | - | - |
| **STC** | 0.999 | 0.13 | 0.43 | 0.999 | 0.15 | 0.50 |
| **T-2** | 0.999 | 0.08 | 0.28 | 0.999 | 0.04 | 0.13 |
| **ZEN** | 0.999 | 0.61 | 2.02 | 0.999 | 0.96 | 3.16 |
| **ZAN** | 0.999 | 0.27 | 0.88 | 0.999 | 0.33 | 1.08 |



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in dairy products such as yogurt at lower concentrations, namely 0.02 and 0.005 ng/g (Jia et al. 2014). The proposed method allows the detection of these levels since the LOD was 0.002 ng/g, while the LOQs were 0.007 and 0.008 ng/g for natural and oat yogurt, respectively; therefore, these limits are adequate to quantify the mycotoxin at the maximum permitted level (EC_2023/915). In the case of the ROQC, it was not possible to calculate the quantification limit in oat yogurt because no blank samples were available.

Several approaches can be used to correct matrix effect including matrix-matched calibration, standard addition, or stable isotopically labeled standards. For instance, isotope-labeled mycotoxins were employed to analyze dairy products (Yang et al. 2021; Zhang et al. 2013). However, the number of toxins for which this kind of standards are available is low, manly regulated mycotoxins. On the other hand, since BEA and ENNs seem to be ubiquitous contaminants, it is difficult to obtain blank matrices to apply matrix-matched calibration (Akinyemi et al. 2022; Pietruszka et al. 2023). Therefore, in this work, the impact of the matrix on the analytical signal was assessed by using the SSE factor. Figure 2 displays the SSE values for each mycotoxin in both natural and oat yogurt; these values were comparable in both matrices. For example, in the case of $AFM_1$, the signal was diminished by 40% in both matrices, aligning with findings reported in dairy products (Xie et al. 2015). However, notable differences were observed for other compounds such as CTN, HT-2, and OTA. Specifically, OTA exhibited a 23% signal reduction in oat yogurt, while in natural yogurt, the analytical signal was suppressed by almost 50%. The less affected compounds were ENNs and BEA, with SSE values varying from 83 to 96%. These emerging mycotoxins had shown a similar behavior in milk (Gonzalez-Jartin, et al. 2021).

Finally, accuracy and precision were evaluated. The accuracy was adequate since, in general, recoveries higher than 70% were obtained for most of target compounds (Fig. 3). In natural yogurt, recoveries ranged from 61 (DON) to 112% (AOH) and from 60 ($AFG_1$) to 120% (ZEN) in oat yogurt. For instance, the $AFM_1$ shows satisfactory recovery in both matrices (81–110%). Error bars in Fig. 3 show the RSD values (intra-day precision) obtained for the different mycotoxins, which are lower than 15%, while the inter-day precision shows RSD values lower than 20% (Table S3). Therefore, this method conforms to the minimum performance characteristic that methods should meet for the analysis of regulated mycotoxins according to European legislation (EC_2023/2782). Emerging mycotoxins such as BEA and ENNs also shows good recoveries, with values ranging from 62 to 97%. The lower fat content in yogurt may account for the reduced matrix effect seen in lipophilic compounds such as ENNs compared to cheese, leading to more accurate results (Rodríguez-Cañás et al. 2023). The yogurts used in this study presented a fat content ranging between 2.5 and 4%. Considering that yogurts with higher fat contents are available, it is necessary to evaluate the method's suitability through SSE and recovery studies before its application to that kind of matrices.

Several methods have been developed for the analysis of dairy products; however, most of them are only validated for milk. For example, a method using QuEChERS extraction followed by UHPLC—Orbitrap—detection was previously proposed for analyzing 58 toxins in dairy products, although the validation was conducted in milk (Jia et al. 2014). Similarly, good performance was achieved by using an extraction method involving a stable isotope dilution assay followed by LC–MS/MS (Yang et al. 2021). Regarding methods specifically developed for yogurt analysis, ELISA technique has been utilized for the detection of $AFM_1$ in yogurt. However, complete validations are not available. The LOD reported for this technique is approximately 0.02 µg/kg (LOQ) (Cano-Sancho et al. 2010; Mason et al. 2015). Therefore, their sensitivity is improved by the proposed method which is 0.008 µg/kg. The detection limits for other AFs (ranging from 0.01 to 0.08 µg/kg) are also lower compared to those achieved by a method based on dispersive liquid-liquid microextraction (DLLME) coupled to HPLC-FLD (ranging from 0.05 to 0.18 µg/kg). The other two methods developed for yogurt analysis are focused on detecting ZEN and its modified forms, with LOQs ranging from 0.9 to 15 µg/kg (D'Orazio et al. 2016; González-Sálamo et al. 2017). The proposed method was validated for the analysis of ZEN and ZAN; however, it did not include the main metabolites from ZEN and alpha- and beta-zearalenol (ZOL). This omission could pose a limitation for the method, as these compounds have been sporadically reported in milk (Prelusky et al. 1990). Therefore, while various methods have been developed for analyzing dairy products, only a few have undergone specific validation for yogurt. Moreover, those focused on this matrix were validated for AFs and ZEN (Table S4). In contrast, the proposed method enables the detection of a broader range of mycotoxins across different classes while maintaining or enhancing sensitivity and accuracy. Additionally, the validation process has been successfully conducted for both natural and oat yogurt samples, yielding comparable results, thus indicating its adaptability to other types of fortified yogurts.

## Analysis of commercial samples

Dairy products are prone to mycotoxin contamination, primarily through the carryover of contaminants from animal feed to milk. Additionally, there is a risk of direct contamination by fungi (Becker-Algeri et al. 2016). On the other hand, it is known that oats are susceptible to contamination with several mycotoxins; therefore, their inclusion in



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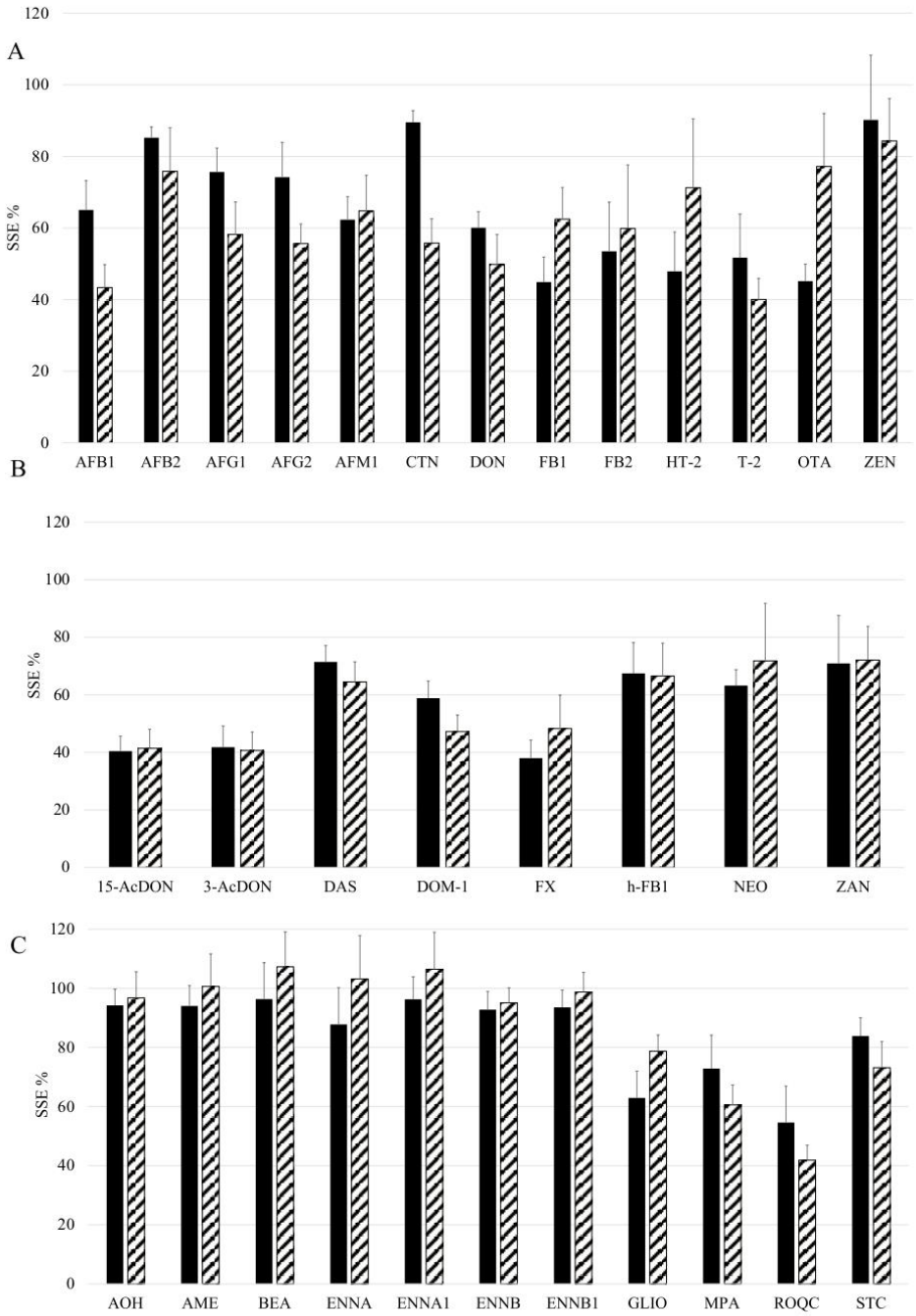



**Fig. 2** Matrix effect of mycotoxins in yogurt. SSE values in natural yogurt (dark columns) and in oat yogurt (diagonal stripes columns). Results for regulated **A**, modified **B**, and emerging **C** toxins



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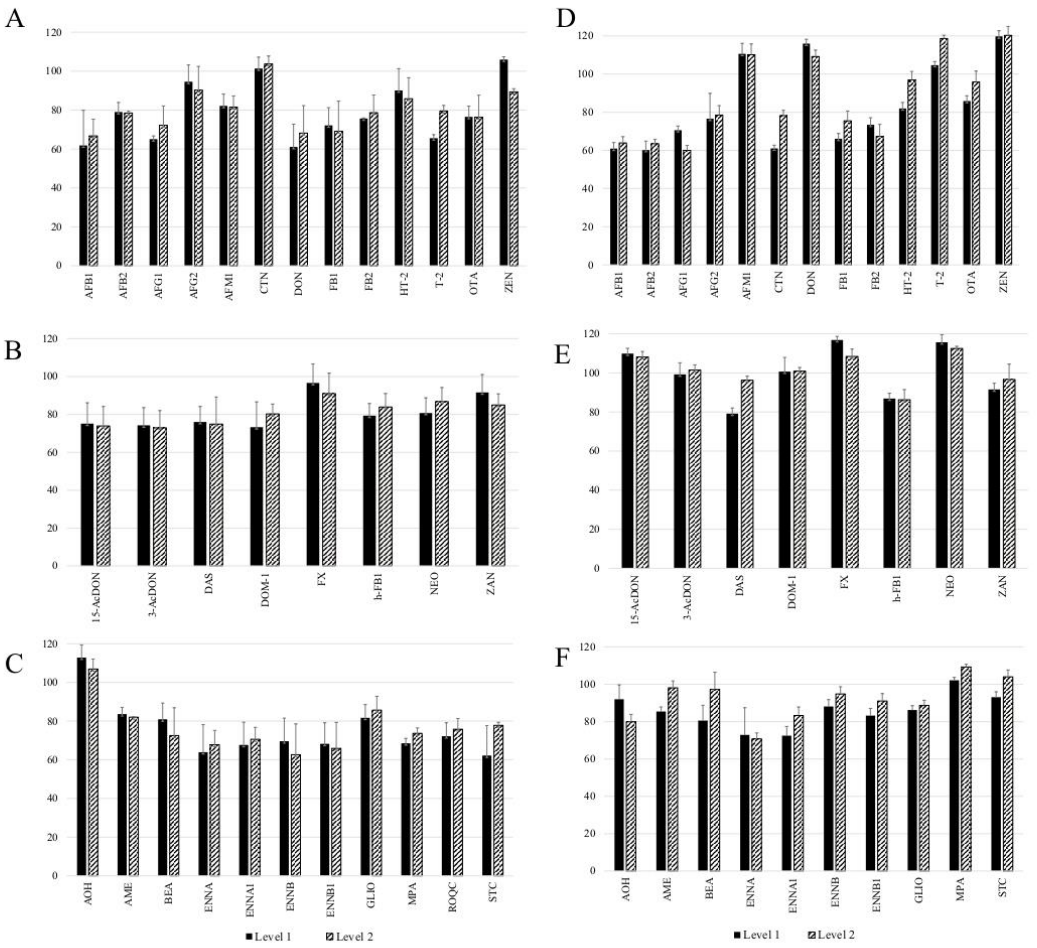



**Fig. 3** Recovery of mycotoxins from yogurt. $R_E$% values for regulated **A**, modified **B**, and emerging **C** toxins in natural yogurt and for regulated **D**, modified **E**, and emerging **F** toxins in oat yogurt

yogurt could cause the direct contamination of these products (Becker-Algeri et al. 2016).

