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TOXICOLOGICAL STUDY IN VITRO, IN VIVO AND TRANSCRIPTOMIC OF MYCOTOXINS

ESTUDIO TOXICOLÓGICO IN VITRO, IN VIVO Y TRANSCRIPTÓMICO DE MICOTOXINAS

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CERTIFICAN QUE:

La licenciada en Farmacia **Laura Escrivá LLorens** ha realizado bajo su dirección el trabajo que lleva por título **TOXICOLOGICAL STUDY IN VITRO, IN VIVO AND TRANSCRIPTOMIC OF MYCOTOXINS** y autorizan su presentación para optar al título de Doctor por la Universitat de València.

Y para que así conste, expiden y firman el siguiente certificado.

Burjassot (Valencia), Marzo, 2018

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Este trabajo ha dado lugar a 12 artículos publicados o que se publicarán en las siguientes revistas científicas:

1. A preliminary repeated dose 28-day oral study in Wistar rats with enniatin A contaminated feed (2014) *Toxicology Mechanisms and Methods 24, 179-90*. Impact Factor: 1.517

2. *In vivo* Toxicity Studies of *Fusarium* Mycotoxins in the last decade: A Review (2015) *Food and Chemical Toxicology* 78, 185-206. Impact Factor: 3.584

3. Quantitation of Enniatins in Biological Samples of Wistar Rats after Oral Administration by LC-MS/MS (2015) *Toxicology Mechanisms and Methods* 25, 552-558. Impact Factor: 1.576

4. Analysis of Trichotechenes in Laboratory Rat Feeds by Gas Chromatography-Tandem Mass spectrometry (2016) *Food Additives and Contaminants: Part A* 33, 329-338. Impact Factor: 2.047

5. Mycotoxin Contamination in Laboratory Rat Feeds and their Implications in Animal Research (2016). *Toxicology Mechanisms and Methods* 26, 529-537. Impact Factor: 1.595

6. Studies on the Presence of Mycotoxins in Biological Samples: an Overview. Toxins (2017) 251, 1-33. Impact Factor: 3.030

7. Effects of Quercetin against Mycotoxin induced Cytotoxicity: a Mini-review (2017) *Current Nutrition and Food Science* 13, 1-7. Impact Factor: 0.55

8. Multi-Mycotoxin Analysis in Human Urine by GC-MS/MS: a Comparative Extraction Study (2017). *Toxins, 330, 1-15.* Impact Factor: 2.047.

9. Alternaria Mycotoxins In Food And Feed: An Overview *(2017)*. *Journal of Food Quality, ID: 1569748.* Impact Factor: 0.968

10. In Vitro Study of Beauvericin and Enniatin B Effects on Human Lymphoblastoid Jurkat T-cell Model (2018). *Food and Chemical Toxicology*. Impact Factor: 3.778

11. Transcriptomic Study of the Toxic Mechanism Triggered by Beauvericin in Jurkat Cells (2018). *Toxicology Letters, 284, 213-221*. Impact Factor: 3.858

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1884
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- Red Nacional de Micotoxinas y Hongos Toxigénicos y sus procesos de descontaminación (MICOFOOD).

En algún lugar, algo increíble está esperando a ser descubierto.

Carl Sagan

A mi familia, por no dejar de creer en mí.

A mi padre, por su apoyo incondicional.

A todos aquellos que me habéis acompañado en este largo camino.

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List of Abbreviations

ACAT	Acil-CoA:colesterol aciltransferasa
ACN	Acetonitrilo
ADME	Absorción, Distribución, Metabolismo y Excreción
AFB1	Aflatoxina B1
AFB2	Aflatoxina B2
AFG1	Aflatoxina G1
AFG2	Aflatoxina G2
AFs	Aflatoxinas
AOH	Alternariol
ALN	Altenuene
AME	Alternariol methyl ether
ASE	Extracción acelerada con disolventes
ATX	Alterotoxinas
BEA	Beauvericina
CAT	Catalasa
CE	Electroforesis capilar
CHCl3	Cloroformo
CIT	Citrinina
d-SPE	Dispersión en fase sólida
DAD	Diodos
DAS	Diacetoxiscirpenol
DEGs	Genes diferencialmente expresados
DLLME	Microextracción líquida-líquida dispersiva
DON	Deoxinivalenol
DON-Glc	Deoxinivalenol-glucósido
EC	Electroquímico
EFSA	Autoridad Europea de Seguridad Alimentaria
ENs	Eniatinas

EtOAc	Acetato de etilo
FAO	Food Agriculture Organization
FDA	Food and Drug Administration
FBs	Fumonisinas B
FD	Fluorescencia
FUS	Fusaproliferina
FUS-X	Fusarenon-X
GC	Cromatografía de gases
GLI	Gliotoxina
GSH	Glutation reducido
HPLC	Cromatografía líquida de alto rendimiento
HT-2	Toxina HT-2
IAC	Columnas de inmunoafinidad
IC	Concentración inhibitoria
LC	Cromatografía líquida
LD50	Dosis letal 50
LLE	Extracción líquido-líquido
LOAEL	Nivel más bajo con efecto adverso observable
LOD	Límite de detección
LOQ	Límite de cuantificación
MAE	Extracción asistida por microondas
MeOH	Metanol
MMP	Potencial de membrana mitocondrial
MON	Moniliformina
MS	Espectrometría de masas
MS/MS	Espectrometría de masas en tándem
NEO	Neosolaniol
NGS	Next generation sequencing
NIV	Nivalenol
NOAEL	Nivel sin efecto adverso observable
OMS	Organización Mundial de la Salud

OTA	Ocratoxina A
PCR	Reacción en cadena de la polimerasa
PLE	Extracción líquida presurizada
QUER	Quercetina
qRT-PCR	PCR cuantitativa transcriptasa inversa
QuEChERS	Quick, Easy, Cheap, Effective Rugged and Safe
RNA-seq	Secuenciación masiva del ARN
ROS	Especies reactivas de oxígeno
SOD	Supeóxido dismutasa
SPE	Extracción en fase sólida
SPME	Microextracción en fase sólida
STE	Sterigmatocistina
T-2	Toxina T-2
TEA	Ácido tenuazónico
TEN	Tentoxina
TLC	Cromatografía de capa fina
UAE	Extracción asistida por ultrasonidos
UPLC	Cromatografía líquida de ultra alto rendimiento
UV	Ultravioleta
ZEN	Zearalenona
15-ADON	15-acetildeoxinivalenol
3-ADON	3-acetildeoxinivalenol

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SUMMARY

Mycotoxins, produced by secondary metabolism of filamentous fungi, are common food and feed contaminants, and they constitute a concern of food safety. The present work studied the toxic effects and mechanisms of action of the emerging mycotoxins Beauvericin (BEA) and Enniatins (ENs) through *in vitro* tests in Jurkat cells, *in vivo* assays in Wistar rats, and transcriptomic approach by RNA-seq; as well as multi-mycotoxin presence in food, feed, and biological samples.

The reviews indicated that (i) the *in vivo* research of fusarotoxins were mainly based on subacute and subchronic toxicity studies with pigs and rats orally exposed to mycotoxins such as Fumonisin B1, Deoxynivalenol (DON) and Zearalenone (ZEN), as well as mycotoxin combinations generally including DON; (ii) the main objectives of mycotoxins analysis in biological samples were method development, biomonitoring studies, and exposure assessment, with human urine as the most analyzed biological sample and Ochratoxin A (OTA), ZEN and DON as the most studied mycotoxins. Despite the high frequency of mycotoxins detected in biological samples, the low quantified levels suggested probable daily intakes generally lower than the permited tolerable intakes; (iii) the most analyzed *Alternaria* mycotoxins in food and feed were Alternariol, Alternariol Methyl Ether, Tentoxinone and Tenuazonic Acid, detecting the highest concentrations in legumes, tomatoes and cereals.

The results of the laboratory rat feed analysis demonstrated high incidence of multi-mycotoxin contamination mainly with ZEN, ENs and DON.

The *in vitro* assays indicated decrease in cell viability, cell cycle arrest, and apoptosis activation with increase of caspase activity after EN B and BEA

exposure to Jurkat cells. BEA demonstrated more pronounced *in vitro* effects, as well as, genotoxic potential. The review about Quercetin showed *in vitro* protective activity against the cytotoxic effect of mycotoxins such as OTA, DON, ZEN, BEA and ENs, suggesting their intake in food as a potential strategy to prevent mycotoxin-induced toxicity.

The *in vivo* studies showed absence of observed adverse effects after subacute administration of EN A and single oral dose administration of ENs in Wistar rats, despite their detection in serum, organs and tissues, thus confirming their intestinal absorption after oral administration.

The transcriptomic study revealed human genome preturbation after Jurkat cells exposure to BEA and EN B, indicating mitochondrial damage with alteration of the oxidative phosphorylation and electron transport chain pathways.

Further studies are needed to evaluate the potential risk of the exposure to these compounds and thus, to protect human and animal health.

RESUMEN

Las micotoxinas, producidas por el metabolismo secundario de hongos filamentosos, se consideran contaminantes comunes de alimentos y piensos y constituyen una preocupación en el ámbito de la seguridad alimentaria. Se han estudiado los efectos tóxicos y mecanismos de acción de las micotoxinas emergentes Beauvericina (BEA) y Eniatinas (ENs) mediante ensayos *in vitro* en células Jurkat, *in vivo* en rata Wistar y transcriptómicos por secuenciación del ARN; así como la presencia de multi-micotoxinas en alimentos, piensos y muestras biológicas.

Las revisiones realizadas ponen de manifiesto que (i) la investigación *in vivo* de fusarotoxinas se basa principalmente en estudios de toxicidad subaguda y subcrónica con cerdos y ratas expuestos por vía oral a micotoxinas como Fumonisina B1, Deoxinivalenol (DON) y Zearalenona (ZEN); (ii) los objetivos principales del análisis de micotoxinas en muestras biológicas son el desarrollo de métodos analíticos, estudios de biomonitorización y evaluación de la exposición, siendo la orina humana la muestra biológica más analizada y destacando Ocratoxina A (OTA), ZEN y DON como las micotoxinas más estudiadas. A pesar de la elevada frecuencia de micotoxinas detectadas en muestras biológicas, los bajos niveles cuantificados indican que las ingestas diarias probables son generalmente inferiores a las ingestas tolerables permitidas; (iii) las micotoxinas de *Alternaria* más analizadas en alimentos y piensos son Alternariol, Alternariol Metil Éter, Tentoxinona y Ácido Tenuazónico, detectando las mayores concentraciones en legumbres, tomates y cereales.

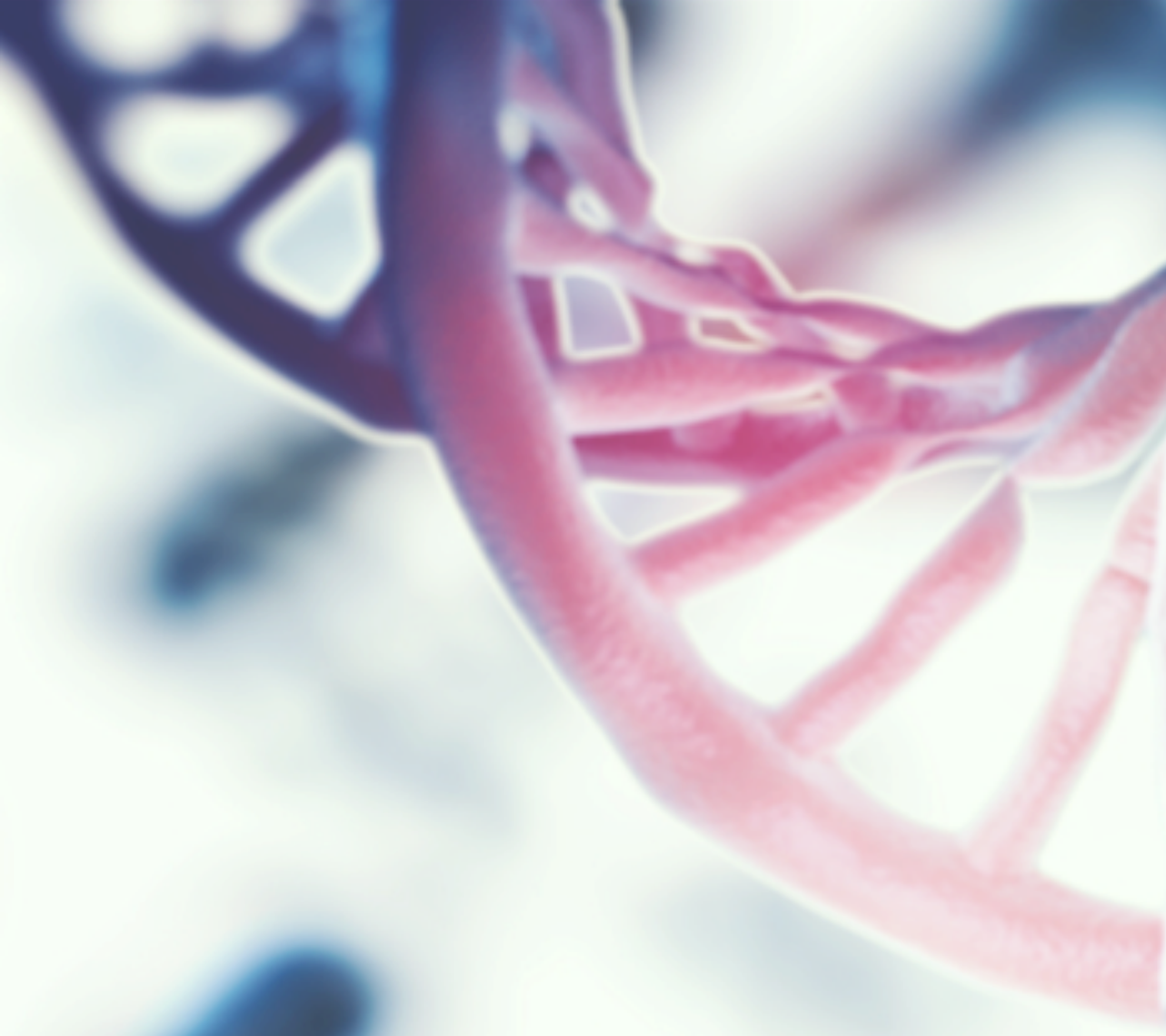
Los resultados del análisis de piensos de rata de laboratorio demuestran una elevada incidencia de contaminación por multi-micotoxinas, principalmente ZEN, ENs y DON.

Los ensayos *in vitro* muestran disminución de la viabilidad celular, arresto del ciclo celular y activación de la apoptosis con aumento de la actividad de las caspasas tras la exposición de células Jurkat a EN B y BEA, demostrando esta última efectos *in vitro* más pronunciados, así como potencial genotóxico. La revisón bibliográfica de la Quercetina revela actividad protectora *in vitro* frente al efecto citotóxico de micotoxinas como OTA, DON, ZEN, BEA y ENs, sugiriendo su ingesta en alimentos como una estrategia potencial para prevenir la toxicidad inducida por micotoxinas.

Los estudios *in vivo* muestran ausencia de efectos adversos observables tras la administración subaguda de EN A y administración única vía oral de ENs en rata Wistar, a pesar de detectar las micotoxinas en suero, órganos y tejidos, confirmando así su absorción intestinal tras administración oral.

El estudio transcriptómico revela alteración del genoma humano tras la exposición de células Jurkat a BEA y EN B, indicando daño mitocondrial con alteración de las vías de fosforilación oxidativa y cadena de transporte de electrones.

Se necesitan nuevos estudios que permitan la evaluación del riesgo potencial de la exposición a estos compuestos para proteger así la salud humana y animal.



1. INTRODUCTION

1. INTRODUCCIÓN

La contaminación de alimentos y piensos con micotoxinas sigue considerándose un problema de seguridad alimentaria y un desafío permanente para la salud humana y animal. Su origen natural y producción ubicua a causa del metabolismo secundario de diversos hongos filamentosos posiciona a estos compuestos como contaminantes inevitables e impredecibles de los alimentos (Hojnik et al., 2017). En este sentido, las micotoxinas plantean un reto difícil en la inocuidad de los alimentos, tal y como indica el informe más reciente de sistema de alerta rápida ante los peligros en seguridad alimentaria (RASFF: Rapid Alert System for Food and Feed), en el que de un total de 3049 notificaciones registradas en 2015, 475 correspondieron a notificaciones relacionadas con la presencia de micotoxinas en alimentos. Estos datos sitúan a la categoría de las micotoxinas en segundo lugar del total de notificaciones, por detrás de las referentes a microorganismos patógenos y seguido de residuos de pesticidas, observándose un aumento considerable respecto al año anterior (RASFF, 2015).

Además de la ingestión directa de micotoxinas a través de alimentos contaminados de origen vegetal existe creciente preocupación sobre la posible ingestión de estos compuestos tóxicos a través alimentos de origen animal que pueden contener residuos de micotoxinas o sus metabolitos (Alshannaq and Yu, 2017). Como consecuencia, los seres humanos están diariamente expuestos a mezclas de micotoxinas a través del consumo de una dieta variada a lo largo de su vida (Figura 1).

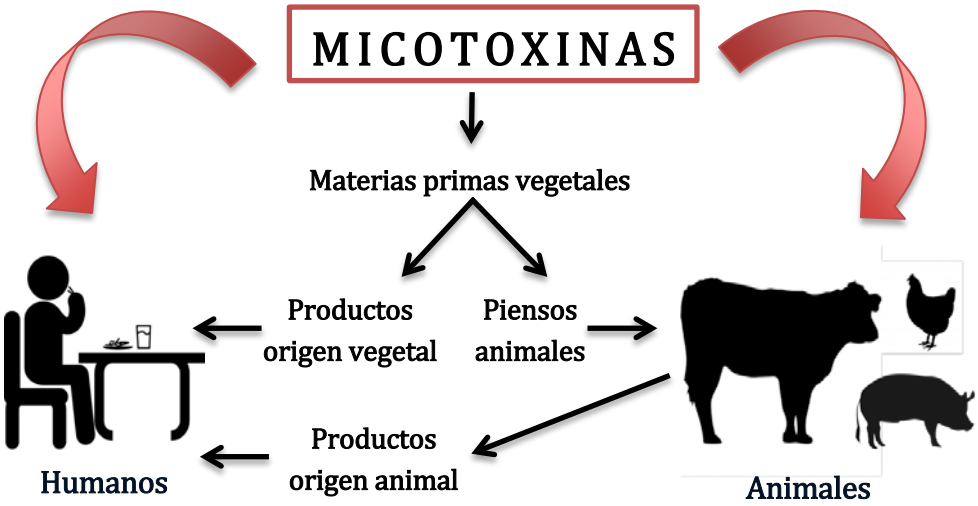


Figura 1. Exposición a micotoxinas a través de los alimentos.

1.1 Toxicidad de las Micotoxinas

La exposición a micotoxinas puede causar intoxicaciones denominadas micotoxicosis. Las micotoxinas pueden ser letales ingeridas en grandes cantidades durante un corto período de tiempo, mientras que la exposición a pequeñas dosis durante períodos prolongados representan un mayor riesgo crónico, pudiendo derivar en un ampila gama de efectos nocivos para la salud humana y animal (Antonissen et al., 2014). La gran variedad de síntomas clínicos derivados de la intoxicación por micotoxinas incluye efectos agudos con alta morbilidad cursando con diarrea y vómitos, así como trastornos crónicos como disminución del crecimiento, inmunosupresión, aumento de la susceptibilidad a enfermedades infecciosas, efectos dérmicos, trastornos reproductivos, daño hepático y renal, edema pulmonar y hemorragias, llegando incluso a ser responsables de efectos graves como cáncer y trastornos neurológicos (Egbuta et al., 2017). La Agencia

Internacional para la Investigación del Cáncer (IARC) ha clasificado a varias micotoxinas como agentes probados carcinógenos para los seres humanos, Grupo 1: Aflatoxinas B1, B2, G1 y G2 (AFB1, AFB2, AFG1, AFG2), o probables, Grupo 2B: Ocratoxina A (OTA), Fumonisinas B1 y B2 (FB1, FB2), y Aflatoxina M1 (AFM1) (IARC, 1993). En la Tabla 1 se muestran los principales grupos de micotoxinas, sus efectos tóxicos y el órgano diana.

La gravedad de los efectos que producen las micotoxinas depende, en gran medida, de la potencialidad tóxica, las cantidades ingeridas, la duración de la exposición y las interacciones que pueden resultar de la ingestión simultánea de diferentes micotoxinas. Una combinación de micotoxinas en bajas concentraciones puede originar efectos nocivos, a pesar de que las concentraciones de micotoxinas individuales estén por debajo de las concentraciones establecidas como tóxicas (Grenier and Oswald, 2011).

La extensa variedad de sintomatología descrita por la exposición a metabolitos secundarios fúngicos se debe principalmente a la heterogeneidad de compuestos que se engloban dentro de esta categoría, formada por compuestos de bajo peso molecular (<1000 daltons) con estructuras químicas muy diversas (Egbuta et al., 2017).

Tabla 1. Principales micotoxinas, efectos tóxicos y órgano diana.

Micotoxina	Efecto tóxico	Órgano diana
Aflatoxinas	Carcinógeno, Hepatotóxico, Inmunosupresor	Hígado
Ocratoxina A	Carcinógeno, Teratógeno, Inmunosupresor, Nefrotóxico	Riñón, Hígado
Fumonisninas	Carcinógeno, Hepatotóxico, Nefrotóxico, Inmunosupresor	Tracto Gastrointestinal, Hígado, Riñón
Deoxinivalenol	Náuseas, Vómitos, Diarrea, Effectos Reproductivos	Órganos Reproductivos, Tracto Gastrointestinal

Nivalenol	Anoréxico, Inmunotóxico, Hematotóxico	Tracto Gastrointestinal, Sistema Inmune
Toxina T-2	Hepatotóxico, Neurotóxico, Inmunosupresor	Tracto Gastrointestinal, Sistema Inmune
Zearalenona	Alteración Hormonal, Efectos Reproductivos	Órganos Reproductivos
Citrinina	Nefrotóxico	Riñón
Patulina	Nefrotóxico, Daño Intestinal	Riñón, Intestino

1.2 Análisis de Micotoxinas

Debido a que la mayoría de las micotoxinas presentan toxicidad a concentraciones muy bajas, se requiere la aplicación de métodos sensibles, eficaces y precisos para su detección. Un método de detección adecuado debe ser robusto y reproducible, con alto grado de aplicabilidad para una amplia gama de compuestos. Sin embargo, debido a la gran diversidad química que presentan las micotoxinas no es posible utilizar una única técnica estándar para detectar todas ellas (Turner et al., 2009).

Con el fin de poder determinar concentraciones muy bajas de micotoxinas, tanto en muestras alimentarias como biológicas, es necesario: (1) la extracción del compuesto o compuestos de interés y purificación de las micotoxinas del extracto inicial, (2) la separación individual de las micotoxinas del resto de componentes del extracto, y (3) la detección por equipos analíticos altamente selectivos y sensibles. Por lo tanto, todos los métodos analíticos pueden dividirse generalmente en las etapas de extracción-purificación, separación y detección.

1.2.1 Extracción y Purificación

La preparación y pre-concentración de la muestra es uno de los pasos cruciales en la determinación de micotoxinas, conociéndose con este fin gran

número de procedimientos de extracción. Sin embargo, en las últimas décadas, se ha puesto énfasis en la minimización del uso de disolventes, especialmente clorados nocivos para el medio ambiente y la salud, así como en la miniaturización de los procedimientos (Cigić y Prosen, 2009).

La *extracción líquido-líquido* (LLE*)* implica aprovechar la diferente solubilidad de las micotoxinas en dos fases inmiscibles para extraer los compuestos diana en un disolvente dejando el resto de la matriz en el otro (Yang et al., 2014). Para tal fin, se debe en primer lugar seleccionar el disolvente de extracción apropiado, considerando tanto la estructura química de las micotoxinas objeto de análisis como la composición de la matriz. Generalmente se utilizan tanto disolventes acuosos como orgánicos y combinaciones de los mismos, siendo los más utilizados el acetonitrilo (ACN), cloroformo (CHCl3), metanol (MeOH), y acetato de etilo (EtOAc) (Turner et al., 2009; Escrivá et al., 2017).

La clásica LLE puede favorecer la eficiencia de extración y reducir el uso de disolventes orgánicos con técnicas como *extracción asistida por microondas* (MAE) o *extracción asistida por ultrasonidos* (UAE) (Kwasniewska et al., 2015). La *extracción líquida presurizada* (PLE), también conocida como *extracción acelerada con solventes* (ASE) se basa en una LLE en la que se aumenta mediante presión el punto de ebullición del disolvente para retener su fase líquida. El disolvente penetra mejor en la muestra en un proceso automatizado, reduciendo el tiempo de extracción (Cao et al., 2013).

La técnica de *microextracción líquida-líquida dispersiva* (DLLME) se basa en un sistema ternario de disolventes en el que la combinación de un disolvente dispersante y uno extractante conduce a la dispersión de finas gotas de extracción en la fase acuosa, aumentando el área de contacto

superficial entre la fase orgánica y acuosa, y facilitando así el alcance del estado de equilibrio (Yan and Wang, 2013). Dependiendo del disolvente de extracción utilizado, el método DLLME puede clasificarse en dos grandes categorías: los que utilizan un disolvente de extracción de baja densidad y los que utilizan uno de alta densidad (Leong et al., 2014).

La adición de una sal inorgánica en una mezcla acuosa con un disolvente orgánico miscible en agua, fuerza la separación del disolvente de la mezcla y la formación de un sistema bifásico en una técnica conocida como *extracción líquido-líquido asistida por sales* (SALLE) (Zhang et al., 2009).

La metodología QuEChERS *(Quick, Easy, Cheap, Effective Rugged and Safe)* consiste en una extracción con ACN y sales, generalmente sulfato de magnesio o sulfato de sodio, seguido de un paso de purificación por dispersión en fase sólida (d-SPE). De esta forma se amplía el intervalo de polaridad de los compuestos susceptibles al análisis, a la vez que permite la purificación de extractos utilizando cantidades más pequeñas de disolventes orgánicos no clorados. Desde su introducción, la técnica QuEChERS ha ido sufriendo modificaciones en cuanto a los reactivos utilizados y las proporciones de los mismos para mejorar la precisión y minimizar los efectos de la matriz (Rejczak and Tuzimski, 2015).

La *extracción en fase sólida* (SPE) es una alternativa más rápida en comparación con la LLE, basada en la retención de analitos en un sorbente especial contenido en una minicolumna con alta afinidad para unirse a moléculas pequeñas (Zheng et al., 2006). Sin embargo, es importante seleccionar la fase sólida más apropiada para las micotoxinas a extraer con el fin de obtener tasas de recuperación altas y estables; siendo comunes los cartuchos de gel de sílice (Saengtienchai et al., 2014), C-18 (Camel et al.,

2012), resinas de polímeros de alta resistencia y materiales de intercambio iónico (Heyndrickx et al., 2015), inmunoadsorbentes y polímeros de impresión molecular (MIP) (de Andrés et al., 2008).

Los cartuchos *MycoSep* son columnas multifuncionales compuestas de varios absorbentes específicamente seleccionados para la recuperación de micotoxinas individuales, siendo un método práctico, portátil y rápido, también utilizado en etapas de purificación. Sin embargo, puesto que las columnas están diseñadas por analito no son útiles en el análisis de multi-micotoxina (Tzanetou and Kasiotis, 2003).

El método de *microextracción en fase sólida* (SPME) emplea fibras recubiertas con diferentes fases estacionarias sobre las que se extraen las micotoxinas a partir de muestras acuosas o gaseosas, lo que permite un análisis fácil y rápido de compuestos orgánicos sin necesidad del uso de disolventes (Vatinno et al., 2008).