In order to carry out a first assessment on the presence of mycotoxins in the Spanish market, a total of 27 natural yogurt samples and 16 oat yogurt samples were obtained in local markets and analyzed with the described method. As shown in Fig. 4, HT-2 was found in an oat yogurt sample (6.25%); this mycotoxin is probable that proceed from the added oat, since it is commonly detected in this cereal (Pettersson et al. 2011). The presence of OTA in 10% of natural yogurt is not surprising, since in previous studies, OTA had been reported in milk and cheese (Keyvan et al. 2018; Rodríguez-Cañás et al. 2023; Sakin et al. 2018; Zhang et al. 2022). Most of the samples tested positive for ENNs and BEA; these ubiquitous *Fusarium* mycotoxins have been found in dairy cow feed as well as milk on a regular basis. Consequently, their occurrence in yogurt seems possible (González-Jartín et al. 2022; Gonzalez-Jartin, et al. 2021; Pietruszka et al. 2023; Rodríguez-Blanco et al. 2020). Fumonisins were primarily detected in natural yogurt, with $FB_1$ found in 40% of samples and $FB_2$ in 26%, albeit in minimal amounts. In the case of oat yogurt, 1 out of the 16 samples showed these toxins at a level lower than the LOQ. The presence of fumonisins in milk has not been widely studied, but it was reported that 80% of commercial milk samples in Italy showed an average contamination level of 0.26 ppbs.



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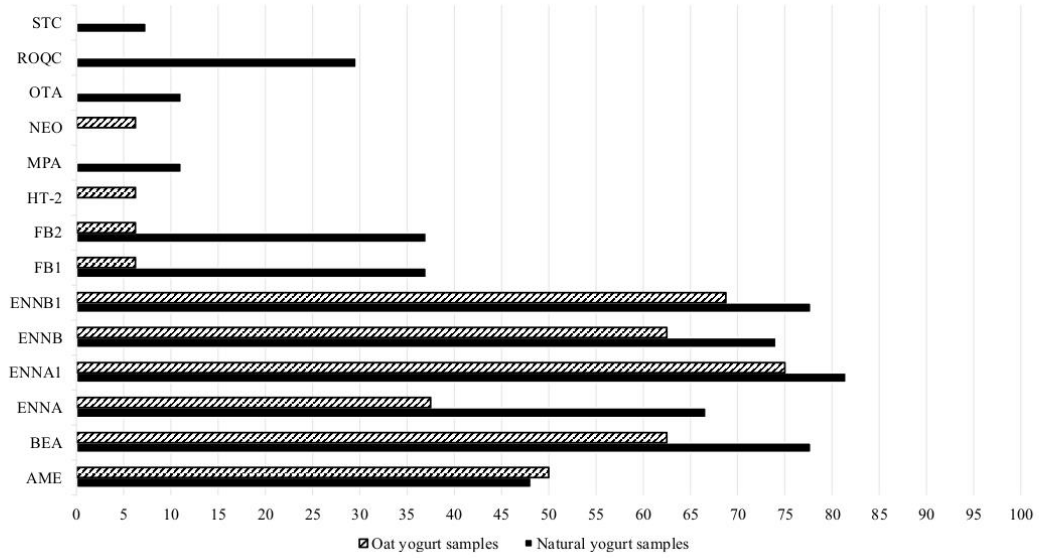



**Fig. 4** Positive samples of natural yogurt and oat yogurt. Percentage of each mycotoxin in samples

Similarly, other studies have reported low levels of fumonisins in milk, while there are no reports of their presence in yogurt (Benkerroum 2016; Gazzotti et al. 2009). Another frequently found toxin was AME, detected in approximately half of the samples of both natural and oat yogurt. *Alternaria* toxins are commonly found in various grains, particularly when there is high moisture content in the field (De Colliet al. 2021). For instance, in Canada, up to 75% of cereal-based samples have tested positive for AME (Scott et al. 2012). The transfer of this toxin to milk has been recently reported, AME was found in 9% of cow milk samples from Nigeria, and it was also detected in camel and goat milk (Akinyemi et al. 2022).

Table 2 shows the average concentrations in samples. For example, of the 81% of natural yogurt samples positive for $ENNA_1$, the average contamination level was 0.90 ng/g. Comparable levels of ENNS have been noted in milk by various researchers. It has been emphasized that these contaminants, albeit detected only in trace quantities, do not imply any immediate hazard to consumers (Pietruszka et al. 2023). In the case of the ROQC, all oat yogurt samples were positive, but showed a signal to noise ratio lower than 10,

**Table 2** Statistic components of the analysis of yogurt samples[a]

| Analyte | Natural yogurt | | | | Oat yogurt | | | |
|---|---|---|---|---|---|---|---|---|
| | 25% percentile | Median | Maximum | Mean | 25% percentile | Median | 75% percentile | Mean |
| AME | 0.75 | 0.77 | 1.48 | 0.88 | 2.67 | 2.67 | 2.67 | 2.67 |
| BEA | 0.09 | 0.11 | 3.11 | 0.43 | 0.09 | 0.11 | 0.20 | 0.13 |
| ENNA | 0.71 | 1.36 | 22.89 | 3.58 | 1.27 | 2.05 | 2.20 | 1.84 |
| $ENNA_1$ | 0.15 | 0.21 | 9.27 | 0.90 | 0.19 | 0.21 | 0.63 | 0.37 |
| ENNB | 0.09 | 0.29 | 8.36 | 0.80 | 0.28 | 0.43 | 0.62 | 0.58 |
| $ENNB_1$ | 0.25 | 0.47 | 8.43 | 1.15 | 0.44 | 0.69 | 1.19 | 0.79 |
| $FB_1$ | 0.76 | 1.99 | 7.39 | 2.69 | - | - | - | - |
| $FB_2$ | 3.22 | 5.05 | 12.28 | 5.65 | - | - | - | - |
| OTA | 1.00 | 1.54 | 4.31 | 2.28 | - | - | - | - |
| ROQC | 0.12 | 0.16 | 0.19 | 0.16 | - | - | - | - |

[a]Concentration data (expressed in ng/g) for samples with levels above the LOQ



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and therefore, they were not quantified. Eight natural yogurt samples were positive and presented an average concentration of 0.16 ng/g. In summary, emerging mycotoxins are present in yogurt at low concentrations but exhibit a high frequency of occurrence.

The present study revealed the susceptibility of yogurt, both natural and oat-based, to mycotoxin contamination. The optimized QuEChERS-type extraction method coupled with UHPLC-MS/MS provided a robust analytical approach capable of detecting and quantifying 32 mycotoxins, and the in-house validation demotes that the method is adequate. Emerging mycotoxins, specifically BEA and ENNs, has a high frequency of appearance in the samples analyzed. Furthermore, OTA has detected three samples of the natural yogurt. In addition, the detection of HT-2 in oat yogurt represents a novel observation. These results underscore the significance of monitoring mycotoxin levels in yogurt products for consumer safety.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s12550-024-00547-0.

**Author contribution** I.R.: investigation and writing—original draft writing; J. M.: investigation, review, and editing; R. A.: investigation; A. A.: review and editing; M.R.: methodology; L.B: funding acquisition and supervision.

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**Data availability** No datasets were generated or analyzed during the current study.

## Declarations

**Competing interest** The authors declare no competing interests.

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## 5. Application of a multi-toxin detect method to analyze mycotoxins occurrence in plant-based beverages

**Resumen:**
En los últimos años, las bebidas de origen vegetal han ganado popularidad en el mercado debido a preocupaciones ambientales y éticas, así como a intolerancias y alergias a la leche. Sin embargo, las materias primas empleadas en la fabricación de estos productos son susceptibles a la contaminación por micotoxinas. Por esta razón, se desarrolló un nuevo método basado en un procedimiento de extracción QuEChERS seguido de la detección mediante cromatografía líquida de ultra alta resolución acoplada a espectrometría de masas en tándem (UHPLC-MS/MS) para el análisis de 29 micotoxinas en bebidas de avena, arroz, soja y almendra. El método fue validado en términos de linealidad, límites de detección y cuantificación, efecto matriz, recuperaciones, exactitud y precisión. Se lograron características de rendimiento satisfactorias, con recuperaciones superiores al 70 % para la mayoría de las micotoxinas. Se analizaron varias muestras comerciales, detectándose frecuentemente aflatoxinas en bebidas de arroz y almendra, mientras que las toxinas T-2 y HT-2 se identificaron en productos a base de avena. Además, micotoxinas emergentes como las eniatinas y la beauvericina fueron detectadas en los cuatro tipos de bebidas.

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**Información revista:**

**Application of a multi-toxin detect method to analyze mycotoxins occurrence in plant-based beverages**

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# Application of a multi-toxin detect method to analyze mycotoxins occurrence in plant-based beverages

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ABSTRACT

In recent years, plant-based beverages have gained popularity on the market due to environmental and ethical concerns, as well as milk intolerances and allergies. However, raw materials employed in the manufacture of these products are susceptible to mycotoxin contamination. For this reason, a new method based on a QuEChERS extraction procedure followed by ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) detection was developed for the analysis of 29 mycotoxins in oat, rice, soy, and almond beverages. The method was validated in terms of linearity, detection and quantification limits, matrix effect, recoveries, accuracy and precision. Satisfactory performance characteristics were achieved, with recoveries above 70% for most mycotoxins. Several commercial samples were analyzed, aflatoxins were frequently detected in rice and almond beverages, while T-2 and HT-2 toxins were identified in oat-based products. In addition, emerging mycotoxins such as enniatins and beauvericin were detected in the four types of beverages.

## 1. Introduction

Plant-based beverages are diluted emulsions obtained from nuts, cereals, legumes, or seeds mixed with water, although they can include other ingredients (salt, sugar, natural additives…) to improve their nutritional value or flavour. In recent years, due to health problems such as intolerances or allergies, there has been an increase in the number of consumers of these types of products that do not include lactose in their composition (Reyes-Jurado, Soto-Reyes, Dávila-Rodríguez, Lorenzo-Leal, Jiménez-Munguía, Mani-López, et al., 2021).

The highest occurrence of mycotoxins tends to arise in agricultural commodities. Fungi can infect crops in the field or during the harvest and/or storage and, if the temperature and moisture conditions are appropriate, the agricultural products will be contaminated by mycotoxins (Lee & Ryu, 2017). Monitoring all stages of the agri-food chain is necessary to preserve food safety. Regulated mycotoxins are analyzed in certain foodstuffs; however, EU regulation does not contemplate plant-based beverages nor legumes or derived products (EC_2023/915, 2023). In addition, climate change may increase the risk related to mycotoxins since these metabolites can appear with more frequency in the matrices employed in the manufacture of plant-based beverages (Medina, Akbar, Baazeem, Rodriguez, & Magan, 2017).

Emerging mycotoxins are compounds for which there is not enough data about their toxicity, although recent studies show a high incidence in different commodities (González-Jartín, Alfonso, Rodríguez, Sainz, Vieytes, & Botana, 2019). These toxins, which are not monitored in routine analysis, include, among others, beauvericin (BEA), enniatins (ENNs), fusaric acid (FA), sterigmatocystin (STC), mycophenolic acid (MPA), and cyclopiazonic acid (CPA) (Gruber-Dorninger, Novak, Nagl, & Berthiller, 2017). However, some emerging toxins namely alternariol (AOH) and alternariol methyl ether (AME) have recently begun to be monitored (EC_2022/553, 2022). There are reports on the incidence of these mycotoxins in raw materials employed in the manufacture of plant-based beverages; for example, BEA and ENNs have been detected in oat, rice, or soybean samples (García & Jarque, 2014; Pérez-Fuentes, Alvariño, Alfonso, González-Jartín, Gegunde, Vieytes, et al., 2021). A cytotoxic effect of emerging mycotoxins in human cells was reported, which suggests that more data about their occurrence and toxicity is needed to carry out a risk characterization (Cimbalo, Alonso-Garrido, Font, & Manyes, 2020). In addition, regulated and emerging mycotoxins can suffer changes in their chemical structure caused by fungi, plant, or animal metabolism, leading to another group of unregulated

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mycotoxins, known as modified mycotoxins, such as 3-acetyl-deoxynivalenol (3-AcDON) or 15-acetyl-deoxynivalenol (15-AcDON) which are modified forms of DON. Data on these mycotoxins are still scarce, and therefore it is necessary to develop methods for the detection of these compounds and study their occurrence in food (Pérez-Fuentes et al., 2021).

In recent decades, ultra-high liquid chromatography coupled mass spectrometry (UHPLC-MS/MS) has become the most employed technique for the analysis of mycotoxins in complex matrices (Medina, Akbar, Baazeem, Rodriguez, & Magan, 2017). This methodology presents some advantages including high selectivity and sensitivity and it also allows the detection of a wide range of toxins (EN17641, 2022; Wang, Zhang, Ma, & Li, 2022). There is not much data available on the presence of mycotoxins in plant-based beverages, Table 1 shows a compilation of the analytical techniques used for the determination of mycotoxins in these matrices (Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019; Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campaña, & Arroyo-Manzanares, 2019; Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017; Miró-Abella, Herrero, Canela, Arola, Borrull, Ras, et al., 2017). The previously available methods are intended for the analysis of specific groups of toxins or allow the detection of a small number of compounds. Therefore, this study aimed to optimize and validate a new method to analyze mycotoxins in soy, oat, rice, and almond beverages, and to evaluate the presence of mycotoxins in these products.

## 2. Materials and methods

### 2.1. Chemicals and materials

The following analytical standards were used in the study: aflatoxin $B_1$ ($AFB_1$), aflatoxin $B_2$ ($AFB_2$), aflatoxin $G_1$ ($AFG_1$), aflatoxin $G_2$ ($AFG_2$), enniatin A (ENNA), enniatin $A_1$ ($ENNA_1$), enniatin B (ENNB), enniatin $B_1$ ($ENNB_1$), gliotoxin (GLIO), and zearalenone (ZEN), which were purchased from Sigma in Madrid, Spain. Ochratoxin A (OTA) standard solution was obtained from Laboratorios CIFGA S.A. in Lugo, Spain, while BEA was supplied by Enzo in Barcelona, Spain, and circumdatin A (CTA) was obtained from Santa Cruz Biotechnology in Santa Cruz, CA. Analytical standards of 3-acetyldeoxynivalenol (3-AcDON), 15-acetyldeoxynivalenol (15-AcDON), alternariol (AOH), alternariol methyl ether (AME), citrinin (CTN), diacetoxyscirpenol (DAS), fumonisin $B_1$ ($FB_1$), fumonisin $B_2$ ($FB_2$), hydrolyzed fumonisin $B_1$ (h-$FB_1$), HT-2 toxin (HT-2), mycophenolic acid (MPA), neosolaniol (NEO), STC, T-2 toxin (T-2), T-2 triol and zearalanone (ZOL) were provided by Romer Labs in Tulln, Austria.

The chemicals used in the study were purchased from the following suppliers: Methanol, acetonitrile, acetic acid glacial 100%, anhydrous magnesium sulphate (MgSO4), and sodium chloride (NaCl) were purchased from Panreac Química S.A. in Barcelona, Spain. Ammonium formate was obtained from Fluka in Buchs, Switzerland, and formic acid from Merck in Madrid, Spain. The PolygoprepTM 60-50 C18 was supplied by Macherey-Nagel in Düren, Germany, and ultrafree-MC Durapore membrane centrifugal filters (0.22 μm pore size) were provided by Millipore in Billerica, USA. A Millipore Milli-Q Plus system (Millipore, USA) was employed to obtain pure water.

### 2.2. Samples

A diverse selection of plant-based beverage samples from different brands was purchased from local supermarkets, namely: 16 oat beverage samples, with oat percentages ranging between 11% and 15%, 6 rice beverage samples (15%–17% of rice), 7 almond beverage samples (2%–14% almond), and finally, 9 soy beverage samples (8%–15% of soy). All samples contain other secondary ingredients such as stabilizers, thickeners, aromas, salt, sugar, and vitamins. All samples were storage at 4 °C until required for sample preparation and analysis.

### 2.3. UHPLC-MS/MS conditions

Sample analysis was conducted using an Agilent 1290 Infinity ultra-high-performance liquid chromatography (UHPLC) system coupled with an Agilent 6460 Triple Quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source, both of which were manufactured by Agilent Technologies in Waldbronn, Germany. Agilent MassHunter LCMS Acquisition Console software was used for system control and data acquisition, while QQQ Quantitative Analysis software was used for data processing.

For chromatographic analysis, an Acquity UPLC HSS T3 column (1.8 μm, 100 mm × 2.1 mm) from Waters was used at a temperature of 40 °C, with a flow rate of 0.3 mL/min. Mobile phase A was composed of water with 5 mM ammonium formate and 0.1% formic acid, while mobile phase B was methanol 100%. The injection volume was set at 5 μL and a gradient elution of 13 min was employed as follows: after 0.5 min at 0% B, the gradient was raised to 14% B within 0.5 min, and maintained for 1.5 min. Then, the percentage of the eluent B was increased to 60% B within 1 min and later held for 0.5 min. Next, it was increased to 100% B within 4.5 min, and maintained for 2 min. Finally, the gradient was back to 0% B in 0.5 min and this percentage was kept for 2.5 min.

Mass spectrometer parameters including the fragmentor voltage (FV), collision energy (CE), cell accelerator voltage (CAV) and mass transitions for each analyte had previously been described (González-Jartín, Rodríguez-Cañás, Alfonso, Sainz, Vieytes, Gomes, et al., 2021). Analysis was carried out in positive and negative modes and dynamic multiple reaction monitoring (dMRM) acquisition. In this sense, two $m/z$ transitions were monitored for each mycotoxin (Table S1). In order to identify a compound, the maximum tolerances for the ion ratio measurements, with respect to their relative intensities, were set at 30% and the retention time retention time (RT) with a tolerance of ±0.1 min in relation to the RT of the analyte in the calibration curve. The ionization source parameters were the following: nebulizer, 45 psi; nebulization

**Table 1**
Available methods for determination of mycotoxins in plant-based beverages.

| Food items | Analyte | Analytical technique | LOQs (μg/L) | Levels (μg/L) | Reference |
|---|---|---|---|---|---|
| Oat, soybean, and rice beverage | *Fusarium* toxins ($FB_1$, $FB_2$, T-2, HT-2, FX, DON, ZEN) | Salting-out-assisted liquid–liquid extraction (SALLE) followed by UHPLC-MS/MS | 3.2–57.7 | 192–263 μg/L (only for DON) | (Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017) |
| Soy, oat and rice beverage | BEA and ENNA, $ENNA_1$, ENNB, $ENNB_1$ | SALLE followed by UHPLC-MS/MS | 0.3–0.8 | 0.4–26 | (Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019) |
| Oat, rice, coconut, almond, birdseed beverages | $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ | Dispersive liquid–liquid microextraction (DMLLE) followed by HPLC-FLD<br>QuEChERS by LC-FLD | 0.5 | – | (Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campaña, & Arroyo-Manzanares, 2019) |
| Soy, oat and rice beverage | 11 mycotoxins (DON, $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, HT-2, $FB_1$, $FB_2$, T-2, OTA, ZEN) | QuEChERS followed by UHPLC-MS/MS | 0.05–15 | 0.1–19 | (Miró-Abella, Herrero, Canela, Arola, Borrull, Ras, et al., 2017) |



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gas flow, 12 L/min; drying gas flow, 8 L/min; nebulization gas temperature 400 °C; drying gas temperature, 350 °C; capillary voltage, 4000 V; nozzle voltage, 0 V. In all cases, the gas used was nitrogen.

### 2.4. Optimization of the procedure

The extraction procedure was optimized following a protocol previously proposed for the analysis of cheese (Rodríguez-Cañás, González-Jartín, Alvariño, Alfonso, Vieytes, & Botana, 2023). In this sense, the feasibility of sample concentration and the use of a clean-up step were investigated, while the effectiveness of different solvents was not evaluated, 0.5% formic acid was employed due to its high efficacy in the extraction of mycotoxins from milk, as previously demonstrated (González-Jartín et al., 2021). Moreover, given the unique nature of the matrix, the contamination levels for optimization were reduced as follows: Five mL of sample were artificially contaminated with 0.19 μg/L of aflatoxins; 6.25 μg/L of BEA, ENNs and h-FB1; 12.5 μg/L of other regulated (DON, $FB_1$, $FB_2$, ZEN, HT-2, T-2, OTA, CTN), emerging and modified (AOH, AME, MPA, NEO, GLIO, STC, DAS, T-2 triol) toxins. Next, the samples were extracted using 5 mL of 0.5% formic acid in acetonitrile. Phase separation was induced by adding 4 g of MgSO4 and 0.6 g of NaCl, followed by agitation and centrifugation (at 3134 × *g* for 10 min). To assess the feasibility of analysing a concentrated sample, the organic phase (1.5 mL) was evaporated to dryness and the resulting extract was redissolved in 1.5, 0.6, 0.3, 0.15, and 0.075 mL of the analysis solvent (a mixture of acetonitrile, water, and acetic acid at a ratio of 49:50:1 v/v/v). The effectiveness of a cleaning step was evaluated by adding 1.125 g of MgSO4 and 0.375 mg of C18 to 2.5 mL of the organic phase. Next, 1 mL of the obtained extract was evaporated to dryness and then redissolved in 0.2 mL of the analysis solvent.

### 2.5. Sample extraction

Five mL of sample were mixed with 5 mL of 0.5% formic acid in acetonitrile in a polypropylene tube using a multi-tube vortex (Nahita Blue, Auxilab, Spain) at 2500 rpm for 5 min. Then, a mixture of 4 g of $MgSO_4$ and 0.6 g of NaCl was added for the partitioning step, which was achieved by shaking using a vortex mixer for 30 s and centrifugation at 3134*g*, 4 °C, for 10 min. An aliquot of 1 mL of the supernatant was evaporated to dryness in a centrifugal evaporator (Jouan RC 1022, Thermo Electron Corp.) and reconstituted with 200 μL of the analysis solvent. Finally, the extract was filtered through 0.22 μm by using centrifugal filters (5 min at 16,000*g*).

### 2.6. Method validation

The method was validated for the target mycotoxins in plant-based beverages in terms of linearity, limits of detection (LODs) quantification (LOQs), matrix effect, accuracy, and precision. Nine-point calibration curves were constructed in the analysis solvent (mixture of acetonitrile/water/acetic acid 49/50/1 v/v/v) and in the blank matrix. In this way, the linearity of the method was evaluated, and the correlation coefficients (R) were calculated. The range of concentrations were: 0.01–3 ng/mL for aflatoxins except for $AFG_2$ (0.05–3 ng/mL); 0.78–100 ng/mL for 15-AcDON, 3-AcDON, BEA, ENNs, and h-FB1; 1.56–200 ng/mL for CTN; 1.95–500 for ZOL, and 0.78–200 ng/mL for other toxins.

LODs and LOQs were calculated according to the EURL guide (Wenzl, Haedrich, Schaechtele, Piotr, Stroka, Eppe, et al., 2016). In this sense, LODs were assessed according to the equation "$LOD = 3.9 \times \frac{Sb}{m}$, where Sb is the standard deviation of the measurement of ten blank samples (plant-based beverage extracts) prepared following the extraction procedure previously described and *m* is the slope of the calibration curve. Next, LOQs were calculated by multiplying the LODs of each mycotoxin by a factor of 3.3.

The matrix effect for each mycotoxin was evaluated in triplicate in each matrix (soy, oat, rice, and almond) by determining the signal suppression/enhancement (SSE) factor. The accuracy and precision of the method were evaluated through recovery experiments by spiking blank samples with mycotoxins at three levels of contamination (Table S2). Recovery experiments were performed in triplicate on the same day to assess repeatability and in three consecutive days, by different operators, to evaluate within-lab reproducibility. The apparent recovery ($R_A$) and the relative standard deviation (RSD) were calculated. Precision was evaluated on the same day using RSDr (intra-day precision) and over three consecutive days using $RSD_R$ (inter-day precision). Finally, the accuracy was assessed by determining the recovery of the extraction ($R_E$). The SSE, $R_A$, RSD, and $R_E$ were calculated according to the previously described equations (Rodríguez-Cañás, González-Jartín, Alvariño, Alfonso, Vieytes, & Botana, 2023). In order to calculate the concentration of toxins in samples, the quantification was conducted using solvent based calibration curves and then the data was corrected with the SSE and $R_E$ factors.

## 3. Results and discussion

Raw materials employed in the manufacture of plant-based beverages include nuts, cereals, legumes, or seeds, all of them can be contaminated in the field or during storage with a wide range of mycotoxins and consequently, a carry-over of toxins to plant-based beverages may occur. The European Regulation stablishes maximum levels of mycotoxins in certain raw materials. For example, it set maximum residue levels of aflatoxins in almonds, cereals, and rice, as well as levels for OTA in soybeans and DON in oat grains. Nevertheless, the presence of mycotoxins in plant-based beverages has not been regulated and there are not many studies dealing with the presence of mycotoxins in the final product, whose consumption has increased significantly in recent years (García & Jarque, 2014; Reyes-Jurado et al., 2021). Consequently, the aim of this study was to develop a method applicable to the analysis of several mycotoxins in the most consumed plant-based beverages and, on the other hand, to carry out a preliminary study on the incidence of toxins in these products.