Por otra parte, la pureza de la muestra afecta a la resolución de la separación y a la sensibilidad de los resultados. En ocasiones, los extractos de muestras alimentarias o biológicas lo constituyen mezclas complejas donde los bajos niveles de micotoxinas pueden ser enmascarados por compuestos interferentes que, o bien dificultan la separación o eluyen al mismo tiempo que el analito de interés (Yang et al., 2014). Es por ello que, en ocasiones, se requiere una etapa de purificación adicional principalmente basada en columnas de inmunoafinidad (IAC), es decir, anticuerpos inmovilizados que ofrecen una excelente recuperación dada su alta especificidad; o alternativas más económicas como cartuchos SPE y columnas MycoSep (Danicke y Winkler, 2015).

1.2.2 Separación y Detección.

Existen gran variedad de técnicas selectivas y sensibles para la separación de micotoxinas, incluyendo varios métodos de cromatografía y electroforesis. La electroforesis capilar (CE) permite la separación eficaz de micotoxinas en base a su carga y masa, sin embargo, a pesar de su versatilidad nunca ha ganado tal popularidad como las técnicas cromatográficas (Cigić y Prosen, 2009). La cromatografía de capa fina (TLC) fue muy común y popular en los primeros años de investigación de las micotoxinas debido a su simplicidad, practicidad y bajo coste, no obstante, a día de hoy ha quedado muy limitada debido a una precisión insatisfactoria y separación deficiente en comparación con la cromatografía líquida de alto/ultra alto rendimiento (HPLC y UPLC) y la cromatografía de gases (CG) (Xie et al., 2016). La HPLC permite su combinación con múltiples sistemas de detección como ultravioleta (UV), diodos (DAD), electroquímicos (EC), fluorescencia (FD), y espectrometría de masas (MS). La detección de FD es por su naturaleza altamente específica y sensible, y comprende mayor simplicidad y menor coste en comparación con la MS. Es por ello que se han establecido métodos HPLC-FD fiables, robustos y sensibles especialmente para la determinación de micotoxinas con fluorescencia natural como la Zearalenona (ZEN), AFs, OTA y Citrinina (CIT) (Cigić y Prosen, 2009). Sin embargo, la aparición de detectores altamente sensibles como la espectrometría de masas en tándem (MS/MS) ha originado que el campo de la cromatografía se expanda rápidamente en las últimas décadas, permitiendo determinar gran número de micotoxinas de forma simultánea con elevado rendimiento y fiabilidad (Turner et al., 2015). La LC-MS/MS se ha convertido en el método de referencia en el campo del análisis de micotoxinas, permitendo la detección altamente precisa y

específica de compuestos polares no volátiles (Yang et al., 2014). Asimismo, la GC-MS/MS es responsable de determinaciones cuanti y cualitativas de compuestos caracterizados por una estabilidad térmica y volatilidad satisfactoria. Debido a que la mayoría de las micotoxinas no son volátiles, se requiere un paso previo de derivatización, es decir, modifcación química para convertir los grupos hidroxilo, carboxilo y amino en sus derivados éster, acilo y alquilo con el fin de obtener materiales volátiles (Suomela et al., 2012).

Finalmente, los métodos de inmunoafinidad basados en la reacción específica antígeno-anticuerpo proporcionan una alternativa conveniente y sensible para la detección de micotoxinas debido a su relativo bajo coste y fácil aplicación. Sin embargo, en ocasiones puede producirse reactividad cruzada entre los anticuerpos y los componentes de la matriz dando señales no específicas y pudiendo obtener falsos positivos o sobreestimación, por lo que se requiere posterior confirmación de los resultados mediante otro método analítico (Meulenberg, 2012).

La gran variedad de metodologías desarrolladas para el análisis de micotoxinas es todavía más extensa, y teniendo en cuenta la complejidad de las muestras susceptibles a contaminación, encontrar la metodología más apropiada plantea, en ocasiones, un arduo reto (Turner et al., 2015). En las últimas décadas, las técnicas de extracción, separación y detección previamente descritas han sido combinadas y aplicadas al análisis de innumerables micotoxinas en todo tipo de matrices alimentarias como cereales y derivados, frutos secos, frutas y sus zumos, vegetales, especias, etc; así como al análisis de micotoxinas en muestras biológicas, desde orina y suero humano, hasta órganos y tejidos de animales. En la Tabla 2 recogen

algunos de estos estudios analíticos indicando las micotoxinas de estudio, la matriz alimentaria o biológica y las técnicas de análisis utilizadas.

Tabla 2. Análisis de micotoxinas en alimentos y muestras biológicas mediante diversas metodologías de extracción, separación y detección.

Micotoxinas	Muestra	Extracción	Separación-Detección	Referencia
ENs, BEA, FUS	Pasta	LLE	HPLC-MS/MS	Serrano et al. 2013
DON-ZEN y metbolitos, NIV, DAS, NEO, FUS-X	Orina humana	SALLE	GC-MS/MS	Rodríguez-Carrasco et al. 2014
AFs, FBs, T-2 y HT-2	Harinas de trigo y maíz, pasta, alimentos horneados, cereales desayuno	QuEChERS	LC-MS/MS	Annunziata et al. 2017
ENs	Harina, pasta, cereales de desayuno y galletas a base de trigo	QuEChERS	LC-MS/MS	Stanciu et al. 2017
DON-ZEN y metbolitos, NIV, FUS-X	Harina de trigo	QuEChERS	UPLC-MS/MS	Zhou et al. 2016
DON, NIV, ZEN, AFs, FBs, T-2, HT-2, OTA, CIT	Arroz	QuEChERS	UHPLC–MS/MS	Koesukwiwat et al. 2014
DON, NIV, ZEN, AFs, FBs, T-2, HT-2, OTA, CIT, GLI,	Mantequilla de sésamo y cacahuete	QuEChERS	HPLC-MS/MS	Liu et al. 2014
STE, etc. DON-ZEN y metbolitos, NEO, AFs, OTA, FBs, HT-2, AME, STE	Especias; pimienta, chile	QuEChERS	HPLC-MS/MS	Yogendrarajah et al. 2013
OTA	Cereales de desayuno	PLE	HPLC-FD	Zinedine et al. 2010
AFs	Avellanas, almendras, nueces, cacahuetes	PLE	HPLC-FD	Campone et al. 2009

Micotoxinas	Muestra	Extracción	Separación-Detección	Referencia
AFs, FBs, OTA, DON-ZEN y metbolitos, NIV, T-2 y HT2, NEO, DAS, etc.	Orina, sangre, heces, saliva, secreciones nasales, hígado, bazo, pulmón, riñón, estómago, colon, cerebro	PLE	HPLC-MS/MS	Cao et al. 2013
AFs, ZEN, OTA	Cacahuetes	DLLME	HPLC-FD	Chen et al. 2017
AHO, AME, TEN	Tomate, productos de tomate	DLLME	HPLC-MS/MS	Rodríguez-Carrasco et al. 2017
CIT, AHO, AME	Zumos de frutas	DLLME	HPLC-FD	Ruan et al., 2016a
DON	Arroz	DLLME	HPLC-DAD	Rahmani et al. 2017
ENs	Agua	DLLME	HPLC-MS/MS	Serrano et al. 2016
AFs, FBs, ZEN, DON, FUS-X, T-2 y HT-2, OTA, CIT, STE	Nueces, cacahuetes, almendras, pistachos, avellanas, semillas de girasol y calabaza	QuEChERS+ DLLME	UHPLC-MS/MS	Arroyo-Manzanares et al., 2013
OTA, CIT	Peras, uvas, manzanas	UAE+DLLME	HPLC-FD	Ruan et al. 2016b
AFs, OTA, ZEN	Trigo, maíz	SPE-C18	UPLC-MS/MS	La Barbera et al. 2017
PAT	Alimentos elaborados con manzana	SPE-MIP	UHPLC–UV	Lucci et al. 2017
AFs	Trigo, maíz, arroz, cebada, avena	SPME	HPLC-FD	Quinto et al. 2009

1.2.3 Validación de Métodos Analíticos

Con el objetivo final de determinar si se requiere una acción regulatoria, la Autoridad Europea de Seguridad Alimentaria (EFSA) se encarga de realizar la evaluación del riesgo de las micotoxinas presentes en alimentos y piensos, mediante la integración del conocimiento sobre la exposición humana a micotoxinas a través de la alimentación, con la información sobre el potencial de estos compuestos para provocar efectos adversos para la salud. A pesar de ello, para poder establecer contenidos máximos de micotoxinas en alimentos y piensos se debe disponer previamente de métodos analíticos que cuenten con el nivel de precisión y sensibilidad adecuados. En esta línea, se han establecido una serie de criterios generales que los métodos analíticos deberán cumplir para ser considerados válidos para el propósito que fueron desarrollados.

A nivel europeo, documentos como SANCO/12571/2013 y Commission Decision 2002/657/EC describen los requisitos de validación de métodos analíticos y el control de calidad necesario para apoyar la validez de los datos obtenidos y utilizados, tanto para establecer límites máximos de micotoxinas como para verificar el cumplimiento de la normativa ya establecida. Los principales parámetros analíticos evaluados para determinar la calidad de un método analítico incluyen: linealidad y rango lineal, efecto de matriz, exactitud, precisión, repetibilidad, reproducibilidad, límite de detección (LOD) y límite de cuantificación (LOQ).

Mediante el cumplimiento de estos requisitos se proporciona una garantía y un control de calidad armonizados y rentables en la Unión Europea, se garantiza la calidad y la posibilidad de comparación de resultados

analíticos, se asegura una precisión aceptable y se garantiza la ausencia de falsos positivos o falsos negativos (SANCO 2013).

1.3 Legislación de Micotoxinas

Desde el descubrimiento de las micotoxinas a comienzos de los años sesenta, las autoridades sanitarias han reconocido los potenciales riesgos para la salud humana y animal originados por la contaminación de alimentos con estos compuestos tóxicos. Por esta razón, se han establecido distintas normativas regulatorias con el fin de proteger al consumidor de los efectos nocivos sobre la salud derivados de la exposición a estos contaminantes, principalmente a través de los alimentos.

En la Unión Europea, el establecimiento de límites o contenidos máximos de micotoxinas en alimentos y piensos es responsabilidad del Comité Científico para la Alimentación Humana y la Sanidad Animal, dependiente de la EFSA. Tras realizar la correspondiente evaluación de riesgo en base a datos toxicológicos y datos sobre la presencia de micotoxinas en materias primas y alimentos; y teniendo en cuenta los hábitos de consumo, la Comisión Europea aprobará, si se requiere, la publicación de dichos límites o contenidos máximos en forma de Directiva, Reglamento o Recomendación para su cumplimiento obligatorio/recomendado por todos los estados miembros. En este sentido, la Unión Europea ha establecido niveles máximos de micotoxinas en diferentes alimentos para AFs (B1, B2, G1, G2, M1), OTA, Patulina, (PAT) Deoxinivalenol (DON), ZEN, y FBs (B1 y B2) a través de la Regulación 2006/1881/EC (European Commission 2006a), y posteriormente valores guía para las toxinas T-2 y HT-2 mediante la Recomendación 2013/165/EC (European Commission 2013). En lo referente a la alimentación

animal, únicamente se han establecido contenidos máximos para la AFB1 mediante la Directiva 2002/32/EC (European Commission 2002) y valores guía para OTA, DON, ZEN, FBs (FB1 y FB2), T-2 y HT-2 en piensos y materias primas destinadas a la alimentación animal, a través de las Recomendaciones 2006/576/EC y 2013/165/EC (European Commission 2006b; 2013). En la Tablas 3a y 3b se muestran los valores máximos y valores guía para dichas micotoxinas en alimentos y piensos, respectivamente.

Tabla 3a. Valores máximos y valores guía para micotoxinas en alimentos. *Reg. (regulación), Recom. (recomendación).*

Micotoxina	Alimentos	Valores máximos/ guía (mg/kg)	Referencia
AFB1	Cereales, derivados de cereales, maíz, frutos secos, especias, alimentos infantiles, alimentos dietétitcos	0.1-8	Reg.1881/2006/EC
Suma AFs B1, B2, G1, G2	Cereales, derivados de cereales, maíz, frutos secos, especias, alimentos infantiles, alimentos dietétitcos	4-15	Reg.1881/2006/EC
AFM1	Leche cruda, alimentos para lactantes	0.025-0.5	Reg.1881/2006/EC
OTA	Cereales, derivados de cereales, café, vino, zumo de uva, frutos secos, cerveza, cacao y derivados, productos cárnicos, especias y regaliz, alimentos infantiles, alimentos dietétitcos	0.5-10	Reg.1881/2006/EC
PAT	Zumos y néctares de frutas, derivados de manzana, alimentos infantiles	10-50	Reg.1881/2006/EC
DON	Cereales, derivados de cereales, alimentos infantiles	200-1750	Reg.1881/2006/EC
ZEN	Cereales, derivados de cereales, maíz, alimentos elaborados a base de maíz, alimentos infantiles	20-200	Reg.1881/2006/EC
Suma FB1, FB2	Maíz y alimentos elaborados a base de maíz	200-2000	Reg.1881/2006/EC
Suma T-2, HT-2	Cereales, derivados de cereales	0.015-1	Recom.2013/165/EU

Tabla 3b. Valores máximos y valores guía para micotoxinas en piensos y materias primas para piensos. *Recom. (recomendación), Dir. (directiva).*

Micotoxina	Valores máximos/ Valores guía (mg/kg)	Referencia
Piensos y materias primas para piensos		
Aflatoxina B1	0,005-0.2	Dir. 2002/32/EC
Ocratoxina A	0.05-0.25	Recom. 2006/576/EC
Deoxinivalenol	0.9-12	Recom. 2006/576/EC
Zearalenona	0.1-3	Recom. 2006/576/EC
Suma FB1 y FB2	5-60	Recom. 2006/576/EC
Suma T-2 y HT-2	0.225-2	Recom. 2013/165/EU

De las más de 400 micotoxinas descritas en la actualidad tan solo unas pocas están legisladas, representando una minoría respecto al total de micotoxinas potencialmente contaminantes de alimentos y piensos. Un claro ejemplo son las micotoxinas emergentes de *Fusarium,* que carecen de normativas regulatorias principalmente debido a la limitada información disponible sobre su toxicidad y toxicocinética, en comparación con las micotoxinas legisladas. La comunidad científica tiene por tanto el deber de proporcionar datos relevantes obtenidos de estudios *in vivo* e *in vitro,* que permitan conocer el riesgo que suponen estos compuestos tóxicos para la salud humana y animal, así como determinar si se precisan nuevas medidas regulatorias (Fraeyman et al., 2017).

1.4 Micotoxinas Emergentes de *Fusarium*

El género *Fusarium* incluye más de 90 especies descritas y produce tres de las clases de micotoxinas más importantes con respecto a la salud humana y animal; los Tricotecenos, las Fumonisinas y las Zearalenonas. Estas micotoxinas se consideran, junto con las Aflatoxinas, las micotoxinas más prevalentes y nocivas para la salud y la productividad animal, así como

responsables de extensas y recurrentes pérdidas económicas. Sin embargo, el género *Fusarium* produce también un grupo de micotoxinas menos estudiadas hasta la fecha y conocidas como micotoxinas emergentes dado su posterior descubrimiento, que incluye Beauvericina (BEA), Eniatinas (ENs), Fusaproliferina (FUS) y Moniliformina (MON) (Escrivá et al., 2015).

1.4.1 Beauvericina

BEA es una micotoxina con estructura cíclica hexadepsipeptídica que contiene de forma alterna tres residuos D-hidroxi-isovaleril- (2-hidroxi-3-metilbutanoico) y tres residuos N-metilfenilalanina unidos por enlaces peptídicos y enlaces de ésteres intramoleculares (Figura 2) (Mallebrera et al., 2014). Las principales especies fúngicas productoras de BEA incluyen *Beaveria bassiana* y varias especies de *Fusarium* como *F. acuminatum, F. armeniacum, F. anthophilum, F. avenaceum, F. beomiforme, F. dlamini, F. equiseti, F. fujikuroi, F. globosum, F. langsethiae, F. longipes, F. nygamai, F. oxysporum, F. poae, F. proliferatum, F. pseudoanthophilum, F. sambucinum, F. semitectum, F. sporotrichioides, y F. subglutinans* (Smith et al., 2016). BEA se encuentra principalmente contaminando cereales y productos derivados de cereales y su presencia ha sido confirmada en diferentes países como Finlandia, Noruega, Suecia, Suiza, Países Bajos, Eslovaquia, Croacia, Alemania, Italia, Marruecos, Túnez y España; así como en EEUU y Sudáfrica. BEA se ha detectado prácticamente en todos los cereales analizados que incluyen trigo, cebada, centeno, arroz, maíz y avena, incluso en alimentos elaborados a base de cereales como pan y alimentos para bebés, en ocasiones en concetraciones elevadas alcanzando los mg/kg (Luz et al., 2017).

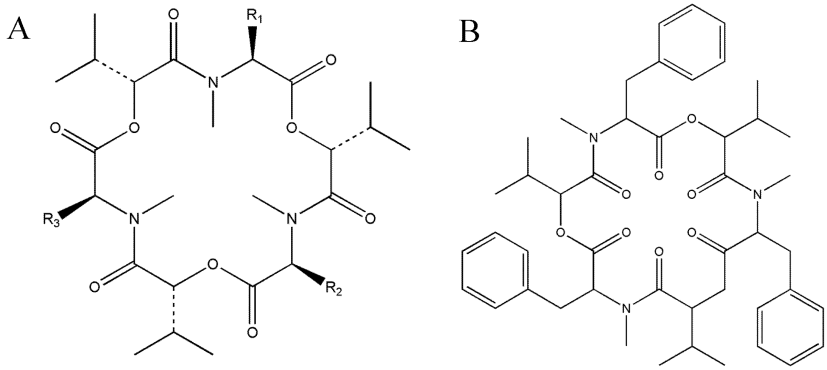
BEA muestra propiedades insecticida y antiviral, y una fuerte actividad antibacteriana frente a bacterias patógenas humanas, animales y vegetales (Wang y Xu, 2012). Como compuesto lipófilo e ionóforo, BEA aumenta la permeabilidad iónica de membranas biológicas formando complejos con cationes esenciales (Ca^{2+}, Na^{+}, K^{+}) y canales selectivos de cationes en las membranas lipídicas, afectando a la homeostasis iónica y desacoplando la fosforilación oxidativa (Tonshin et al., 2010). BEA perturba principalmente el equilibrio iónico y el pH citoplásmico en la membrana mitocondrial por la acumulación de Ca^{2+}, lo que conduce a un aumento de los niveles intracelulares de especies reactivas de oxígeno (ROS) y disminución de glutation reducido (GSH) como signos de estrés oxidativo. La pérdida de la integridad de la membrana mitocondrial puede finalmente conducir a la degeneración celular, necrosis y/o apoptosis (Schoevers et al., 2016).

1.4.2 Eniatinas

Las ENs son un grupo de compuestos estructuralmente relacionados con estructura química similar a BEA; hexadepsipéptidos cíclicos que alternan residuos de ácido D-hidroxi-isovaleril- (2-hidroxi-3-metilbutanoico) con differentes residuos N-metilaminoácido como valina e isoleucina. Las especies de *Fusarium* productoras de ENs incluyen *F. acuminatum, F. avenaceum, F. langsethiae, F. lateritium, F. poae, F. proliferatum, F. sambucinum, F. sporotrichioides,* y *F. tricinctum* (Smith et al., 2016). Se conocen al menos 29 análogos de las ENs agrupados en diferentes series (A, B, C, D, E, F, G, H, I, J, K, L, M, N, O y P) en función de los grupos funcionales que contenga su estructura. Sin embargo, tan sólo siete ENs han sido detectadas en productos alimentarios de forma natural, presentando una mayor incidencia

las ENs A, A1, a B y B1, consideradas por tanto de mayor interés. Mientras que la EN A y la EN B presentan tres residuos de isoleucina y tres residuos de valina, respectivamente, las variantes A1 y B1 sustituyen uno de los residuos por el aminoácido del grupo opuesto (Figura 2).

Las ENs A, A1, B y B1 se han detectado en todos los cereales investigados como trigo, cebada, centeno y avena en países que incluyen Finlandia, Noruega, Alemania, Italia, España y Marruecos, siendo las ENs B y B1 las más frecuentes y detectadas en mayores concentraciones, con niveles máximos superiores a 18 y 5 mg/kg, respectivamente (EFSA, 2014). La presencia de ENs ha sido confirmada en productos derivados de cereales, así como en piensos y materias primas para la elaboración de piensos, detectándose con muy elevada frecuencia y en concentraciones superiores a los 5 mg/kg (Streit et al., 2013). Concentraciones traza de ENs y BEA han sido también reportadas en alimentos de origen animal como carnes de ave y huevos (Gruber-Dorninger et al., 2017).



Enniatin	R_1	R_2	R_3
A	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$
A_1	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)_2$
B	$-CH(CH_3)_2$	$-CH(CH_3)_2$	$-CH(CH_3)_2$
B_1	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$	$-CH(CH_3)CH_2CH_3$

Figura 2. Estructura química de la ENs A, A1, a B y B1 (A) y BEA (B).

Las ENs han demostrado actividad fitotóxica, citotóxica y apoptótica, con propiedades antibacterianas, antihelmínticas, antifúngicas e insecticidas (Kamyar et al., 2004). Las principales acciones tóxicas de las ENs se deben a sus propiedades lipófilas e ionofóricas. Del mismo modo que BEA, las ENs son capaces de promover el transporte de cationes mono y divalentes a través de membranas biológicas conduciendo acciones tóxicas mediante la perturbación de las concentraciones fisiológicas normales de estos iones (Tonshin et al., 2010). Las ENs han demostrado ser citotóxicas en diferentes líneas celulares en concentraciones micromolares, pudiendo originar muerte celular por apoptosis, bien a través de la vía mitocondrial o por la inducción de la necrosis vinculada al daño lisosómico. Sin embargo, existen datos contradictorios sobre si las ENs desencadenan la producción de ROS, así como de sus efectos genotóxicos (Gruber-Dorninger et al., 2017).

La toxicidad potencial de BEA y ENs y su presencia en altas concentraciones en alimentos condujo a la EFSA a elaborar una opinión científica en 2014 sobre los riesgos para la salud humana y animal relacionados con la presencia de estas micotoxinas emergentes en alimentos y piensos. Sin embargo, debido a la falta de datos de toxicidad disponibles no fue posible realizar la evaluación del riesgo. Según indicó la EFSA, son necesarios datos relevantes de toxicidad *in vivo* para llevar a cabo una evaluación del riesgo en humanos. Desde entonces, aunque se han publicado nuevos estudios centrados en la presencia, la toxicocinética y el metabolismo de estas micotoxinas emergentes, la EFSA sigue recogiendo información científica relevante sobre estos compuestos tóxicos (Escrivá et al 2015b).

1.5 Estudios *in vitro* de Beauvericina y Eniatinas

La toxicidad *in vitro* de BEA y ENs ha sido investigada en varias líneas celulares incluyendo células intestinales porcinas; IPEC-J2 (Springler et al., 2016), renales porcinas; PK15 (Klaric et al., 2006; Klaric et al., 2008), ovario de hámster chino; CHO-K1 (Lu et al., 2013; Mallebrera et al., 2016; Zouaoui et al., 2016), sanguíneas periféricas de pavo (Dombrink-Kurtzman et al., 2003), hepatoma y glioma rata; H4IIE y C6 (Watjen et al., 2014), adrenales humanas; H295R (Kalayou et al., 2015), pulmonares humanas NSCLC A549 (Lin et al., 2005; Lu et al., 2016), carcinoma de colon humano; Caco-2, HT-29, HCT116 (Meca et al., 2011; Prosperini et al., 2013a; 2013b; Watjen et al., 2014), carcinoma hepático humano; Hep-G2 (Meca et al., 2011; Juan-García et al., 2015), leucemia aguda humana; CCRF-CEM (Jow et al., 2004), carcinoma de cérvix humano; KB, KBv200 y KB-3-1 (Tao et al., 2015), leucocitos y linfocitos humanos (Klaric et al., 2010; Celik et al., 2010), células dendríticas y macrófagos humanos (Ficheux et al., 2013).

Está generalmente aceptado que el mecanismo principal de toxicidad de BEA y ENs se relaciona con sus propiedades ionóforas, las cuales facilitan su inserción en la membrana celular y formación de poros selectivos a cationes afectando a la homeostasis iónica celular. El aumento del Ca^{2+} intracelular origina la pérdida del potencial de membrana mitocondrial (MMP) desencadenando la liberación del citocromo c al citosol, induciendo una mayor actividad de la caspasa-3 y resultando finalmente en muerte celular por apoptosis y/o necrosis (Fraeyman et al., 2017). Sin embargo, son varios los mecanismos específicos a nivel celular que han sido descritos para estas micotoxinas emergentes y que siguen siendo debatidos por la comunidad científica (Figura 3).

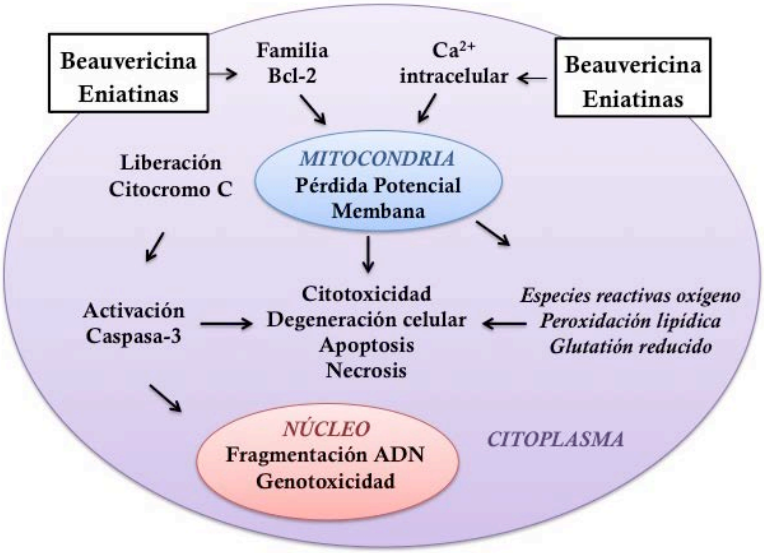


Figura 3. Mecanismos de citotoxicidad propuestos para BEA y ENs.

La exposición a BEA (8 μM; 72 h) en linfocitos sanguíneos periféricos de pavo originó la fragmentación del ADN internucleosomal y características morfológicas de la apoptosis como condensación citoplásmica (Dombrink-Kurtzman et al., 2003). BEA (1 μM; 24 h) incrementó la fragmentación nuclear y formación de células apoptóticas con liberación de citocromo c, y aumento de caspasa-3 de manera dosis y tiempo dependiente en células de leucemia humana (CCRF-CEM) (Jow et al., 2004). BEA (0.05-5 μg/ml; 24-48 h) también condujo a apoptosis en células pulmonares humanas NSCLC A549 de manera dosis y tiempo dependiente con reducción del MMP, aumento de la liberación del citocromo c, y activación de la caspasa-3, junto con regulación positiva de Bax, Bak y Bad y negativa de Bcl-2. Además, el tratamiento con el inhibidor de la caspasa-3 previno la actividad de la caspasa-3 y la muerte celular, demostrando que la apoptosis inducida por BEA implica múltiples

vías celulares y moleculares (Lin et al., 2005). Posteriormente, el tratamiento con BEA (0.05-5 μg/ml; 24-48 h) en la misma línea celular pulmonar (A549) condujo a apoptosis, activación la vía de señalización MEK1/2 (proteína quinasa quinasa activada por mitógeno)-ERK42/44 (quinasas reguladas por señal extracelular)-90RSK (ribosomal s6 quinasa). El tratamiento con el inhibidor de MEK1/2 atenuó el cambio morfológico apoptótico inducido por BEA, disminuyó las células apoptóticas y la vía de señalización MEK1/2-ERK42/44-90RSK, confirmando las múltiples vías y mecanismos de acción en la apoptosis inducida por BEA (Lu et al., 2016). Sin embargo, la regulación de Bcl-2 o Bax no pareció estar implicada en el proceso apoptosis tras la exposición a BEA (3-12 μM; 24 h) en células de carcinoma de cérvix humano (KB, KBv200 y KB-3-1), observándose pérdida del MMP, liberación de citocromo c, activación de caspasas -9 y -3, y escisión de PARP (Tao et al., 2015).