### 3.1. Optimization of extraction procedure

In the present study, a QuEChERS based extraction was applied. This technique has ever increased relevance for the analysis of different contaminants, including mycotoxins since it was first proposed in 2003 for the analysis of pesticides (Xu, Xu, Han, Qiu, & Hou, 2019). This methodology, which allows the simultaneous extraction of analytes from complex matrices, consists of a solid–liquid or liquid–liquid extraction, followed by a phase partition which is achieved by the addition of a mixture of salts, commonly $MgSO_4$ and NaCl since they have shown an improved extraction efficiency. A clean-up step such as a dispersive solid phase extraction (d-SPE) is sometimes applied, although many authors have ruled out this step since it reduces the recovery of some mycotoxins or does not produce significant improvements in the matrices effect (Koesukwiwat, Sanguankaew, & Leepipatpiboon, 2014). The effectivity of the QuEChERS extraction is affected by several factors, such as the amount of acid that is added or the relation between the amount of solvent and matrix. A buffered QuEChERS extraction employing potassium dihydrogen phosphate and acetonitrile with 5% formic acid was previously proposed to determine aflatoxins in plant-based beverages although low recoveries were achieved (Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campaña, & Arroyo-Manzanares, 2019). Another method using acetonitrile with 1% formic acid was applied to the analysis of 11 regulated mycotoxins in oat, soy and rice based products (Miró-Abella et al., 2017).

In order to develop a new extraction procedure applicable to regulated and emerging toxins, conditions previously used for cow milk analysis were chosen since the method achieved good results (González-



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Jartín et al., 2021). In this sense, acetonitrile acidified with 0.5% formic acid was employed to extract 5 mL of plant-based beverages as described in Material and Methods. The extract was evaporated to dryness and redissolved in analysis solvent to reach a final extract 0, 2.5, 5, 10 and 20 times concentrated. In this way, the $R_A$ was evaluated in five different conditions, with a different concentration of the final extract. As shown in Fig. S1, in general, when the concentration of the extract increases, the $R_A$% decreases, and therefore there is a suppression of the analytical signal caused by some compounds present in the matrix. However, rice and almond beverages show higher $R_A$% values when the sample was analysed 2.5 times concentrated. Therefore, there is an enhancement of the analytical signal in comparison with the not concentrate extract. In soybean and oat beverages, although there is an enhancement of the signal for some toxins such as AME or DAS, most of the analysed compounds do not show this effect. In the case of lipophilic toxins, namely BEA and ENNs the highest $R_A$% values were achieved when the extract was not concentrated especially in oat, rice, and almond beverages. For certain mycotoxins, such as T-2 triol the suppression is very pronounced in all matrices. For analysis, a five times concentrated sample was chosen as the compromise level which allows the detection of low mycotoxin levels, and, at the same time, the matrix effect is not too pronounced.

Next, it was studied whether the application of an additional clean-up step was effective in reducing the matrix effect. The adsorbent employed was C18, because is commonly employed in QuEChERS procedures (Zhao, Liu, Zhang, Zhou, & Yang, 2021). As shown in Fig. S2, in general, there is a suppression of the signal although for certain mycotoxins such as h-FB1 or ZEN an enhancement was observed. When the d-SPE was applied, in the case of almond beverages the differences between the extraction without and with clean-up steps were low. A reduction in the matrix effect was observed when the C18 was employed, especially for BEA and ENNs. For example, in the case of rice beverage, $ENNB_1$ gave an SSE value of 63% in comparison with a value of 8% without the clean-up step. On the contrary, the cleaned extract yielded higher suppression rates for AFs, especially in the case of oat beverage. Therefore, due to the great relevance of aflatoxins and the need to have methods especially applicable to their detection the extraction without a clean-up step was chosen.

### 3.2. Method validation

The linearity was studied by using calibration curves constructed at nine levels of concentration. Linearities of the method were acceptable since most of the R values were higher than 0.997 in the studied concentration range (Table 2). In addition, it was check that the back-calculated concentration was in the range of 80–120%.

LODs and LOQs were calculated following the guidance of the EU-RL by the measurement of ten solvents or blank samples (Wenzl et al., 2016). In the case of oat beverage, LODs an LOQs are indicated in Table 2, while data for rice, soy and almond beverages are indicated in Tables S3, S4 and S5, respectively. The LOQs in oat beverage were from 0.007 μg/L ($AFG_2$) to 10.7 μg/L (DON), in soy beverage were from 0.03 μg/L ($AFB_2$) to 29 μg/L (CTN), in almond beverage were from 0.003 μg/L (BEA and $ENNB_1$) to 1.1 μg/L (CTN) and in rice beverage were from 0.001 μg/L ($AFG_1$ and $ENNB_1$) to 2 μg/L (CTN). The obtained LOQs improve those previously reported in the literature for some mycotoxins. For instance, LOQs of 10 μg/L and 0.5 μg/L were reported for HT-2 and T-2 in oat beverage, while the LOQs achieved by the present method are 0.4 μg/L and 0.1 μg/L, respectively (Miró-Abella et al., 2017). Although the way that has been employed to calculate LOQ has been widely used in the bibliography, currently, the latest SANTE document establishes this level as the lowest one that has been validated by applying the complete analytical method (SANTE, 2021).

The matrix effect was calculated as the suppression or enhancement of the signal caused by the presence of coeluting compounds. This phenomenon can affect the accuracy of the method and therefore it is necessary to correct the data; for instance, using the SSE factor, internal standards, or matrix-matched calibration (Fabregat-Cabello, Zomer,

**Table 2**
**Performance characteristics of the method in oat beverage.** [a, b] Expressed as μg/L. [1]Relative Standard Deviation evaluated in terms of reproducibility (inter-day precision) calculated from the results of the extraction recovery obtained with the 3rd level of contamination.

| Analyte | LOD[a] | LOQ[b] | $r^2$ | SSE | Recovery % | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Level 1 | $RSD_r$ % | Level 2 | $RSD_r$ % | Level 3 | $RSD_r$ % | $RSD_R$ (%)[1] |
| 15-AcDON | 2.44 | 8.06 | 0.997 | 46 | 84 | 4 | 77 | 5 | 94 | 6 | 15 |
| 3-AcDON | 0.11 | 0.37 | 0.995 | 46 | 70 | 19 | 84 | 12 | 79 | 6 | 10 |
| $AFB_1$ | 0.013 | 0.042 | 0.999 | 51 | 81 | 2 | 95 | 14 | 87 | 5 | 12 |
| $AFB_2$ | 0.008 | 0.028 | 0.998 | 54 | 85 | 4 | 79 | 8 | 92 | 2 | 13 |
| $AFG_1$ | 0.008 | 0.025 | 0.997 | 37 | 92 | 13 | 103 | 2 | 98 | 8 | 11 |
| $AFG_2$ | 0.002 | 0.007 | 0.995 | 35 | 84 | 10 | 85 | 4 | 84 | 6 | 13 |
| $FB_1$ | 0.59 | 1.94 | 0.999 | 143 | 80 | 9 | 83 | 9 | 86 | 10 | 15 |
| $FB_2$ | 0.06 | 0.21 | 0.997 | 121 | 93 | 3 | 98 | 8 | 88 | 14 | 20 |
| HT-2 | 0.14 | 0.47 | 0.999 | 44 | 101 | 5 | 105 | 4 | 103 | 13 | 17 |
| T-2 | 0.03 | 0.12 | 0.999 | 73 | 104 | 7 | 95 | 10 | 99 | 2 | 7 |
| CTN | 0.21 | 0.70 | 0.998 | 47 | 114 | 12 | 93 | 11 | 87 | 13 | 15 |
| DON | 3.24 | 10.68 | 0.998 | 21 | 127 | 20 | 136 | 6 | 100 | 17 | 19 |
| OTA | 0.02 | 0.06 | 0.999 | 98 | 106 | 4 | 98 | 3 | 101 | 4 | 6 |
| ZEN | 0.01 | 0.04 | 0.999 | 83 | 112 | 5 | 101 | 8 | 101 | 2 | 15 |
| GLIO | 0.40 | 1.32 | 0.999 | 112 | 122 | 7 | 118 | 9 | 98 | 8 | 18 |
| MPA | 0.03 | 0.11 | 0.999 | 106 | 101 | 5 | 103 | 6 | 94 | 2 | 17 |
| AOH | 0.10 | 0.32 | 0.999 | 68 | 110 | 2 | 99 | 6 | 102 | 7 | 11 |
| AME | 0.005 | 0.02 | 0.999 | 99 | 109 | 2 | 96 | 2 | 93 | 3 | 14 |
| NEO | 0.25 | 0.86 | 0.999 | 38 | 108 | 4 | 99 | 5 | 99 | 3 | 19 |
| DAS | 0.01 | 0.04 | 0.999 | 89 | 92 | 1 | 95 | 2 | 91 | 10 | 16 |
| STC | 0.002 | 0.009 | 0.999 | 80 | 93 | 0 | 100 | 1 | 110 | 1 | 22 |
| T-2 TRIOL | 0.028 | 0.09 | 0.999 | 27 | 108 | 4 | 83 | 5 | 74 | 8 | 13 |
| ZOL | 0.03 | 0.08 | 0.997 | 92 | 74 | 6 | 80 | 1 | 90 | 4 | 10 |
| BEA | 0.006 | 0.019 | 0.999 | 49 | 71 | 4 | 86 | 2 | 84 | 11 | 13 |
| ENNA | 0.004 | 0.015 | 0.999 | 103 | 88 | 4 | 89 | 3 | 87 | 6 | 22 |
| $ENNA_1$ | 0.007 | 0.023 | 0.999 | 57 | 80 | 3 | 75 | 6 | 92 | 15 | 19 |
| ENNB | 0.004 | 0.013 | 0.998 | 25 | 87 | 13 | 65 | 2 | 66 | 7 | 18 |
| $ENNB_1$ | 0.005 | 0.018 | 0.998 | 43 | 86 | 10 | 73 | 5 | 100 | 3 | 20 |
| h-$FB_1$ | 0.03 | 0.10 | 0.999 | 209 | 82 | 2 | 79 | 2 | 72 | 9 | 9 |



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Sancho, Roig-Navaroo, & Mol, 2016). In this work, the SSE factor was calculated for all mycotoxins (Fig. 1). In general, signal suppression was observed, in the rice beverage the SSE factor vary from 11% for $ENNB_1$ to 125% for ZOL, in almond beverage from 33% for DON to 153% for h-$FB_1$, in oat beverage from 20% for DON to 209% for h-$FB_1$ and in soy beverage from 19% for T2 triol to 218% for h-$FB_1$. Therefore, a signal enhancement is usually observed for $FB_1$, $FB_2$, and h-FB1. In the case of DON, signal suppression occurred in all matrices. These results were in agreement with other data previously reported in rice and soy beverages (Miró-Abella et al., 2017). The matrix effect for HT-2 is more pronounced in oat, rice, and soy beverages (SSE values from 44% to 58%) than in almond (SSE of 85%). Previously, a high matrix effect was also observed for HT-2 and DON, with a signal suppression of 39% was observed in oat, soy, rice, and bird seed beverage (Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017).

Next, the accuracy of the method was studied through of recovery at three concentration levels (Table S2). Recoveries are indicated in Fig. 2 and Table 3, S3, S4, and S5. Recoveries vary according to the matrix and the level of contamination, but, in general, values higher than 80% were achieved (Fig. 2), although there are some exceptions as HT-2 and T-2 triol in soy beverage, with values of 45% and 48%, respectively; and CTN in rice beverage with a 21% of recovery. In the case of AFs, values higher than 65% were obtained for all matrices in the three levels of contamination (0.02, 0.09 and 0.19 μg/L). In literature, good recoveries have also been obtained using a method based on a dispersive liquid–liquid microextraction followed by high-performance liquid chromatography with fluorescence detection (DLLME-HPLC-FL) (Hamed, Abdel-Hamid, Gámiz-Gracia, García-Campaña, & Arroyo-Manzanares, 2019). In the case of lipophilic toxins, BEA and ENNs, the recoveries were satisfactory (64%–107%) when samples were spiked at concentrations between 0.6 μg/L and 2.5 μg/L. A salting-out assisted liquid–liquid extraction (SALLE) was applied to extract BEA and ENNs of oat, rice and soy beverages, the obtained recoveries varied from 84% to 97% when samples were spiked at 1, 10 and 50 μg/L (Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019). Similarly, a method to determinate *Fusarium* toxins in plant-based beverages was previously proposed showing recoveries around 80% in two levels of contamination of up to 300 μg/L (Hamed, Arroyo-Manzanares, García-Campaña, & Gámiz-Gracia, 2017). The proposed method also achieves good recoveries for these toxins and their modified forms at much lower contamination levels, more similar to the levels at which the toxins are present in samples. As described above, precision was studied through of RSD of recoveries obtained by triplicate in the same day and in three days consecutives. Intra-day and inter-day precision was high, with RSD values lower than 20% in both cases.

Levels of contamination used for the study of the recovery and their RSD were lower than those used in previously published methods, but still, a high accuracy and precision were obtained. In this sense, the method meets the performance criteria of the Commission Regulation (EC) N° 401/2006. Therefore, although the proposed method greatly broadens the target toxins, it maintains adequate performance characteristic.

### 3.3. Application to real samples

As mentioned before, previously available methods were intended to the analysis of specific groups of toxins and allow the detection of a small number of compounds. Consequently, in order to know the impact of mycotoxins in plant-based beverages, first a new method applicable to the analysis of several mycotoxins was validated and next it was applied to the analysis of 38 plant-based beverages including 9 of soy, 6 of rice, 16 of oat and 7 of almond. Samples were collected in the local market and belong to different brands or batches. The results are indicated in the Table 3, a high incidence of emerging mycotoxins was found, especially for BEA and ENNs. These results are in concordance with previously reported data (Arroyo-Manzanares, Hamed, García-Campaña, & Gámiz-Gracia, 2019). Similarly, the AME was found in the four varieties of the plant-based beverages, the STC was detected in rice, soy and oat, while MPA was found in almond, soy and oat. Regarding regulated toxins, aflatoxins were mainly detected in almond and rice,

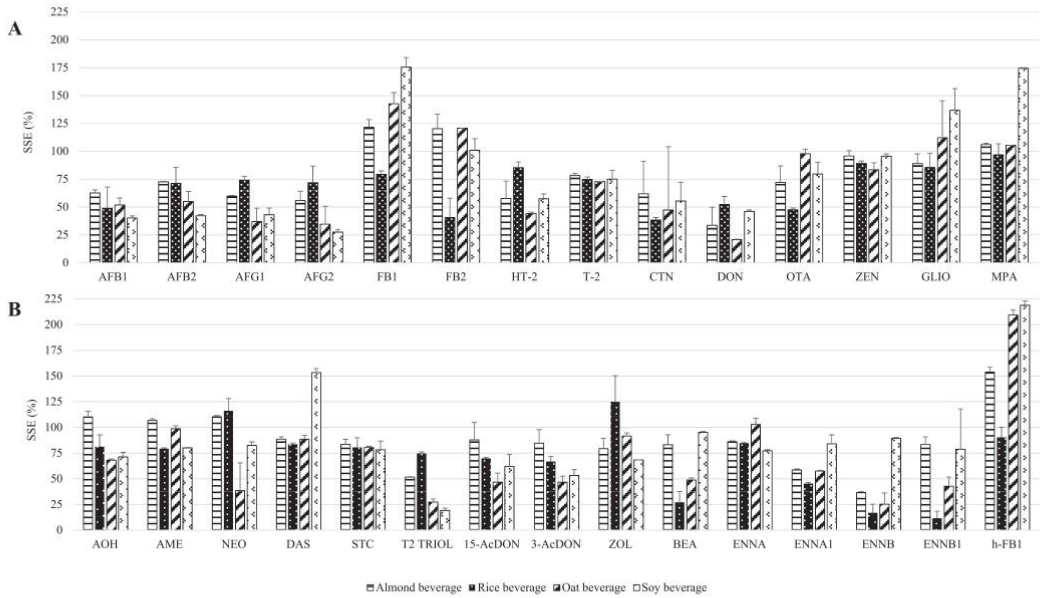



**Fig. 1. Matrix effect of the analysis method.** SSE value of oat, rice, soy, and almond beverages obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B).



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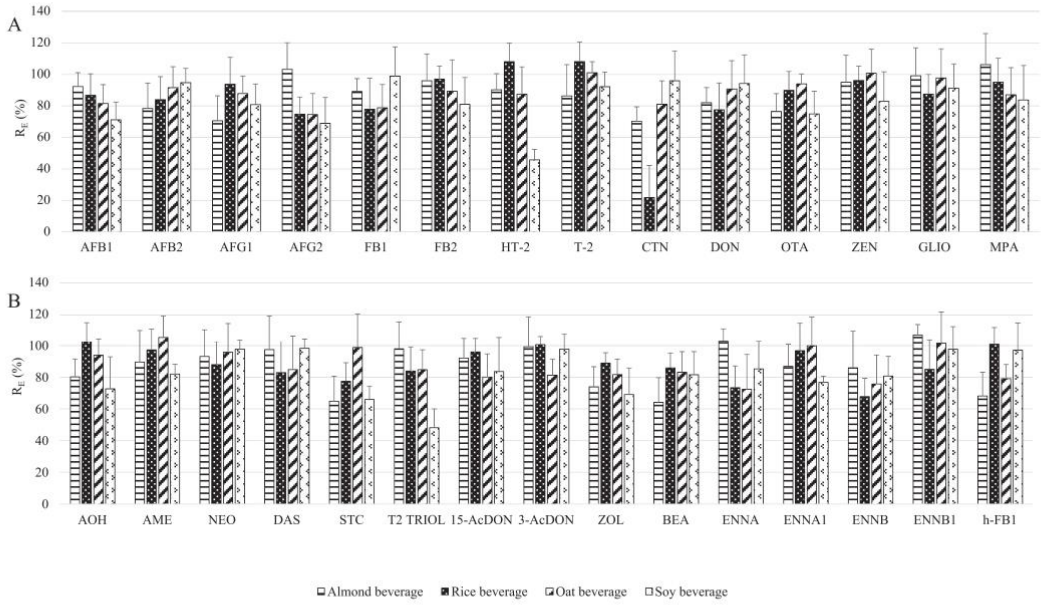



**Fig. 2. Accuracy of the analysis method.** $R_E$ value of oat, rice, soy, and almond beverages obtained for regulated mycotoxins (A) and emerging/modified mycotoxins (B).

**Table 3**
Analysis of commercial samples. Percentage of positive samples (%). mean concentration and range of mycotoxins expressed as μg/L.

| Toxin | Almond | | Oat | | Rice | | Soy | |
|---|---|---|---|---|---|---|---|---|
| | % | Mean (range) μg/L | % | Mean (range) μg/L | % | Mean (range) μg/L | % | Mean (range) μg/L |
| $AFB_1$ | 28 | 0.03 (<LOQ – 0.04) | 0 | | 33 | 0.05 (<LOQ – 0.09) | 11 | 0.05 |
| $AFB_2$ | 0 | | 0 | | 33 | <LOQ | 0 | |
| AME | 100 | 0.3 (<LOQ – 0.32) | 87 | 0.22 (<LOQ – 0.31) | 100 | 0.28 (0.17–0.33) | 44 | 0.33 (0.29–0.37) |
| BEA | 100 | 0.36 (0.05–2) | 94 | 0.08 (0.03–0.5) | 83 | 0.18 (0.08–0.33) | 66 | 0.39 (<LOQ – 2.47) |
| ENNA | 85 | 0.08 (<LOQ – 0.1) | 100 | 0.07 (0.03–0.14) | 66 | 0.05 (0.03–0.07) | 11 | 0.19 |
| $ENNA_1$ | 85 | 0.14 (<LOQ – 0.2) | 81 | 0.09 (<LOQ – 0.2) | 66 | 0.07 (<LOQ – 0.1) | 22 | 0.19 (0.12–0.23) |
| ENNB | 100 | 0.16 (0.07–0.3) | 100 | 0.07 (0.01–0.22) | 66 | 0.08 (<LOQ – 0.2) | 11 | 0.50 |
| ENNB1 | 100 | 0.08 (0.04–0.12) | 100 | 0.1 (<LOQ – 0.34) | 83 | 0.22 (<LOQ – 0.3) | 11 | 0.29 |
| $FB_1$ | 42 | 0.8 (0.72–0.84) | 6 | 5.99 | 0 | | 0 | |
| FB2 | 42 | 1 (0.75–1.3) | 6 | 0.72 | 0 | | 0 | |
| HT-2 | 0 | | 37 | 0.65 (<LOQ – 0.9) | 0 | | 0 | |
| T2 | 0 | | 50 | 0.6 (<LOQ – 0.7) | 0 | | 0 | |
| MPA | 28 | 0.2 (<LOQ – 0.27) | 25 | 0.2 (<LOQ – 0.22) | 0 | | 22 | 0.12 (0.11–0.13) |
| OTA | 14 | 0.186 (<LOQ – 0.186) | 6 | 0.74 | 0 | | 0 | |
| STC | 0 | | 43 | 0.11 (<LOQ – 0.125) | 100 | 0.19 (<LOQ – 0.25) | 66 | 0.16 (<LOQ – 0.19) |

FBs were in almond while HT-2 and T-2 toxins were present in oat samples.

In the case of oat beverages, AME, BEA, ENNAs, FBs, HT-2, T-2, MPA, OTA and STC were found. With respect to T-2, it was identified in 7 samples with a concentration ranging from 0.55 to 0.74 μg/L, while the HT-2 was found in 9 samples (concentration ranging from 0.49 to 0.87 μg/L). These mycotoxins were only detected in this matrix. In addition, there are previous reports on the presence of these type-A trichothecenes in oat beverage (Miró-Abella et al., 2017). T-2 and HT-2 usually contaminate oat crops, and therefore an important carry-over is observed (Edwards, Barrier-Guillot, Clasen, Hietaniemi, & Pettersson, 2009; Janavičienė, Mankevičienė, Kochiieru, & Venslovas, 2022). In this matrix, the OTA was detected in one sample at 0.741 μg/L, which is close to the maximum amount of this toxin allowed in some products like wine (EC_2023/915, 2023).

In the rice beverage, $AFB_1$ was detected in five samples in a concentration that ranged from the LOQ to 0.09 μg/L, STC, an aflatoxin biosynthetic precursor, was detected and in all samples (Anjorin, Ariyo, Peter, Sulyok, & Krska, 2021). In the case of soy beverage, the $AFB_1$ was detected in one sample at 0.06 μg/L, the emerging compounds NEO and MPA were also detected at trace levels. These results are in agreement with previous data that reported the contamination of soy beverage with $AFB_1$ in the range of 1.6 to 3.9 ng/L (Rezaeefar, Nemati, Farajzadeh, Afshar Mogaddam, & Lotfipour, 2022). Regarding the almond beverage, OTA was present in three samples, but in only one with concentration above the LOQ. $AFB_1$ was found in five samples, in a concentration lower than 0.04 μg/L and only one sample was contaminated with $AFB_2$ with levels below the LOQ. In this case, there can also happened a carry-over from raw materials since almonds are usually contaminated with aflatoxins. The observed levels are close to the maximum amount of



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aflatoxin $M_1$ allowed in milk (EC_2023/915, 2023). Therefore, the obtained data revealed that soy beverage is the matrix with the lowest levels of contamination, and, in order to avoid risks to consumers, $AFB_1$ should be monitored in rice and almond beverage, and T-2 and HT-2 toxin in oats.

## 4. Conclusions

A new method based on a QuEChERS extraction procedure followed by UHPLC-MS/MS detection was developed for the simultaneous analysis of 29 mycotoxins in plant-based beverages. The method was validated for the determination of mycotoxins in oat, rice, soy and almond beverages, and shows good performance characteristics including linearity, repeatability, accuracy, and precision. This study revealed that plant-based beverages are susceptible to mycotoxin contamination, especially beauvericin and enniatins, T-2 and HT-2 were found in oat beverage samples, and aflatoxins in rice and almond. Therefore, further investigation is needed to provide incidence data and evaluate the risk for consumers.

## CRediT authorship contribution statement

**Inés Rodríguez-Cañás:** Investigation. **Jesús M. González-Jartín:** Investigation, Writing – original draft, Writing – review & editing. **Amparo Alfonso:** Methodology, Writing – review & editing. **Rebeca Alvariño:** Investigation. **Mercedes R. Vieytes:** Methodology. **Luis M. Botana:** Funding acquisition, Supervision.

## Declaration of Competing Interest

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

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.137427.

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# DISCUSIÓN

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# 5. DISCUSIÓN

Las micotoxinas son compuestos tóxicos producidos por la actividad metabólica de ciertos hongos filamentosos. Estos contaminantes representan un riesgo para la salud humana y animal, ya sea por ingestión o por inhalación. Los hongos colonizan alimentos y piensos, provocándoles un deterioro que, en algunos casos, no es perceptible a simple vista. Los niveles máximos de contaminantes en alimentos destinados al consumo humano, así como las materias primas, estuvieron regulados inicialmente por el Reglamento (CE) Nº 1881/2006, que ha sido recientemente derogado por el Reglamento (UE) 2023/915 el cual establece niveles máximos permitidos, entre otros contaminantes, de las siguientes micotoxinas: AFs, OTA, PAT, DON, ZEA, $FB_1$, $FB_2$, CTN, HT-2 y T-2, así como también para esclerocios y alcaloides de cornezuelo (14).