BEA (0.5-25 μM; 4, 48 h) mostró ser altamente citotóxica en varias líneas celulares (HepG2, C6, Hct116 y H4IIE), originando muerte celular por necrosis en células de glioma C6, y apoptosis en células de hepatoma H4IIE con actividad de caspasa-3/7 y fragmentación nuclear. En las células H4IIE, BEA produjo fuerte inhibición de la señalización de NF-kB, disminución de la fosforilación de las proteínas quinasas activadas MAPKy ERK con aumento de JNK, e inhibición selectiva de 21 proteínas quinasas implicadas en las vías de transducción de señales, proliferación celular, supervivencia, angiogénesis y metástasis, incluyendo el proto-oncogén-proteína tirosina quinasa src (Watjen et al., 2014). BEA (1-10 μM; 24-168 h) mostró efectos en la maduración de ovocitos porcinos y en el desarrollo embrionario preimplantacional, así como citotoxicidad en células HT29, sugiriendo

diferentes vías y mecanismos de acción e indicando toxicidad potencial en el sistema reproductivo (Schoevers et al., 2016). La exposición a BEA (0.05-5 μg/ml; 24-48 h) en células porcinas PK15 redujo la viabilidad celular, aumentó la peroxidación lipídica (LPO) y disminuyó el GSH (Klaric et al., 2006) con cambios en la LDH, actividad de la caspasa-3 e índice apoptótico, de manera dosis y tiempo dependiente (Klaric et al., 2008). BEA (0.5 μM; 24h) provocó daño significativo del ADN en la misma línea celular porcina PK15 y en menor grado en leucocitos humanos de manera dependiente de la dosis (Klaric et al., 2010). La exposición a BEA (1.25-10 μM; 24-48 h) en linfocitos humanos disminuyó significativamente el índice mitótico, provocó aberraciones cromosómicas, intercambios entre cromátidas hermanas y micronúcleos, indicando genotoxicidad (Celik et al., 2010). En células CHO-K1 BEA (0.6-20 μM; 24, 48, 72 h) mostró citotoxicidad, interrupción de actividad enzimática mitocondrial y apoptosis, produciendo además roturas del ADN y aumento de la actividad de las enzimas superóxido dismutasa (SOD) y catalasa (CAT) (Zouaoui et al., 2016; Mallebrera et al., 2016).

Por otra parte, las ENs A, A1, B y B1 (0.6-5 μM; 24, 48, 72 h) también han mostrado efectos citotóxicos de forma dosis dependiente en en células CHO-K1 (Lu et al., 2013) y en tres líneas celulares tumorales humanas; adenocarcinoma colorrectal epitelial humano (Caco-2), carcinoma de colon humano (HT-29) y carcinoma hepático humano (Hep-G2), reportando la EN A1 como la más citotóxica entre las ENs estudiadas; A, A1, B, B1 y B4 y J3 (15-30 μM; 24-48 h) (Meca et al., 2011). La EN B (0.1-100 μM; 24, 48, 72 h) mostró citotoxicidad en células H295R mediante reducción de la viabilidad y alteración de la distribución del ciclo celular. Además originó la reducción de

hormonas y modulación de genes a dosis no citotóxicas indicando posible toxicidad endocrina suprarrenal (Kalayou et al., 2015)

La exposición a BEA y ENs (0.5-25 μM; 24, 48, 72 h) en células Caco-2 produjo disminución de GSH, aumento del glutatión oxidado, incremento y generación temprana de ROS, aumento de LPO, muerte celular apoptótica con pérdida del MMP, necrosis, daño en el ADN (12 μM) y reducción de la fase G0/G1 con arresto en G2/M (24h) y S (72h) (Prosperini et al., 2013a; 2013b). Concentraciones similares de BEA y ENs (1-3 μM; 24, 48, 72 h) mostraron bloqueo en G1, disminución del porcentaje de células necróticas/apoptóticas y alteración del MMP en células de hepatocarcinoma humano HepG2 (Juan-García et al., 2015). Contrariamente, otros estudios indican que ni el incremento ROS ni el daño en el ADN son elementos clave en la citotoxicidad mediada por BEA y ENs (0.1-10 μM; 24, 72 h) en células A549, GLC-4, KB-3-1, HL-60, aunque reportan inhibición de la actividad enzimática de las topoisomerasas I y II, implicadas en la replicación, transcripción y recombinación del ADN (Dornetshuber et al., 2009a), e interacción con glicoproteinas (ABCB1) y proteínas de transporte resistentes a múltiples fármacos como ABCC1 y ABCG2 pudiendo alterar la biodisponibilidad, farmacocinética y la toxicidad de fármacos (Dornetshuber et al., 2009b). BEA y ENs son además inhibidores específicos de la acil-CoA:colesterol aciltransferasa (ACAT), enzima encargada de la esterificación del colesterol celular e implicada en su absorción intestinal y en la aterogénesis (Marín et al., 2013), y muestran capacidad de unión a la calmodulina e inhibición de la 3'5'-ciclo-nucleotido fosfodiesterasa (Gruber-Dorninger et al., 2017).

BEA y ENs (5-10 μM; 24-48, 72 h) redujeron significativamente la resistencia eléctrica transepitelial sin afectar a la viabilidad en células

epiteliales porcinas diferenciadas IPEC-J2 (Springler et al., 2016), mientras que en células dendríticas y macrófagos humanos la exposición a BEA y EN B1 (0.1-32 μM) originó alteraciones en los procesos de diferenciación y maduración, indicando posibles trastornos inmunológicos en seres humanos (Ficheux et al., 2013), y mielotoxicidad en células progenitoras hematopoyéticas humanas; glóbulos blancos (CFU-GM); plaquetas (CFU-MK) y glóbulos rojos (BFU-E) tras la exposición a BEA (6.4-64 μM) y EN B (1-6 μM) y (Ficheux et al., 2012).

Por otra parte, dado que la presencia simultánea de BEA y ENs es muy frecuente, diversos autores han estudiado los efectos originados por mezclas de micotoxinas emergentes llegando a diversas conclusiones. Combinaciones binarias, ternarias y cuaternarias de BEA y ENs han sido estudiadas en células CFU-GM y CHO-K1 observándose efectos sinérgicos a niveles bajos (IC25) y aditivos a niveles medios y altos (IC50-IC90) (Ficheux et al., 2012; Lu et al., 2013; Prosperini et al., 2014). Sin embargo, en células CHO-K1 se observó efecto antagónico y aditivo a niveles bajos y altos, respectivamente (Lu et al., 2013; Prosperini et al., 2014).

Los resultados derivados de la combinación de micotoxinas emergentes con otras fusarotoxinas no son más uniformes. La exposición simultánea de BEA-FB1 en células PK15 originó efectos aditivos y sinérgicos a dosis bajas y altas, respectivamente (Klaric et al., 2006). La combinación BEA-DON produjo efectos antagónicos en células CHO-K1 y Vero (Ruiz et al., 2011a; 2011b) y efectos sinérgicos en células CFU-GM (Ficheux et al., 2012). La combinación BEA-T-2 originó resultados opuestos (sinergismo y antagonismo) bajo las mismas condiciones de estudio en células CHO-K1 y Vero (Ruiz et al., 2011a; 2011b).

Se demuestra así la complejidad de las interacciones entre micotoxinas y la influencia de los modelos celulares utilizados para su estudio, capaces de originar efectos en ocasiones opuestos, no necesariamente dependientes de la dosis y el tiempo de exposición.

En la Tabla 4 se muestran los estudios de BEA y ENs realizados en líneas celulares así como los principales efectos observados.

Tabla 4. Estudios *in vitro* de BEA y ENs, especie y línea celular, dosis, tiempo de exposición, efectos principales y referencias.

Micotoxina	Especie	Línea celular	Dosis (µM)	Tiempo exposición	Principales Efectos	Referencia
BEA	Ave	Sanguíneas periféricas	8	72h	Apoptosis, Daño ADN	Dombrink-Kurtzman et al. 2003
BEA	Humano	CCRF-CEM	1	24 h	Apoptosis, Daño nuclear	Jow et al. 2004
BEA	Humano	NSCLC A549	1-10	24 h	Apoptosis	Lin et al. 2005; Lu et al. 2016
BEA	Humano	KB; KBv200, KB-3-1	3-12	24 h	Apoptosis	Tao et al. 2015
BEA	Humano, Humano, Rata, Rata	HepG2, HCT116, C6, H4IIE	1.5-25	4-48 h	Citotixicidad, Apoptosis, Necrosis	Watjen et al. 2014
BEA	Cerdo	Oocitos	1-10	24-168 h	Citotoxicidad	Schoevers et al. 2016
BEA	Cerdo	PK15	0.5-5 µg/ml	24-48 h	Citotoxicidad, Apoptosis, LPO, GHS	Klaric et al. 2006; 2008
BEA	Humano, Cerdo	Leucocitos, PK15	0.1-0.5	24 h	Genotoxicidad	Klaric et al. 2010
BEA	Humano	Linfocitos	1.25-10	24-48 h	Genotoxicidad	Celik et al. 2010
BEA	Hámster	CHO-K1	0.6-20	24-48-72 h	Citotoxicidad, Apoptosis, Daño ADN	Zouaoui et al. 2016; Mallebrera et al. 2016
ENs A, A1, B, B1	Hámster	CHO-K1	0.6-5	24-48-72 h	Citotoxicidad	Lu et al. 2013

Micotoxina	Especie	Línea celular	Dosis (μM)	Tiempo exposición	Principales Efectos	Referencia
ENs A, A1, A2, B, B1, B4, J3	Humano	Caco-2, HT-29, Hep-G2	15-30	24-48 h	Citotoxicidad	Meca et al. 2011
EN B	Humano	H295R	0.1-100	24-48-72 h	Citotoxicidad	Kalayou et al. 2015
ENs A, A1, B, B1, BEA	Humano	Caco-2	0.5-25	24-48-72 h	Apoptosis, Necrosis, ROS, LPO, Daño DNA	Prosperini et al. 2013a; 2013b
ENs A1, B1, BEA	Humano	Hep-G2	1.5-3	24-48-72 h	Alteración MMP	Juan-García et al. 2015
ENs A, A1, B, B1, BEA	Humano	A549, GLC-4, KB-3-1, HL-60	0.1-10	24-72 h	Apoptosis, Inhibición topoisomerasas y glicoproteinas	Dornetshuber et al. 2009a; 2009b
ENs A, A1, B, B1, BEA	Cerdo	IPEC-J2	5-10	24-48-72 h	Reducción resistencia eléctrica transepitelial	Springler et al. 2016
EN B1, BEA	Humano	Dendríticas y macrófagos	0.1-32	-	Alteración inmunológica	Ficheux et al. 2013
BEA, EN B	Humano	CFU-GM, CFU-MK, BFU-E	1-64	-	Mielotoxicidad	Ficheux et al. 2012

1.6 Estudios *in vivo* de Beauvericina y Eniatinas

1.6.1 Toxicidad Aguda

Los estudios *in vivo* sobre las micotoxinas emergentes de *Fusarium* son escasos. A día de hoy no se ha informado de brotes de micotoxicosis en humanos y animales debido a BEA y ENs, por lo que la toxicidad aguda no parece ser la mayor preocupación con respecto a la exposición a estas micotoxinas (Jestoi, 2008). La toxicidad aguda de las ENs tan solo se ha observado en ratones con deficiencia inmune tras la administración intraperitoneal de una mezcla de ENs (1-40 mg/kg pc) en un intervalo de 8 h. Mientras las dosis mayores fueron letales, la administración de bajas dosis únicamente provocó pérdida de peso (McKee et al., 1997). Las ENs presentaron una dosis letal 50 (LD50) de 300 mg/Kg pc en ratas tras la administración oral de fusafungina, un antibiótico y antiinflamatorio elaborado con una mezcla de ENs para el tratamiento de la infección respiratoria superior (Yuca et al., 2006). Recientemente, la Agencia Europea de Medicamentos de la Unión Europea ha recomendado revocar la autorización de la fusafungina debido a reacciones alérgicas graves y a la limitada evidencia de su eficacia (European Medicines Agency, 2016)

La LD50 de BEA se ha determinado en ratas y ratones por vía intraperitoneal y oral con un valor de 10 y 100 mg/Kg pc, respectivamente (Omura et al., 1991; EFSA 2014). Otros estudios en rata indican la ausencia de efectos adversos observables tras la administración oral de ENs (1-50 mg/kg pc) (Bosch et al., 1989; Escrivá et al., 2015b).

1.6.2 Toxicidad Subcrónica y Biodisponibilidad

La administrción oral de EN A (20.9 mg/kg pc) en ratas durante 28 días no provocó variaciones en el peso corporal y ni en la morfología de los órganos, no se observaron alteraciónes histológicas del tejido duodenal ni en los parámetros bioquímicos sanguíneos (Manyes et al., 2014) ni signos visibles de enfermedad (Juan et al., 2014). Recientemente, la exposisción intraperitoneal de EN B y BEA (5 mg/kg pc) durante 9 días en ratones no provocó alteraciones en el peso corporal, ni en el comportamiento o la ingesta de alimentos (Rodríguez-Carrasco et al., 2016).

A pesar de la ausencia de efectos adversos observables, estos estudios demuestran que BEA y ENs se absorben a nivel intestinal tras su administración oral en roedores, llegando al torrente sanguíneo en concentraciones detectables y apuntanto al yeyuno como el lugar de absorción intestinal de ENs (Manyes et al., 2014). Se indica además una bioacumulación en diversos órganos, con tendencia hacia componentes mayormente lipófilos como hígado y tejido graso (Rodríguez-Carrasco et al., 2016). Se ha demostrado que BEA y ENs son capaces de transpasar la barrera hematoencefálica en ratones distribuyéndose considerablemente en el parénquima cerebral, por lo que podrían ejercer efectos adversos a nivel del sistema nervioso central (Taevernier et al., 2016).