En el caso de la alimentación animal, el Reglamento de la Comisión (UE) Nº 574/2011 que modifica el Anexo I de la Directiva 2002/32/CE sobre sustancias indeseables en alimentación animal establece, entre otros contaminantes, el contenido máximo permitido de $AFB_1$ y cornezuelo de centeno en materias primas y en piensos para animales destinados al consumo humano. Además, la Recomendación 2006/576/CE de la Comisión establece valores orientativos en materias primas de piensos y piensos compuestos para las siguientes micotoxinas: DON, ZEA, OTA, $FB_1$, $FB_2$, HT-2 y T-2 (16, 118).

La toxicidad e incidencia de las toxinas reguladas está bien establecida, sin embargo, se conocen más de 500 metabolitos producidos por hongos. De estos, se consideran micotoxinas emergentes aquellas que no están reguladas, pero han demostrado algún tipo de toxicidad *in vitro.* Además, todos estos compuestos pueden sufrir modificaciones en su estructura como resultado de la acción metabólica de animales, plantas u hongos. La detección de micotoxinas es compleja debido al gran número de matrices que pueden estar afectadas, el elevado número de compuestos y su variabilidad en cuanto a propiedades químicas. Por lo tanto, surge la necesidad de desarrollar métodos que permitan la detección y cuantificación fiable de estos contaminantes en alimentos y piensos (25, 119).

En la presente tesis doctoral, se han optimizado métodos de extracción y detección de micotoxinas con el fin de disponer de nuevas herramientas que permitan analizar múltiples matrices a lo largo de toda la cadena alimentaria, y dentro de esta, en el sector lácteo. En este sentido, se han desarrollado métodos dirigidos de UHPLC-MS/MS para evaluar la presencia de micotoxinas en materias primas obtenidas en distintas granjas y en productos finales adquiridos en supermercados locales. En primer lugar, se evaluaron productos destinados a la alimentación del ganado lechero, concretamente TMR (120). Por otro lado, se han desarrollado métodos destinados al análisis de queso y yogur, además de un método para el análisis de bebidas vegetales, empleadas como sustituto de productos lácteos. Por otro lado, se ha determinado la capacidad de *Gnomoniopsis smithogilvyi,* un patógeno causante de la podredumbre parda de la castaña, para producir micotoxinas y otros metabolitos secundarios, empleando un método no dirigido de UHPLC-MS-IT-TOF (121).

La Eurorregión formada por Galicia y el Norte de Portugal destaca por ser la principal zona de producción de leche en la península ibérica. En la industria agroalimentaria española, el sector lácteo es fundamental debido al gran volumen de consumo de leche y productos lácteos. Galicia lidera la producción de leche en ámbito nacional, porque supone un 55 % de las explotaciones. Así mismo, el sector lácteo es también uno de los pilares de la economía del Norte de Portugal. La producción de leche en la Eurorregión se incrementó debido a la ampliación de granjas y modernización de instalaciones impulsadas por la Política Agraria Común (122).

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Uno de los principales riesgos del sector lácteo es la presencia de contaminantes. Por lo tanto, es importante conocer la incidencia de micotoxinas en los productos destinados a la alimentación animal. En este sentido, el TMR consiste en una mezcla de distintas proporciones de forrajes, subproductos, vitaminas y aditivos que proporciona al ganado una dieta equilibrada y rica en nutrientes. El objetivo principal de la dieta TMR es asegurar que cada vaca pueda consumir los nutrientes necesarios en la cantidad adecuada. La composición del TMR varía según la zona, pero en la Eurorregión Galicia-Norte de Portugal está compuesta principalmente por silo (75 %), concentrado (20 %) que incluye maíz, cebada, soja, colza, DDG (granos de destilería desecados) y otros productos, y en menor proporción por forrajes secos como paja y alfalfa. Todos estos productos son susceptibles de ser contaminados por micotoxinas, por lo que se evaluó la presencia de estos contaminantes en muestras de TMR del Norte de Portugal recolectadas entre el 2019 y 2022. Asimismo, se estudió la posible transferencia de las micotoxinas presentes en el TMR a la leche animal. En primer lugar, se estudió si era posible la extensión de un método previamente validado para el análisis de silos a TMR. El método de extracción empleado, junto con el resto de los optimizados en la presente tesis doctoral, se ha basado en la metodología de extracción denominada bajo el acrónimo QuEChERS. Originalmente fue diseñado para el análisis de pesticidas, pero se ha demostrado que su uso es eficaz en el análisis de micotoxinas y otros contaminantes presentes en matrices complejas (123). El método original está formado por dos etapas. La primera consiste en una extracción sólido-líquido en la que normalmente se emplean disolventes orgánicos como acetona, acetato de etilo o acetonitrilo, este último es el más empleado porque es capaz de extraer compuestos con un amplio rango de polaridades. Si la muestra no posee un porcentaje elevado de agua (80-85 %) es recomendable adicionarla previamente. A continuación, mediante la adición de sales inorgánicas, se consiguen separar la fase acuosa y la fase orgánica. Las sales inorgánicas que se suelen utilizar son $MgSO_4$, $MgCl_2$, $NaNO_3$, NaCl, $Na_2SO_4$ y LiCl. La mezcla de $MgSO_4$ con NaCl logra una separación de fases adecuada y una buena forma de los picos cromatográficos. La segunda etapa consiste en una limpieza adicional del extracto, a través de una d-SPE, utilizando adsorbentes como N-propiletildiamina, PSA, carbon negro grafitizado o C18. El objetivo del adsorbente es eliminar interferencias como ácidos grasos, pigmentos y azúcares (123). El método empleado para el análisis de TMR había sido previamente optimizado para silos (124). En este sentido, se realizó una humectación de 2,5 g de muestra con 10 mL de una disolución acuosa de ácido acético al 1 %. Seguidamente, se añadieron 20 mL de acetonitrilo y se realizó la separación de las fases empleando 8 g de $MgSO_4$ combinados con 2 g de NaCl. Del extracto obtenido se evaporaron 0,1 mL y se redisolvieron con 0,4 mL de disolvente (acetonitrilo/agua/ácido acético [49/50/1 (v/v/v)]). Las alícuotas se filtraron (0,22 μm) previamente antes del análisis por UHPLC-MS/MS. Empleando el método de extracción descrito, se realizó una validación en términos de LOQ, precisión y exactitud para la determinación de 29 micotoxinas. Los LOQs de los métodos empleados en la tesis toctoral fueron calculados siguiendo la guías de EU-RL (125). En general los LOQ están en el rango de los obtenidos para el análisis de los silos (126). En el presente método de análisis se mantuvieron todas las toxinas reguladas y emergentes, pero se redujo el número de toxinas modificadas, eliminando principalmente toxinas derivadas de ZEA ya que no se han encontrado previamente en materias primas y proceden principalmente de la metabolización animal. De esta forma se consiguió mejorar la sensibilidad para algunos metabolitos como las AFs. En todos los casos, los LOQs están por debajo de los niveles máximos

permitidos o recomendados y por lo tanto la sensibilidad del método es adecuada. Por ejemplo, en el caso de $AFB_1$, el LOQ fue de 0,9 μg/kg, siendo el límite máximo permitido de 20 μg/kg.

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A continuación, se evaluó el efecto matriz que se define como la interferencia en la respuesta analítica debida a la presencia de compuestos secundarios distintos al analito que están presentes en la muestra. El factor SSE (signal suppression/enhancement) se calculó aplicando la siguiente ecuación:

$$\text{SSE}\ (\%) = \frac{\text{pendiente recta de calibrado en matriz blanca}}{\text{pendiente recta de calibrado en disolvente}} \cdot 100.$$

En este sentido, el efecto matriz se considera como la relación entre la respuesta del pico del analito en presencia de iones de la matriz y la respuesta del pico del analito en ausencia de iones de la matriz (127). Para el estudio del SSE se compararon las pendientes de las rectas de calibrado construidas en disolvente con las pendientes de las rectas de calibrado construidas en matriz.

La obtención de un valor superior al 100 % significa que existe una sobreestimación de la señal, mientras que un valor inferior al 100 % indica que existe supresión. Las toxinas mostraron valores dentro del rango 78-125 %. En definitiva, el efecto matriz fue bajo y por lo tanto, se evitan problemas de reproducibilidad, dando lugar a análisis más precisos y robustos.

Con el fin de evaluar la exactitud del método se estudió la $R_E$. Para ello, en primer lugar, se calcularon las $R_A$ mediante la fórmula $R_A\% = 100 \cdot \frac{\text{área experimental}}{\text{área teórica}}$. Se contaminaron artificialmente muestras blancas y se midió la cantidad de toxina extraída en base a una curva de calibración construida en disolvente. Los datos de $R_A$ se corrigieron con el factor SSE obteniendo de esta manera la $R_E$ del método, $R_E\,\% = 100 \cdot \frac{R_A}{\text{SSE}}$. Los valores fueron satisfactorios, cercanos al 70 %, variando entre el 63 % y el 94 %. La precisión se evaluó en base a la repetibilidad ($RSD_r$). La ecuación empleada fue la siguiente $\text{RSD}\,\% = \frac{S}{\bar{X}} \cdot 100$. La precisión del método fue alta ya que los valores de RSD estuvieron por debajo del 10 % para la mayoría de las toxinas, cumpliéndose los criterios de precisión y exactitud establecidos tanto en el Reglamento (CE) Nº 401/2006 como los nuevos criterios marcados en el Reglamento de Ejecución (UE) Nº 2023/278 (99, 117).

Aunque anteriormente se habían publicado algunos métodos validados para el análisis de micotoxinas en TMR, la mayoría de ellos han sido desarrollados para analizar una o un grupo reducido de toxinas (128-130). De forma contraria, el método propuesto en la presente tesis doctoral se ha validado para evaluar múltiples toxinas reguladas y emergentes de manera simultánea. Se analizaron 87 muestras de TMR, obtenidas en explotaciones lecheras en Vila do Conde, ubicado en el norte de Portugal, entre noviembre de 2019 y abril de 2022. De las 16 micotoxinas encontradas en las muestras, las FBs fueron las toxinas más frecuentes, ya que estaban presentes en el 74 % de las muestras. Por el contrario, la menos detectada fue la $AFB_1$ identificada en el 2 % de las muestras, y la OTA en un 9 %, mientras que DON y ZEA se detectaron en un 35 %. El concentrado es uno de los componentes del TMR y está formado, a su vez, por cereales como el maíz que suele contener niveles altos de DON, lo que justifica su amento en TMR (131).

Estudios previos realizados en TMR han revelado un perfil similar de micotoxinas, aunque la incidencia varía según la ubicación geográfica. Por ejemplo, un estudio llevado a cabo en el sur de Portugal detectó $AFB_1$ en el 8 % de las materias primas utilizadas en la producción de TMR. En contraste, la presencia de ZEA y DON fue notablemente mayor, aunque se evaluó un número limitado de muestras (132). De forma similar, una investigación previa que evaluó micotoxinas en TMR procedente de España reportó una prevalencia del 15 % para $AFB_1$, 17 % para DON, 16 % para ZEA y 34 % para FBs. La elevada aparición de AFs puede explicarse por la alta sensibilidad que presentaba el método UHPLC-FLD empleado para su análisis y por el

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

hecho de que el muestreo se realizó en explotaciones que habían presentado problemas relacionados con AFs en años anteriores (133). Por lo tanto, los resultados obtenidos coinciden con otros estudios realizados en la misma zona geográfica mientras que es inferior al de otros países como México o China (134, 135). En cuanto a las toxinas emergentes producidas por *Fusarium*, en concreto BEA y ENNs, han sido detectadas en un 88 % de las muestras, lo que coincide con un estudio previo realizado en México y con la alta incidencia observada en las muestras de silo. Por otro lado, toxinas producidas por *Aspergillus* y *Penicillium* se encuentran en porcentajes menores (124, 136).

El perfil de micotoxinas observado en TMR puede variar en función de la época del año en la que se realiza la toma de muestras. En este sentido, la baja prevalencia de FBs, ENNs y BEA en el año 2019 puede ser debida a que las muestras fueron recogidas en otoño. En cuanto al DON, se observó una notable reducción en 2021, cuando las 24 muestras se obtuvieron principalmente durante la primavera. En cambio, durante esta estación se observa un notable aumento en las muestras contaminadas con ZEA y ROQC. En cuanto a los niveles de contaminación medidos mediante UHPLC-MS/MS, se observó una concentración máxima de $AFB_1$ de 2,9 µg/kg, la cual está por debajo del límite permitido de 5 µg/kg en las materias primas destinadas a la alimentación animal. Los niveles de otras micotoxinas también se mantuvieron por debajo de los límites máximos recomendados para el ganado lechero, excepto en el caso de una muestra de ZEA que contenía 817 µg/kg, superando el límite de 500 µg/kg. En cuanto a las toxinas emergentes, como BEA y ENNs, se encontraron en concentraciones inferiores a 38 y 96 µg/kg, respectivamente. Por lo tanto, los datos obtenidos evidencian que la presencia de micotoxinas en TMR puede variar en función de la época del año, pero también puede variar en función del ciclo de producción ya que la composición del TMR varía en función de las distintas necesidades de la vaca. En cualquier caso, la presencia de toxinas reguladas como las FBs o emergentes como la BEA o ENNs es constante.

Con el fin de determinar el impacto que estas podrían tener en los humanos se evaluó el riesgo de que las toxinas pudieran ser arrastradas del TMR a la leche. Para ello se recogieron muestras de leche 24 horas después de alimentar al ganado con TMR. Las muestras de leche se analizaron empleando un método validado para esta matriz y que incluye 40 micotoxinas (72). En la leche solo se encontraron ROQC, BEA y ENNs. Se calculo la tasa de transferencia con el método de Guo et al. (137), siendo para la ROQC de un 2,8 %, para la BEA de un 1,06 % y para las ENNs la tasa se encontró en un rango de 4,85 % a 10,10 %. En este estudio se determinó por primera vez el parámetro de transferencia para estas toxinas. Hasta el momento, se había investigado solamente el arrastre de AFs con valores reportados entre el 1 % y el 6 %. También se había reportado la transferencia de T-2 y $FB_1$ con tasas inferiores al 2 % y al 0,05 % respectivamente (137, 138).

Este estudio muestra cómo se produce un arrastre de micotoxinas a lo largo de la cadena alimentaria, en concreto, del TMR a la leche. Por lo tanto, surge como nueva preocupación la contaminación indirecta de productos de origen animal, como los lácteos o incluso la carne. Como se ha mencionado en el trabajo anterior, en la leche se pueden detectar varias micotoxinas en niveles bajos de concentración. No obstante, la leche se emplea como materia prima para la elaboración de distintos productos lácteos, como el queso y el yogur, en los que se concentra a través de varios procesos tecnológicos. Por ejemplo, si la leche que se emplea para la elaboración del queso está contaminada con $AFM_1$, en el producto final la concentración será de 2,4 a 4,9 veces mayor que en la leche original. En este sentido, el uso de leche contaminada con 0,05 µg/kg de $AFM_1$, da lugar a quesos con niveles de concentración comprendidos entre 0,1 y 0,2 µg/kg (139).

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

El queso es uno de los productos lácteos más consumidos en Europa (140). En su elaboración se pueden emplear diferentes tipos de leche y una amplia gama de tecnologías para la fabricación de distintas variedades de quesos. En cuanto a la $AFM_1$, no existen límites regulados en queso, y en este caso se elige de referencia el nivel máximo permitido en leche y en productos derivados, es decir, 0,05 µg/kg. Además de las micotoxinas que pueden aparecer como consecuencia de usar leche contaminada, estos productos lácteos están expuestos al crecimiento de microorganismos, tanto de bacterias como de levaduras y hongos filamentosos (141). En algunos casos, la contaminación fúngica ocurre de manera intencionada ya que, con el fin de obtener unas características organolépticas específicas, en la fabricación y maduración de algunos quesos se utilizan ciertas especies de hongos filamentosos (142). Las especies empleadas suelen pertenecer al género *Penicillium*, como *P. roqueforti* y *P. camemberti*, que se utilizan en la elaboración de quesos azules y blancos, respectivamente (64). Por lo tanto, los quesos podrían ser una fuente de exposición a múltiples micotoxinas. Por ello, se optimizó y se validó un método UHPLC-MS/MS para determinar micotoxinas reguladas, emergentes y modificadas en queso, concretamente, en variedades de queso emmental, azul, brie y camembert.

El método se desarrolló a partir de un protocolo previo diseñado para analizar micotoxinas en leche (143). Sin embargo, se realizaron ciertas modificaciones debido a que la especie *P. camemberti* produce CPA, por lo tanto, esta micotoxina fue incluida en el método junto con la GLIO que también se había encontrado previamente en queso (144). El CPA es una toxina inestable y su detección es compleja. De hecho, algunos autores han descartado previamente la inclusión de CPA en métodos multi-micotoxina ya que no pudieron obtener curvas de calibración lineales. La inestabilidad del CPA puede deberse a su bajo pKa (2,94) (145). Con el fin de desarrollar unas condiciones cromatográficas que permitan su detección junto con el resto de toxinas, se evaluaron diez fases móviles. En todos los casos, la fase móvil A estaba compuesta por agua y la fase móvil B por mezclas de metanol/agua, conteniendo en ambos casos formiato de amonio o acetato de amonio y ácido fórmico o ácido acético. Se determinó que la presencia de ácido fórmico en cantidades iguales o superiores al 0,5 % era esencial para obtener una señal analítica adecuada. Añadir este aditivo al 1% provocó un incremento significativo en la señal, pero porcentajes superiores al 2 % resultaron en un ensanchamiento del pico. Empleando ácido fórmico al 1 %, se probaron distintas concentraciones de formiato de amonio, encontrando la mayor intensidad de señal a una concentración de 5 mM. Por consiguiente, se estableció una fase móvil A compuesta por agua con 1 % de ácido fórmico y formiato de amonio 5 mM, pH de 2,5, y una fase móvil B compuesta por metanol/agua/ácido fórmico 97/2/1 (v/v/v) con un pH de 4. Con las nuevas condiciones se obtuvo un pico de CPA bien definido mientras se mantenía la sensibilidad para la mayoría de los analitos incluidos en el método. Sin embargo, la sensibilidad a algunos compuestos como el DON y sus análogos DOM-1 y DON-3-Glu se vio disminuida, la probabilidad de encontrar estas toxinas en el queso es muy baja por lo que fueron eliminados del método. En cuanto al proceso de extracción, se basó en la metodología QuEChERS que se optimizó para conseguir una reducción del efecto matriz y la máxima extracción de las toxinas. Se observó que al emplear C18 para la limpieza de la muestra durante la etapa de d-SPE, las $R_A$ de las toxinas emergentes BEA y ENNs disminuían. Dado que estos compuestos son lipofílicos, existe la posibilidad de que la eliminación de lípidos por parte del C18 también elimine algunas micotoxinas que están adheridas a esa fracción. Por ello, se omitió la etapa de d-SPE. Además, dado que la $R_A$ de algunos compuestos era baja, se optimizó el porcentaje de ácido empleado en la extracción. En general, la solución acuosa que contenía ácido acético dio lugar a $R_A$ más altas que el ácido fórmico. Por ello, se eligió la solución de ácido acético al 2 % para la extracción. Finalmente,

USC DE COMPOSTELA

se estudió cómo la concentración de la muestra modifica el efecto matriz. Las muestras se extrajeron utilizando el método descrito anteriormente y se concentraron 0, 2, 5, 10 y 20 veces. En general, la concentración de la muestra conduce a una disminución de la $R_A$ debido a un aumento progresivo del efecto matriz. Incluso cuando la muestra no estaba concentrada, hubo una baja $R_A$ para muchas toxinas, lo que sugiere un alto efecto matriz. Por lo tanto, se evaluó el empleo de un extracto diluido (1/1 v/v) con una mezcla de acetonitrilo/agua/ácido acético 49/50/1 (v/v/v), observándose una importante reducción del factor SSE. Por este motivo, se eligió el extracto diluido para el análisis.

El método se validó y fue adecuado en términos de linealidad, LODs, LOQs, efecto matriz, exactitud y precisión. Según la legislación europea, la $AFM_1$ está regulada en leche en un nivel máximo permitido de 0,05 µg/kg en leche y productos derivados. Los LOQs obtenidos para las distintas variedades de queso fueron 0,282 µg/kg (emmental), 0,244 µg/kg (azul), 0,232 µg/kg (brie) y 0,224 µg/kg (camembert). En cuanto al efecto matriz, aun empleando una muestra diluida 1/1 (v/v) existe una fuerte supresión de la señal. La $_{RE}$ de las toxinas reguladas fue superior al 80% en las cuatro variedades de quesos y, por lo tanto, el método cumple con las regulaciones europeas. En cuanto a las toxinas emergentes y modificadas, las recuperaciones también estuvieron, en la mayoría de los casos, por encima del 80 %. En el caso de la BEA y ENNs, las $R_E$ solo fueron satisfactorias en queso emmental y en azul.

El método propuesto es capaz de detectar 32 micotoxinas en quesos comerciales, lo que mejora la metodología previa (146). Una vez validado, se empleó en el análisis de 38 muestras comerciales de queso. En general, mostraron una alta incidencia de toxinas emergentes de *Fusarium*, en concreto, BEA y ENNs, aunque fueron detectadas en niveles bajos, en el rango de concentración de 0,7 µg/kg a 29 µg/kg en el caso de la BEA, mientras que la concentración de las ENNs fue aún menor. Estos datos refuerzan la idea de que existe una importante transferencia de micotoxinas a lo largo de la cadena alimentaria.

Por otro lado, todas las muestras de queso azul contenían ROQC en concentraciones mayores a 250 µg/kg. Esto es debido a que en el proceso de maduración del queso se emplean cepas de *Penicillium*. Aunque se ha sugerido que la presencia de esta micotoxina en queso a los niveles habituales no supone un peligro para la salud pública, es necesario realizar más estudios (147). Además, serían necesarias más investigaciones que aporten datos de su toxicidad cuando se encuentra en combinación con otras toxinas, pues podrían existir efectos sinérgicos que aumenten la toxicidad.

Otra micotoxina presente en las muestras de queso brie, azul y camembert es la OTA, que fue detectada en concentraciones situadas entre 4,8 y 9,6 µg/kg. La OTA es considerada genotóxica y carcinógena del riñón. En el año 2020, la EFSA publicó un dictamen científico sobre los riesgos que presenta para la salud pública, concluyendo que el rumen de la vaca degrada esta toxina, por lo que los niveles en leche animal son bajos. No obstante, se requieren más datos sobre la presencia de OTA en alimentos, y en concreto, en queso (40).

Siguiendo la línea de los productos lácteos, se ha validado un método para el análisis de micotoxinas en yogur, un producto lácteo elaborado mediante un proceso de fermentación facilitado por bacterias ácido-lácticas. Este producto no solo se ha asociado con la prevención de enfermedades intestinales y la reducción de los niveles de colesterol, sino que también ofrece la versatilidad de poder ser fortificado con ingredientes funcionales bioactivos. Dentro de estos se encuentra una amplia gama de productos como frutas frescas, cereales, extractos de aloe vera o aceites esenciales (148). Por lo tanto, es crucial tener en cuenta que, además de la contaminación que puede proceder de la leche, existe la posibilidad de que las micotoxinas sean arrastradas desde los cereales o las frutas empleadas en la elaboración de yogures fortificados.

USC UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Con el objetivo de determinar el riesgo asociado a este producto lácteo, se ha optimizado y validado un nuevo método para el análisis de dos variedades de yogur: natural y de avena. La naturaleza de este alimento es completamente distinta a las anteriores matrices, principalmente por su mayor contenido en agua. Con el objetivo de desarrollar un nuevo método de extracción, el primer paso fue evaluar las condiciones ácidas ya que en los estudios previos se observó que tanto el tipo de ácido como su concentración pueden afectar la eficiencia de extracción. Dentro de las condiciones probadas, se decidió utilizar una concentración de ácido acético al 2 % ya que mostró un ligero aumento en la señal para ENNs, que previamente han mostrado bajas $R_E$ en productos lácteos como el queso.

El siguiente paso fue probar diferentes volúmenes de agua y acetonitrilo. La utilización de cantidades más altas de acetonitrilo que de agua (10 mL de acetonitrilo frente a 5 mL de agua) condujo a mayores recuperaciones de toxinas reguladas como DON, OTA y ZEA. Por el contrario, para ENNs y BEA, la recuperación se vio favorecida con mayores proporciones de agua. Por consiguiente, se optó por emplear una extracción con 10 mL de agua y 10 mL de acetonitrilo como la opción de compromiso para la extracción de todas las toxinas. Además, se observó que la utilización de altas cantidades de NaCl en el proceso de separación de las fases acuosa y orgánica mejora la $R_A$ de las AFs. Por lo tanto, se optó por emplear 4 g de $MgSO_4$ y 2 g de NaCl. El último paso del proceso de optimización consistió en estudiar la concentración más adecuada para el extracto. De entre todas las condiciones probadas, se seleccionó una muestra concentrada 5 veces para el análisis, ya que logra un equilibrio entre una mayor sensibilidad y los posibles efectos adversos del efecto matriz.

Una vez optimizado el proceso de extracción, el método se validó internamente utilizando muestras contaminadas artificialmente. Los LOQs fueron bajos, siendo de 0,007 µg/kg y 0,008 µg/kg para el yogur natural y de avena respectivamente, lo que indica que el método es lo suficientemente sensible como para detectar este contaminante a concentraciones inferiores al nivel máximo permitido (0,05 µg/kg) en la UE (99). Además, el método se encuentra dentro del rango de otros métodos previamente desarrollados para el análisis de micotoxinas, como la $AFM_1$ o la OTA, con la ventaja adicional de que en este caso es posible la detección simultánea de múltiples contaminantes (149).