La información disponible sobre la toxicocinética de BEA y ENs es también escasa pero varios estudios coinciden en una rápida absorción y eliminación, mostrando además elevada biodisponibilidad oral (91%) en cerdos (Devreese et al., 2013; 2014b). Los autores proponen la rápida metabolización, en lugar una baja biodisponibilidad, como posible explicación de la escasa toxicidad de ENs observada *in vivo*.

Además de rápida, la metabolización de estos compuestos es extensa, y aunque los metabolitos no han sido ampliamente estudiados se han detectado derivados monooxigenados, hidroxilados, carboxilados, carbonilizados, dioxinados y desmetilados en microsomas hepáticos de rata, perro, cerdo, pollo y humano (Faeste et al., 2011, Ivanova et al., 2011; 2014; 2017), plasma, hígado y huevos de pollo (Ivanova et al., 2014), e hígado y colon de ratón (Rodríguez-Carrasco et al., 2016). Recientemente, al estudiar los metabolitos de EN B1 detectados en plasma de cerdos tras una única administración oral e intravenosa, se observó que la formación de metabólitos fue mayor cuando EN B1 se absorbía desde el intestino en comparación con la administración intravenosa, apuntando al metabolismo pre-sistémico tras la absorción oral (Ivanova et al., 2017).

En la Tabla 5 se recogen los estudios *in vivo* de BEA y ENs indicando los principales efectos observados, así como la especie animal, vía y dosis de administración y duración del experimento.

Tabla 5. Estudios *in vivo* de BEA y ENs, especie, dosis, vía de administración, duración, efectos principales y referencias.

Micotoxina	Especie	Dosis (mg/kg pc)	Administración	Duración	Principales efectos	Referencia
ENs	Ratón	50	Oral	5 días	No efectos adversos	Bosch et al. 1989
BEA	Ratón	1-100	Oral Intraperitoneal	-	Toxicidad aguda	Omura et al. 1991
ENs	Ratón	10-40	Intraperitoneal	5 días	Toxicidad aguda	McKee et al. 1997
ENs	Ratón	1% (500 μg)	Inhalatoria	10 días	Alteración histopatológica	Yuca et al. 2006
BEA, ENs	Cerdo	0.05	Oral	4 horas	Rápida absorción y eliminación	Devreese et al. 2013
EN B1	Cerdo	0.05	Oral Intravenosa	2 horas	Rápida absorción, distribución y eliminación. Elevada biodisponibilidad oral	Devreese et al. 2014
EN A	Rata	20.91	Oral	28 días	No efectos adversos Absorción intestinal Bioacumulación tejidos	Manyes et al. 2014
EN A	Rata	20.91	Oral	28 días	Alteración inmune	Juan et al. 2014
EN B	Ave de corral	11.2-12.7 mg/kg	Oral	12 días	Extensa metabolización Bioacumulación en tejidos	Ivanova et al. 2014
ENs	Rata	1-2.2	Oral	8 horas	No efectos adversos	Escrivá et al. 2015b
BEA	Raton	5	Intraperitoneal	4 días	No efectos adversos. Absorción intestinal. Biocumulación en tejidos	Rodríguez-Carrasco et al. 2016
BEA	Ratón	33.2 μg/mL	Intravenosa	15 min.	Paso barrera hematoencefálica Distribución parénquima cerebral	Taevernier et al. 2016
EN B1	Cerdo	0.05	Oral, Intravenosa	2 horas	Extensa metabolización	Ivanova et al. 2017

1.7 Toxicogenómica

La secuenciación del genoma humano en 2003 (Zwart, 2015) ha dado paso a la aparición a las denominadas 'ciencias omicas' impulsadas por los recientes avances y desarrollos tecnológicos en el campo de la biología molecular. La Genómica se ocupa de la caracterización molecular de genomas completos aportando información sobre la secuencia y función de los genes en diferentes situaciones y condiciones ambientales, así como de los mecanismos implicados en la regulación de la expresión e interacción génica. De su aplicación a la Toxicología surge la Toxicogenómica, que estudia la respuesta genómica de los organismos expuestos a tóxicos y permite identificar genes de interés e interpretar los procesos biológicos y mecanismos moleculares de toxicidad desencadenados (Li et al., 2016). Mediante el análisis de los patrones normales de expresión de genes en un organismo determinado, es posible detectar cambios en la expresión génica que pueden predecir, influir o facilitar la comprensión de la respuesta tóxica. El enfoque toxicogenómico ya ha sido utilizado con éxito para predecir la genotoxicidad y la carcinogenicidad de diversos compuestos, entre ellos algunas micotoxinas (Wilson et al., 2013).

1.7.1 Transcriptómica

La Transcriptómica, una de las ramas de la Toxicogenómica, se centra en el estudio funcional del genoma a nivel de la transcripción, es decir a nivel de la formación del ARN mensajero a partir del ADN (Figura 4) (Joseph et al., 2017).

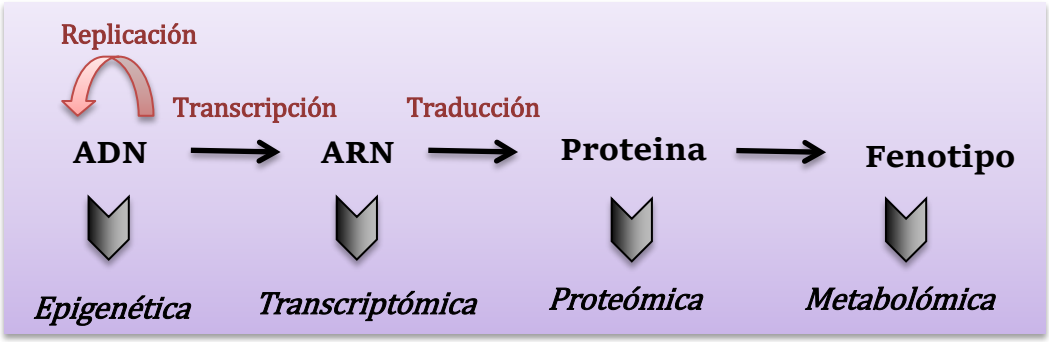


Figura 4. Esquema de las ciéncias ómicas básicas.

Las técnicas de transcripción más empleadas en la actualidad son la reacción en cadena de la polimerasa (PCR) en su variante cuantitativa con transcriptasa inversa (qRT-PCR), los microarrays, y la secuenciación masiva. Si el objetivo es determinar con precisión el perfil de expresión de un número limitado de genes la qRT-PCR es generalmente el método de elección, ya que genera un gran número de copias de un fragmento de ARN concreto permitiendo su identificación y cuantificación mediante (1) la retrotranscripción a partir del ARN formando ADN complementario (ADNc), (2) la amplificación a partir de la primera hebra de ADNc y (3) la amplificación por PCR convencional (Figura 5).

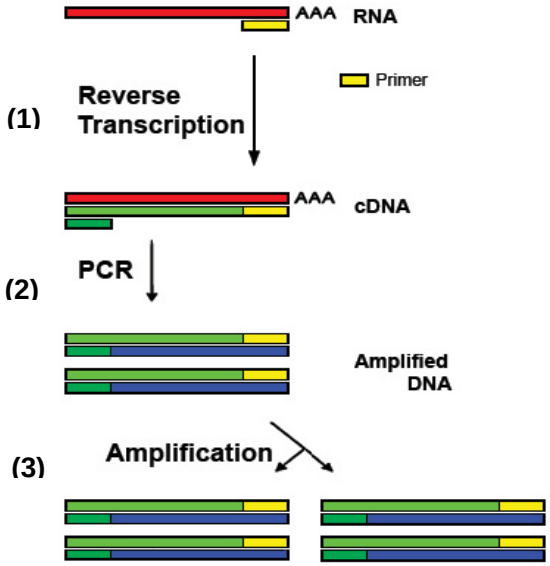


Figura 5. Esquema del proceso de RT-PCR. (1) Retrotranscripción del ARN, (2) Amplificación primera hebra ADNc, (3) PCR convencional.

Sin embargo, desde la perspectiva global de la toxicología de sistemas donde se busca obtener el perfil completo del transcriptoma, la qRT-PCR no es práctica ni económica, por lo que se emplean técnicas hibridación como microarrays, y técnicas de secuenciación, también conocidas como Next Generation Sequencing (NGS) (Hurd and Nelson, 2009; Raddatz et al., 2017). Con el fin de verificar los resultados obtenidos con ambas técnicas (de hibridación y de secuenciación), es conveniente realizar la confirmación mediante qRT-PCR con una pequeña selección de genes considerados relevantes y representativos (Joseph et al., 2017).

1.7.2 Microarrays

Los microarrays, microchips que contienen millones de sondas específicas de un único gen previamente conocido (Figura 6), han sido aplicados con éxito en las últimas décadas al estudio de algunas micotoxinas como DON, ZEN, Fusarenon-X (FUS-X), AFs, OTA, Nivalenol (NIV), T-2 y HT-2 y EN B, empleando como modelo biológico diversas líneas celulares como células hepáticas (HepG2), intestinales (Caco-2, IPEC-1, IPEC-J2), pulmonares (A549), linfoides (Jurkat, MTEC1) y sanguíneas (PBMCs); o bien células extraídas de tejidos como explantes intestinales, riñón y bazo de cerdo, hígado, cerebro y huesos vertebrales de roedor, hígado y yeyuno de pollo y cartílago humano (Tabla 6).

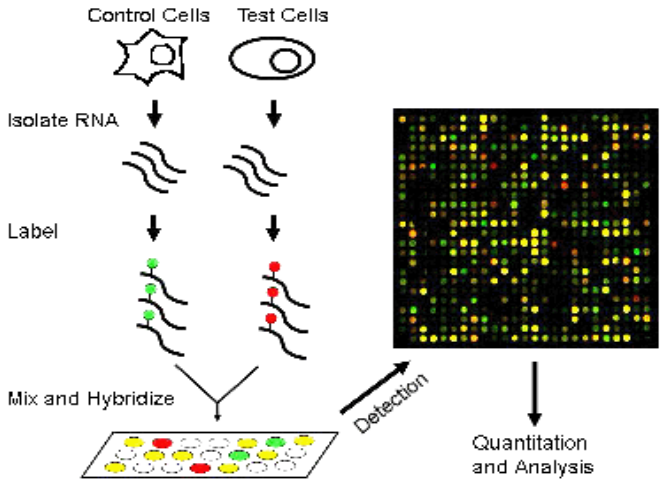


Figura 6. Esquema del funcionamiento de los Microarrays.

De esta forma, el enfoque transcriptómico ha sido útil en el estudio de DON en células pulmonares (Toyotome et al., 2016) y células epiteliales intestinales IPEC-J2 administrado solo o junto con lipopolisacáridos (Kuess et al., 2015), así como en la identificación de genes candidatos implicados en los

mecanismos de toxicidad desencadenados por DON (Diesing et al., 2012). Los efectos de DON sobre la viabilidad, la proliferación y el ciclo celular han sido estudiados en células epiteliales tímicas de ratón MTEC1 (Li et al., 2013), y los mecanismos moleculares implicados en la inmunotoxicidad inducida por DON han sido evaluados en células T linfoblásticas Jurkat y células mononucleares de sangre periférica humanas (PBMCs) observándose efectos similares en ambas líneas celulares (Katika et al., 2012). Los efectos tóxicos de DON en el desarrollo esquelético fetal y malformaciones óseas fetales se han estudiado a nivel genómico en ratones (Zhao et al., 2012), así como los efectos de bajas dosis de DON administrado de forma crónica en pollos mediante el estudio genómico de hígado y células intestinales (Dietrich et al., 2012). La toxicidad de DON y su metabolito DON-glucósido (DON-Glc) ha sido investigada en células intestinales humanas Caco-2 y explantes intestinales de cerdo (Pierron et al., 2016).

Respecto a la ZEN, las diferencias en el genoma tras la exposición a bajas concentraciones de micotoxina (Taranu et al., 2015), y el impacto de la contaminación simultánea de ZEN y *Eschericha coli* ha sido evaluada a nivel transcriptómico en células epiteliales intestinales porcinas IPEC-1 (Braicu et al., 2016). La toxicidad de ZEN se investigó en bazo porcino como órgano crítico para la respuesta inmune innata y adaptativa, mediante la integración de la genómica y la proteómica (Pistol et al., 2015).

Tambíen las AFs han sido objeto de estudio mediante microarrays, aplicándose a la evaluación de los mecanismos y biomarcadores potenciales de hepatotoxicidad aguda inducida por AFB1 en el tejido hepático de ratas, a través del análisis de cambios en la expresión génica y en el metabolismo tras la administración de la micotoxina (Lu et al., 2013). La capacidad de las AFs

para activar el receptor pregnane X (PXR), receptor de hormonas nucleares que regula el metabolismo xenobiótico y endobiótico en el hígado, ha sido estudiado en células hepáticas humanas HepG2 (Ratajewski et al., 2011).

OTA ha sido estudiada en cerdos en base a la identificación de los marcadores toxicológicos tras exposición crónica a bajas concentraciones (Marín et al., 2017), mientras que la apoptosis inducida por FUS-X y los mecanismos genéticos reguladores de la apoptosis en el cerebro de fetos de ratones ha sido investigada mediante el tratamiento de ratones embarazadas y el posterior análisis transcriptómico (Sutjarit et al., 2014). Los efectos tóxicos de la EN B sobre el metabolismo energético, la proliferación y muerte celular, e inducción a apoptosis han sido recientemente estudiados mediante el análisis del perfil genómico de fibroblastos de embrión de ratón Balb 3T3, células hepáticas humanas HepG2 y hepatocitos primarios de rata tras la exposición a la micotoxina emergente (Jonsson et al., 2016).