A continuación, se evaluó el efecto matriz en la señal analítica. Los valores de SSE obtenidos en el yogur natural y de avena son similares. Por ejemplo, en el caso de la $AFM_1$, la señal disminuyó en un 40 % en ambas matrices, lo que concuerda con los hallazgos reportados en productos lácteos (150). Sin embargo, se observaron diferencias notables para otros compuestos como CTN, HT-2 y OTA. Los compuestos menos afectados fueron las ENNs y la BEA, con valores de SSE entre el 83 % al 96 %. Estas micotoxinas emergentes habían mostrado un comportamiento similar en leche (143).

Finalmente, se evaluó la exactitud y precisión a través del promedio y la RSD de la $R_E$ del proceso de extracción. La exactitud del método fue adecuada, ya que, en general, se obtuvieron $R_E$ superiores al 70 % para la mayoría de los compuestos. En el caso del yogur natural, las $R_E$ oscilaron entre 61 % (DON) y 112 % (AOH). Para el yogur de avena, las $R_E$ variaron entre 60 % ($AFG_1$) y 120 % (ZEA). Además, se observó una buena precisión del método, con valores de RSD inferiores al 15 % para los análisis realizados el mismo día e inferiores al 20 % para los análisis realizados en distintos días. Por lo tanto, este método se ajusta a las características mínimas de funcionamiento que deben cumplir los métodos para el análisis de micotoxinas reguladas según la legislación europea (99). Anteriormente se obtuvo un resultado similar utilizando un método de extracción que implica un paso de dilución y análisis por LC-MS/MS empleando un patrón interno marcado isotópicamente (151). Las micotoxinas emergentes como BEA y ENNs también mostraron buenas $R_E$, con valores que oscilaron entre el 62 % y el 97 %.

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Para realizar una primera evaluación de la presencia de micotoxinas en el mercado español, se obtuvieron 27 muestras de yogur natural y 16 muestras de yogur de avena en supermercados locales y se analizaron con el método descrito. El análisis reveló, al igual que en las anteriores matrices, una alta incidencia de las toxinas emergentes BEA y ENNs, aunque a bajas concentraciones. La incidencia de estas micotoxinas plantea la necesidad de obtener más datos de su toxicidad por si fuese necesario establecer límites máximos. Por otro lado, se detectó HT-2 en una muestra de yogur de avena, lo que podría indicar un arrastre a partir de la avena empleada en la elaboración del yogur, ya que este cereal es especialmente sensible a la contaminación por tricotecenos del tipo A, como la T-2 y la HT-2. Además, se encontró OTA en un 10 % de las muestras de yogur natural, lo que sugiere una posible transferencia de la leche al yogur, dado que estudios previos han reportado la presencia de OTA en leche y queso (152, 153). Finalmente, también se detectó ROQC, de hecho, todas las muestras de yogur de avena fueron positivas, aunque mostraron una relación señal-ruido inferior a 10, por lo que no fueron cuantificadas, mientras que 8 muestras de yogur natural fueron positivas y presentaron una concentración promedio de 0,16 µg/kg. Estos resultados resaltan la importancia de controlar los niveles de micotoxinas en los productos lácteos como el yogur para garantizar el máximo nivel de seguridad para los consumidores.

Por otra parte, se ha llevado a cabo una investigación sobre la incidencia de micotoxinas en productos que pueden ser utilizados como alternativas a los productos lácteos, específicamente en las bebidas vegetales. Estos nuevos productos son emulsiones diluidas obtenidas a partir de frutos secos, cereales, legumbres o semillas mezcladas con agua, aunque pueden incluir otros ingredientes (sal, azúcar, aditivos naturales…) para mejorar su valor nutricional o sabor. En los últimos años, debido a problemas de salud como intolerancias o alergias, se ha producido un aumento en el número de consumidores de este tipo de productos que no incluyen lactosa en su composición. Sin embargo, están formulados con ingredientes muy susceptibles a la contaminación por micotoxinas. Por lo tanto, es necesario disponer de métodos que permitan el control de los productos finales. Con este fin se optimizó y validó un nuevo método para analizar micotoxinas en bebidas de soja, avena, arroz y almendras. Además, se evaluó la presencia de micotoxinas en estos productos.

En primer lugar, para proceder al proceso de extracción se partió del método previamente optimizado para determinar micotoxinas en leche animal, por lo tanto, se empleó acetonitrilo con un 0,5 % de ácido fórmico para la extracción de 5 mL de muestra (72). Bajo estas condiciones, se estudió la concentración del extracto final en 0, 2,5, 5, 10 y 20 veces. En general, se observó que, al aumentar la concentración del extracto, la recuperación aparente disminuye, lo que indica una supresión de la señal analítica causada por componentes de la matriz. Sin embargo, las bebidas de arroz y almendras mostraron valores más altos cuando la muestra se analizó 2,5 veces concentrada. En el caso de las toxinas lipofílicas, concretamente BEA y ENNs, los valores más altos se alcanzaron cuando el extracto no estaba concentrado, especialmente en bebidas de avena, arroz y almendras. Para el análisis, se eligió una muestra cinco veces concentrada. A continuación, se investigó si la aplicación de un paso de limpieza adicional era efectiva para reducir el efecto matriz. Para ello se utilizó el adsorbente C18, ampliamente empleado en los procedimientos QuEChERS (154). Sin embargo, no se observaron mejoras significativas, por lo que se optó por realizar una extracción sin el paso de limpieza d-SPE.

Una vez optimizado el proceso de extracción, se estudiaron las características de funcionamiento del método. Se obtuvo una alta sensibilidad con LOQs en el rango de 0,019 a 5,16 µg/L, los cuales mejoran los reportados previamente en la literatura para algunas micotoxinas en bebidas de avena (155). En general, la matriz causó una supresión de señal que

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varía en función de la toxina y de la bebida, encontrándose la mayor supresión en un 11 % para $ENNB_1$ en bebida de arroz. Sin embargo, se observó una mejora de la señal para el grupo de las FBs. Estos resultados coincidieron con otros datos reportados previamente en bebidas de arroz y soja (155). El efecto de matriz para la HT-2 es más marcado en las bebidas de avena, arroz y soja (valores de SSE del 44 % al 58 %) en comparación con las de almendra (SSE del 85 %). De forma similar, se ha observado anteriormente un alto efecto de matriz para HT-2 y DON, con una supresión de señal del 39 % en bebidas de avena, soja, arroz y alpiste (156).

A continuación, se estudió la precisión del método mediante la recuperación en tres niveles de concentración. Las recuperaciones varían según la matriz y el nivel de contaminación, pero en general se alcanzaron valores superiores al 80 %. Sin embargo, existen algunas excepciones, como la de HT-2 y T-2 triol en bebida de soja, con valores del 45 % y 48 %, respectivamente, y de la CTN en bebida de arroz, con una recuperación del 21 %. En el caso de las AFs, se obtuvieron valores superiores al 65 % para todas las matrices en los tres niveles de contaminación (0,02, 0,09 y 0,19 µg/L). Por lo tanto, el método propuesto logra buenas recuperaciones para los distintos grupos de micotoxinas. La precisión se estudió mediante RSD de recuperaciones obtenidas por triplicado en el mismo día y en tres días consecutivos, obteniendo en ambos casos valores de RSD inferiores al 20 %. En este sentido, el método cumple con los criterios de funcionamiento establecidos en la legislación (99, 117). Por lo tanto, aunque el método propuesto incluye múltiples toxinas, mantiene unas características de rendimiento adecuadas.

Una vez validado, el método fue aplicado al análisis de 38 bebidas vegetales, concretamente: 9 de soja, 6 de arroz, 16 de avena y 7 de almendra. Se encontró una alta incidencia de micotoxinas emergentes, especialmente para BEA y ENNs. Estos resultados concuerdan con datos reportados previamente (157). Además, se detectaron HT-2 y T-2 en bebida de avena. Estas dos toxinas suelen infestar los cultivos de este cereal, por lo tanto, es posible que exista un arrastre desde la materia prima hacia la elaboración de la bebida vegetal. También se encontró OTA a un nivel de 0,741 µg/L en una muestra de bebida de avena, mientras que la $AFB_1$ se detectó en bebida de arroz (0,042-0,09 µg/L) y en una muestra de bebida de almendra, pero con una concentración inferior a 0,04 µg/L. De los cuatro tipos de bebidas vegetales analizados, la de soja resultó ser la matriz con niveles más bajos de contaminación. Por lo tanto, se necesita más investigación para proporcionar datos de incidencia y evaluar el riesgo para los consumidores.

Además de estudiar la presencia de micotoxinas mediante métodos dirigidos, en la presente tesis doctoral también se empleó un método no-dirigido por UHPLC-MS-IT-TOF con el fin de establecer si *G. smithogilvyi* era productor de micotoxinas. Este hongo es el principal agente causal de la pudrición parda del castaño a nivel mundial. En 2021 se produjo un importante brote de podredumbre del castaño en el sur de Galicia. Este hongo puede vivir endofíticamente y causar lesiones que pueden pasar desapercibidas, por lo que los humanos pueden consumir castañas afectadas por este hongo (158). Por ello, es importante determinar si esta especie de hongo es capaz de producir micotoxinas para determinar las consecuencias del consumo humano y animal. Con este fin se estudió el perfil de metabolitos secundarios producidos por *G. smithogilvyi*

Para el análisis del hongo se obtuvieron tres tapones de agar (6 mm de diámetro) de aislados cultivados en medio PDA. Estos se extrajeron con una mezcla de acetonitrilo/agua/ácido acético [49:50:1 (v/v/v)] y el extracto resultante se filtró y analizó mediante UHPLC-MS-IT-TOF en modo scan. De esta forma se identificaron varios picos que no estaban presentes en la muestra control (PDA sin hongo). En este sentido, se detectó un pico *m/z* 268,1051 a un tiempo de retención de 3,4 minutos y se identificó tentativamente como adenosina. La presencia de este

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compuesto se confirmó empleando un estándar analítico. Por otro lado, se estudió un pico *m/z* 360,2166 que eluyó a los 5,8 min. El compuesto se identificó tentativamente con la oxatesina, ya que la fórmula más probable y el patrón de fragmentación coincidió con este compuesto. De forma similar, el pico *m/z* 318,2992 que eluyó a 8 minutos fue identificado como fitoesfingosina (159). Por lo tanto, este estudio demostró que *G. smithogilvvi* no es una especie micotoxigénica, pero produce metabolitos con interesantes propiedades farmacológicas y fitotoxicológicas.

En resumen, en la presente tesis doctoral se han desarrollado métodos analíticos para la detección de micotoxinas en matrices alimentarias que no están controladas por las autoridades competentes, debido a que la legislación europea no las contempla todavía. En vista a los resultados obtenidos, las micotoxinas son contaminantes presentes en la cadena alimentaria y que, por transferencia, pueden pasar sufrir un arrastre desde una materia prima a un producto derivado. Este suceso podría suponer un riesgo en término de salud humana y animal. Es conveniente recopilar datos de incidencia sobre micotoxinas en nuevas matrices alimentarias para evaluar si es necesaria su regulación.

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# CONCLUSIONES

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# 6. CONCLUSIONES

1. La extracción tipo QuEChERS con la detección por LC-MS permite la cuantificación de hasta 32 micotoxinas en TMR, queso, yogur y bebidas vegetales.
2. La detección por LC-MS de CPA requiere la adición de ácido fórmico 0,03 M en las fases móviles.
3. *Gnomoniopsis smithogilvyi* produce adenosina, oxasetina y fitosfingosina.
4. Entre el 2 % y el 10 % de la BEA, ENNs y ROQC presente en el TMR es arrastrado a la leche.
5. La BEA y las ENNs se transfieren a lo largo de la cadena alimentaria a productos lácteos y bebidas vegetales.
6. En bebidas vegetales y yogures con base de avena se detectan las micotoxinas T-2 y HT-2.

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# BIBLIOGRAFÍA

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UNIVERSIDADE DE SANTIAGO DE COMPOSTELA

Las micotoxinas representan un riesgo para la salud humana y animal. En la presente tesis doctoral se desarrollan métodos analíticos basados en la cromatografía de líquidos acoplada a la espectrometría de masas. Los métodos creados permiten la detección y cuantificación de estos contaminantes en materias primas destinadas a la alimentación animal, en productos lácteos y en bebidas vegetales. En general, se detecta un alto porcentaje de muestras contaminadas con micotoxinas emergentes y, se demuestra su arrastre a lo largo de la cadena alimentaria. Además, se establece el perfil metabólico de Gnomoniopsis smithogilvyi, hongo responsable de la podredumbre en la castaña, descartando la producción de micotoxinas.