Por otra parte, además de evaluar los efectos toxicológicos y mecanismos moleculares originados por la exposición a micotoxinas, mediante el enfoque transcriptómico con microarrays se ha conseguido identificar los mecanismos moleculares que subyacen a la inmunotoxicidad directa identificando clasificadores funcionales basados en transcriptomas. En esta línea, tras investigar los cambios de expresión génica en células Jurkat en respuesta a una amplia gama de compuestos que incluyen inmunotóxicos directos (entre ellos algunas micotoxinas como DON, NIV, AFB1 y OTA), fármacos inmunosupresores y sustancias control no inmunotóxicas, se consiguió identificar un conjunto de genes capaces de predecir con 85% de precisión los efectos inmunotóxicos de compuestos, considerándose un adecuado indicador *in vitro* para detectar posibles propiedades

inmunotóxicas de compuestos, y categorizar estos inmunotoxicantes directos en distintas subclases funcionales (Shao et al., 2013; 2014).

Finalmente, los microarrays se han empleado en el estudio del papel de algunas micotoxinas en enfermedades como Kashin-Beck, identificando los principales genes implicados en las lesiones de cartílago inducidas por DON, T-2 y HT-2 en dicha patología, y revelando el mecanismo molecular desencadenado por estas micotoxinas en la alteración de la expresión génica en la enfermedad de Kashin-Beck (Zhang et al., 2012; Yu et al., 2016). En la Tabla 6 se recogen los estudios transcriptómicos realizados mediante microarrays y confirmación por qRT-PCR, indicando la micotoxina de estudio, el modelo biológico, así como los principales resultados obtenidos.

Tabla 6. Estudios transcriptómicos realizados con micotoxinas mediante microarrays y confirmación por qRT-PCR.

Micotoxina	Organismo Biológico	Alteración de la expresión de genes	Referencia
DON	IPEC-J2	Sobre-expresión de genes ß-actina and ZO-1	Kuess et al. 2015
DON	IPEC-J2	Sobre-expresión de genes relacionados con el metabolismo energético celular	Diesing et al. 2012
DON	A549	21 genes y RNAs no codificantes alterados con diversasd funciones	Toyotome et al. 2016
DON	MTEC1	Alteración de genes implicados en proceso metabólico, ciclo celular, oxidación-reducción y apoptosis, sugiriendo importante rol de la vía p53 en la inhibición de la proliferación celular	Li et al. 2013
DON	Jurkat PBMCs	Sobre-expresión de genes implicados en estructura y función ribosómica, síntesis y procesamiento de ARN/proteína, estrés del retículo endoplasmático, señalización mediada por calcio, función mitocondrial, estrés oxidativo, activación de células T y apoptosis.	Katika et al. 2012
DON	Huesos vertebrales de ratón	134 genes sobre-expresados y 148 reprimidos relacionados con el desarrollo keletal, carcinogénesis, trastornos nerviosos, desarrollo de espermatozoides y embriogénesis, e inflamación	Zhao et al. 2012
DON	Hígado y yeyuno de pollo	Alteración de genes relacionados con iniciación de la traducción, genes de estabilización de mRNA y relacionados con las proteínas de reparación de ADN indicando posibles efectos mutagénicos	Dietrich et al. 2012
DON DON-Gluc	Caco-2 Explantes intestinales	Sobre-expresión de genes de citoquinas proinflmatorias, genes implicados en inflamación y respuesta inmune, estrés oxidativo, activación de NFkB, regulación del ciclo celular y apoptosis	Pierron et al. 2016
ZEN	IPEC-1	190 genes sobre-expresados relacionados con enzimas glutation peroxidasa, y epresión de genes supresores de tumores demostrando potencial carcinogénico	Taranu et al. 2015
ZEN	IPEC-1	303 genes sobre-expresados y 49 reprimidos relacionados con factor de	Braicu et al. 2016

Micotoxina	Organismo Biológico	Alteración de la expresión de genes	Referencia
		transcripción, señalización y proliferación celular, respuesta inflamatoria, citoquina, interleucina y factor de crecimiento	
ZEN	Bazo de cerdo	Alteración de genes relacionados con vías de señalización celular, citoquinas y respuesta inflamatoria indicando actividad de disruptor inmune	Pistol et al. 2015
AFs	HepG2	Sobre-expresión del del metabolismo xenobiótico y genes dependientes de PXR, y represión de genes implicados en colesterologénesis	Ratajewski et al. 2011
AFB1	Hígado de rata	Inducción de la vía p53 por daño oxidativo, originando gluconeogénesis y trastorno del metabolismo lipídico	Lu et al. 2013
OTA	Riñón de cerdo	105 genes alterados relacionados con respuesta inmune, estrés oxidativo y carcinogénesis	Marín et al. 2017
FUS-X	Cerebro de ratón	Sobre-expresión de Bax, Trp53, Casp9, and represión de Bcl2 sugiriendo apoptosis regulada por Bax, Bcl2, Trp53 y Casp9 o a través de la vía apoptótica intrínseca	Sutjarit et al. 2014
EN B	Balb 3T3 HepG2 Hepatocitos de rata	Alteración genes relacionados con metabolismo energético celular, membrana plasmática, necrosis, organización mitocondrial, disfunción mitocondrial, y cadena de tranporte de electrones principalmente complejo I	Jonsson et al. 2016
DON AFB1 NIV OTA	Jurkat	Identificación y validación de 25 genes capaces de predecir efectos inmunotóxicos y categorizar los inmunotoxicantes directos *in vitro.* Alteración de genes implicados en citoprotección, metabolismo lipídico y producción de interferón I por OTA	Shao et al. 2014; 2013
DON T-2, HT-2	Cartílago humano	Inducción de la vía de señalización p53. Posible contribución de las micotoxinas al desarrollo de la enfermedad de Kashin-Becka a través alteraciones en el colágeno, apoptosis, crecimiento y desarrollo del cartílago	Zhang et al. 2012 Yu et al. 2016

1.7.3 Next Generation Sequencing (NGS)

Las técnicas NGS se están convirtiendo en la actualidad en el estándar para la comprensión de los mecanismos moleculares de toxicidad, y están revolucionando el campo de la genómica debido a la generación de datos con rendimiento y precisión sin precedentes (Caiment et al., 2015). NGS tiene mayor alcance que los microarrays al no limitar el rango de detección a genes previamente descritos y permite la producción de datos a gran escala reduciendo enormemente los costes de secuenciación en los últimos años, identificando de forma simultánea patrones de expresión de miles de genes en respuesta a diferentes estímulos (Cubillos et al., 2014). Aunque su primera aplicación fue la secuenciación del ADN, su gran versatilidad permite la secuenciación de todas las moléculas de ARN, transcritas a ADNc, presentes en una muestra, y por tanto, del transcriptoma completo. Esta técnica se conoce como secuenciación masiva del ARN (RNA-seq) que permite catalogar y cuantificar los transcritos expresados por una célula en condiciones específicas e identificar así los genes diferencialmente expresados (DEGs) en dichas condiciones.

A día de hoy, RNA-seq está sustituyendo a las técnicas de microarrays para investigar la función detallada de grupos de genes implicados en la patogenicidad de hongos micotoxigénicos (Magan and Medina, 2016), así como para elucidar los mecanismos moleculares de toxicidad, detectar la exposición y/o toxicidad en órganos diana, incluso para la predicción y detección temprana de toxicidad mediante la obtención de un perfil de genes característicos que pueden ser utilizados como biomarcadores tras una validación adecuada (Joseph et al., 2016).

A pesar de su reciente aparición, la técnica RNA-seq ha sido ya aplicada al estudio de algunas micotoxinas como DON y sus metabolitos 3-ADON y 15-ADON, NIV, ZEN, AFB1, OTA, y sporidesmina (SPO), utilizando como modelo biológico bacterias como *E. coli*, cepas fúngicas como *F. graminearum, A. carbonarius, y A. flavus,* células del estroma endometrial (ESC), y células obtenidas de riñon de cerdo y pollo; hígado de cerdo, rata, pato, oveja y camarón; y bazo de pavo (Tabla 7).

A través de RNA-seq se han estudiado las respuestas biológicas y mecanismos bacterianos de resistencia a DON y NIV (Park et al., 2014). Los efectos de DON y ZEN sobre la expresión de citoquinas pro-inflamatorias y genes relacionados con el sistema inmune se han investigado en hígado y riñón de cerdo (Reddy et al. 2017a; 2017b), mientras que los efectos tóxicos de ZEN, AFB1 y SPO han sido estudiados en células embriónicas de ratón (Xie et al., 2016), células hepáticas de patos (Zhang et al., 2016), y oveja (Zhang et al., 2014b), respectivamente. Los efectos subcrónicos de AFB1, previos a la aparición de lesiones histopatológicas o tumores, se han estudiado en el transcriptoma de hígado de rata (Merrick et al., 2013), así como los mecanismos moleculares de inmunotoxicidad en bazo de pavo y la capacidad de un probiótico de Lactobacillus para proteger contra la aflatoxicosis (Monson et al., 2015). Para mejorar la comprensión de la interacción huésped-AFB1 se ha estudiado la inmunidad del camarón en respuesta a la micotoxina mediante el análisis del transcriptoma hepático (Zhao et al., 2017). La toxicidad renal reportada para OTA en aves ha sido corroborada a nivel de transcriptoma en células renales de pollo, indicando varios mecanismos de acción para OTA en línea con los efectos cancerígenos y tumorigénicos previamente descritos (Zeferino et al., 2015; 2017).

Las técnicas RNA-seq han tenido también aplicación en el estudio de hongos micotoxigénicos como *F. gramineum,* para investigar la resistencia a la infección y la acumulación de DON (Hofstad et al., 2017), los mecanismos moleculares durante la infección fúngica y la identificación de genes relacionados con la agresividad y la producción de micotoxinas (Puri et al, 2016). La ruta de biosíntesis de OTA se ha estudiado en *A. carbonarius* mediante la identificación de genes específicos relacionados con la activación de vías metabólicas de biosíntesis de OTA y su posible conexión con otros procesos biológicos como el crecimiento, esporulación y pigmentación (Gerin et al., 2016). El transcriptoma de *A. flavus* en diferentes condiciones de temperatura (Yu et al., 2011) y actividad de agua (Zhang et al., 2014a) ha sido de gran utilidad para estudiar el efecto de las condiciones ambientales en la en la producción de AFs (Medina et al., 2017).

En la Tabla 7 se muestran los estudios realizados con micotoxinas mediante RNA-seq y confirmación por qRT-PCR, indicando los principales resultados obtenidos.

Tabla 7. Estudios transcriptómicos realizados con micotoxinas mediante RNA-seq y confirmación por qRT-PC.

Micotoxina	Organismo Biológico	Resultados	Referencia
DON NIV	E. coli	4124 genes alterados implicados en el metabolismo de fenilalanina, ciclo de glioxilato y sistemas de citocromo o ubiquinol oxidasa	Park et al. 2014
DON ZEN	Hígado de cerdo	99 genes sobre-expresados y 150 reprimidos relacionados con la respuesta inmune, los procesos celulares y metabólicos, citocinas inflamatorias	Reddy et al. 2017a
DON ZEN	Riñón de cerdo	120 genes sobre-expresados y 66 reprimidos relacionados con la respuesta inmune, citoquinas inflamatorias, receptor citoquina-citoquina, procesos celulares y metabólicos, vía de señalización quimioquina	Reddy et al. 2017b
ZEN	ESC	3846 genes alterados relacionados con el ciclo celular, apoptosis y el desarrollo embriológico	Xie et al. 2016
AFB1	Hígado de pato	749 genes alterados relacionados con el metabolismo de fase I, detoxificación de fase II, oxidación-reducción, carcinogénesis, apoptosis, ciclo celular y metabolismo de ácidos grasos	Zhang et al. 2016
AFB1	Hígado de rata	1026 genes alterados implicados en la señalización de Ahr, Nrf2, GSH, xenobióticos, ciclo celular, la matriz extracelular, diferenciación celular, estructura del cinetocoro, montaje del huso mitótico y remodelación tisular	Merrick et al. 2013
AFB1	Bazo de pavo	982 genes alterados con represión de genes de sistema inmune innato y sobre-expresión de genes linfotactina, granzima A, perforina 1 y E3 ubiquitina-proteína ligasa, indicando aumento del potencial citotóxico o muerte celular	Monson et al. 2015
AFB1	Hígado de camarón	1024 genes alterados relacionados en el metabolismo de peroxidasas, transducción de señales, control de la transcripción, apoptosis, proteolisis, endocitosis, adhesión y unión celular	Zhao et al. 2017

Micotoxina	Organismo Biológico	Resultados	Referencia
OTA	Riñon de pollo	Sobre-expresión de genes implicados en la toxicidad renal	Zeferino et al. 2017*
OTA	Riñon de pollo	Alteración génica dependiente del tiempo asociada al a biodegradación xenobiótica, metabolismo de carbohidratos, aminoácidos y lípidos; y sobre-expresión de genes del sistema inmune.	Zeferino et al. 2015*
SPO	Hígado de oveja	1974 genes alterados relacionados con el metabolismo de cafeína, fármacos y xenobióticos mediante el citocromo P450.	Zhang et al. 2014b
DON	*F. graminearum*	Alteración de genes que responden a DON y Fhb1, implicados en la resistencia del trigo a micotoxinas.	Hofstad et al. 2017
3- y 15-AcilDON	*F. graminearum*	479 genes sobre-expresados y 801 reprimidos implicados en compuestos C, metabolismo de carbohidratos y metabolismo de polisacáridos	Puri et al. 2016
OTA	*A. carbonarius*	3705 genes alterados relacionados con el metabolismo de carbohidratos y aminoácidos, transporte, respuesta a estrés y esporulación, con sobre-expresión de genes codificntes de enzimas implicados en biosíntesis de metabolitos secundarios, oxidorreductasas, enzimas transportadoras y factores de transcripción	Gerin et al. 2016
AFs	*A. flavus*	5632 genes alterados, 16 reprimidos a baja actividad de agua relacionados con la producción de AFs y 11 sobre-expresados implicados en el desarrollo	Zhang et al. 2014a
AFs	*A. flavus*	1153 genes alterados con fuerte sobre-expresión de genes implicados en la biosíntesis de AFs, especialmente a baja temperatura	Yu et al. 2011*
AFB1	*A. flavus*	Alteración genómica por cambios en temperatura y actividad del agua, incluyendo genes reguladores y activadores de la transcripción de AFB1	Medina et al. 2017*

**sin confirmación qRT-PCR*

1.7.4 Análisis Bioinformático

La secuenciación RNA-seq puede realizarse utilizando cuatro tipos diferentes de plataformas: SOLiD, 454, Ion Torrent, e Illumina; considerándose ésta última una de las tecnologías más utilizadas (Mardis, 2017). Una vez obtenidos los datos de secuenciación en forma de lecturas brutas, se debe realizar un profundo análisis bioinformático mediante la (1) preparación de las lecturas, (2) mapeo de lecturas a un genoma de referencia, (3) ensamblaje y estimación de las abundancias de los transcritos, (4) normalización, (5) identificación de transcritos/genes diferencialmente expresados, y (6) análisis de procesos y vías metabólicas (Cubillos et al., 2014). El análisis bioinformático de estos datos permite asociar grupos de genes que se expresan de forma coordinada y proporciona información relevante sobre la función de los mismos. Sin embargo, dada la magnitud de los datos obtenidos, existe una creciente demanda de métodos estadísticos y herramientas bioinformáticas capaces de gestionar, procesar y analizar la enorme cantidad de información generada, convirtiendo los análisis computacionales en una necesidad absoluta (Backofen et al., 2017).

El primer paso para el análisis bioinformático de datos RNA-seq consite en realizar un control de calidad de los archivos brutos de secuenciación con heramientas como FastQC o similares. A continuación, realiza el mapeo de las lecturas RNA-seq en un genoma de referencia mediante herramientas de alineación como Bowtie, TopHat, HTSeq o STAR que encuentran coincidencias perfectas (casi perfectas), pequeñas inserciones y deleciones, y filtran secuencias repetidas que alinean el genoma en ubicaciones diferentes. La alineación generalmente es el paso más exigente desde el punto de vista informático y requiere tiempos prolongados en un

conjunto de ordenadores de alto rendimiento (Brown and Goecks, 2015). Una vez alineadas en el genoma de referencia, la expresión génica en cada muestra se determina cuantificando el número de lecturas de cada gen o transcrito mediante herramientas como Cufflinks y HTSeq-count. Aunque la sensibilidad y especificidad de RNA-seq es muy elevada, las transcripciones más largas de ARNm producen más lecturas ya que contribuyen con más fragmentos a la biblioteca de secuenciación, aumentando la estadística para detectar DEGs, por lo que es necesario escalar los datos mediante métodos de normalización para conseguir una cuantificación precisa de la expresión génica diferencial (Choi, 2016). Softwares como *EdgeR, DESeq, BaySeq, o PoissonSeq* aplican normalizaciones basándose en suposiciones que asumen que: a) las células producen cantidades similares totales de ARN, b) la mayoría de los genes no se expresan diferencialmente y c) los números de genes regulados positiva y negativamente son similares (Ma and Zhang, 2017). La identificación de los DEGs es una de las áreas más controvertidas en NGS ya que, a pesar de que se han desarrollado diversos softwares basados en diferentes algoritmos, no existe consenso en cuanto a cuál de ellos proporciona el mejor enfoque. Sin embargo, la cuantificación de las diferencias de expresión génica se ha estandarizado mediante el Fold Change (FC), parámetro que indica la relación de cambio de un gen en diferentes condiciones (un fold change 1 correspondería a un cambio del 100%). A nivel práctico se utiliza el log2 Fold Change (log2FC), que proporciona un valor positivo para genes sobreexpresados y negativo para genes reprimidos. Para obtener los valores de expresión diferencial existen diversas herramientas destacando paquetes de R/Bioconductor como *EdgeR*, *DESeq2 y HTseq2.* Finalmente, una vez identificados los DEGs, se determina la correlación funcional de los mismos

mediante métodos de enriquecimiento de aplicando el análisis de ontología de genes (GO) que proporciona información sobre las procesos biológicos, las funciones moleculares y componentes celulares involucrados en la alteración del patrón de expresión génica (Li et al., 2017). Plataformas como *Gene Set Enrichment Analysis* (GSEA), *ConsensusPathDB-human* (CPDB), e *Integrative Genomics Viewer* (IGV) integran la información disponible en enormes bases de datos genómicos destacando *Database for Annotation, Visualization and Integrated Discovery* (DAVID), *Kyoto Encyclopedia of Genes and Genomes* (KEGG), *BioCarta, y Reactome* (Dona et al., 2017). Finalmente, es importante determinar las vías metabólicas en las que estos conjuntos de DEGs podrían estar implicados, así como la visualización de dichas vías mediante complejas redes y diagramas que aportan valiosa información biológica. Algunos de los softwares anteriores incluyen esta función, además de existir otros específicamente desarrollados para tal fin como *PathVisio* y *Cytoscape*. La Figura 7 muestra un esquema del proceso de secuenciación mediante RNA-seq y el posterior análisis estadístico y bioinformático requerido.

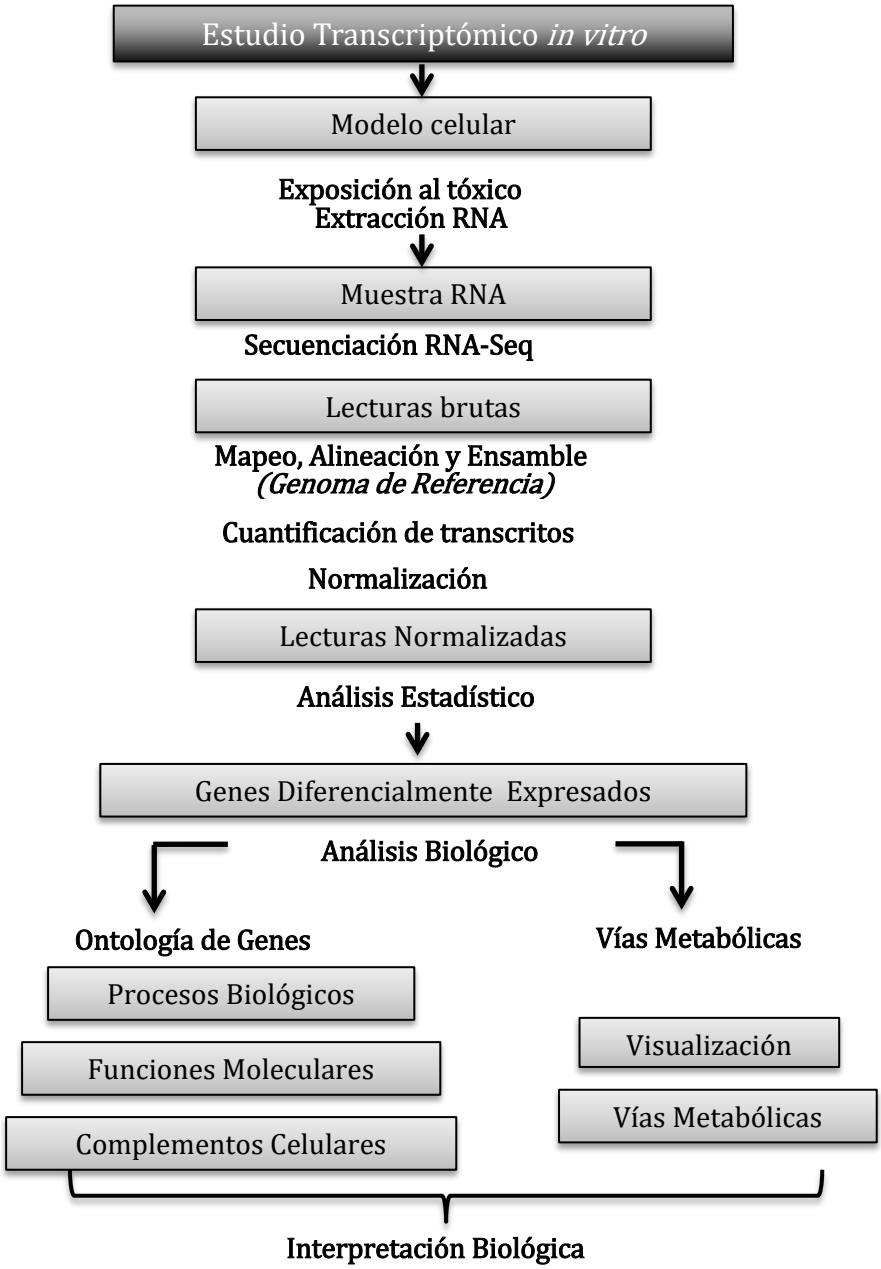


Figura 7. Diagrama de un estudio transcritómico RNA-seq *in vitro.*

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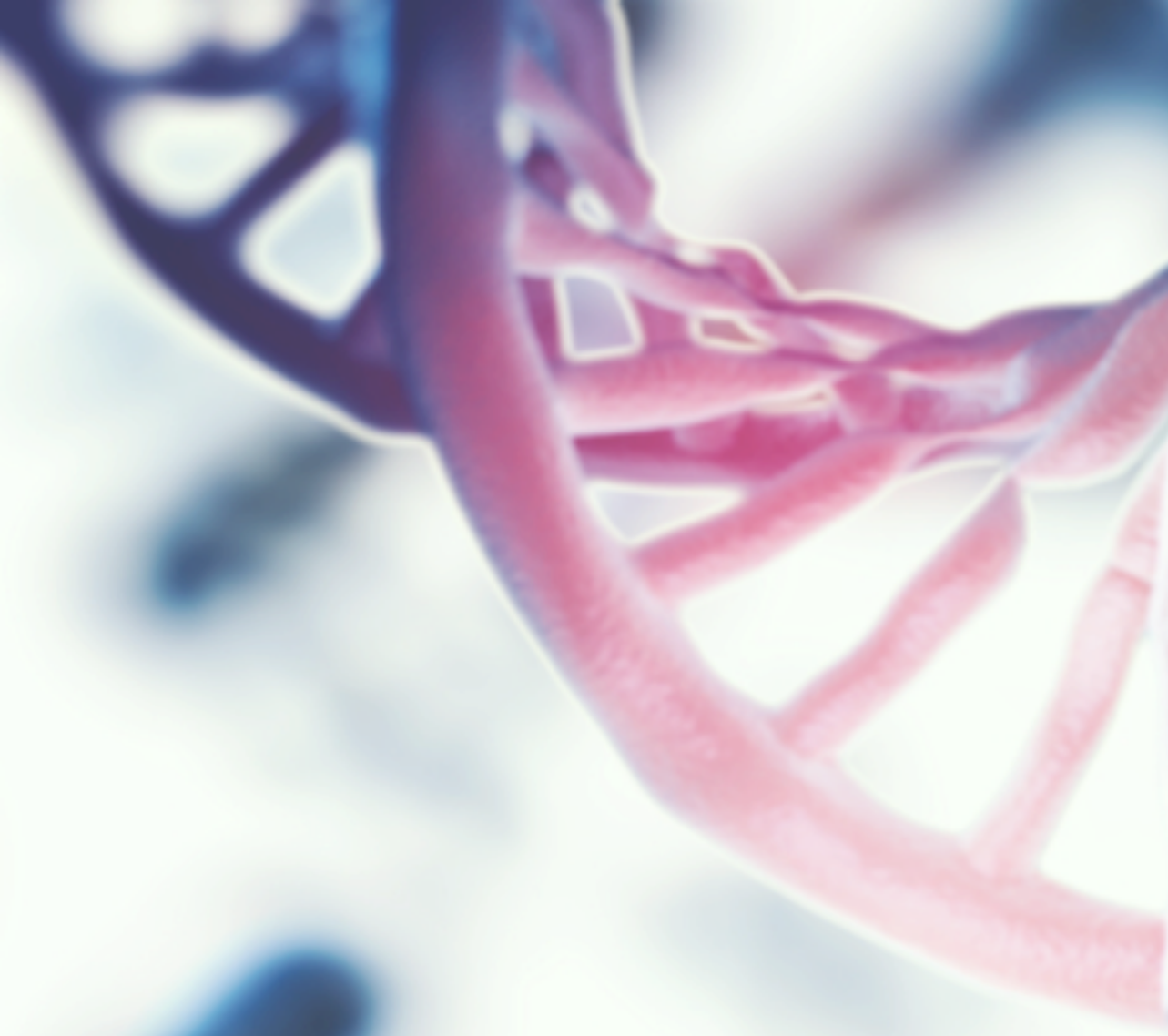


2. OBJECTIVES

OBJECTIVES

The **overall objective** of this research was to evaluate the mechanisms of action and toxic effects of emerging *Fusarium* mycotoxins through *in vitro, in vivo* and transcriptomic techniques, as well as, the presence of mycotoxins in food, feed and biological samples.

To achieve this purpose, the following **specific objectives** were proposed:

1. To review the bibliographic literature about mycotoxins toxicity and their presence in food, feed and biological samples, as well as, the cytoprotective effect of quercetin.

2. To analize multi-mycotoxin in human urine samples, rat biological samples and feed by chromatography-mass spectrometry.

3. To evaluate the cytotoxicity, genotoxicity, oxydative stress and cellular cycle alteration of Beauvericin and Enniatin B in human lymphoblastic Jurkat T cells.

4. To perform a single oral dose toxicity study of Enniatins A, A1, B and B1 in Wistar rat.

5. To perform a subacute toxicity study of Enniatin A in Wistar rat after 28 days of oral exposure.

6. To study the transcriptomic response and mechanisms of action of Beauvericin and Enniatin B in human lymphoblastic Jurkat T cells by RNA-seq and bioinformatics analysis.

OBJETIVOS

El **objetivo general** de la presente investigación es profundizar en el conocimiento de los mecanismos de acción y los efectos tóxicos de las micotoxinas emergentes de *Fusarium* mediante métodos *in vitro, in vivo* y transcriptómicos, así como la presencia de micotoxinas en alimentos, piensos y muestras biológicas.

Para llevar a cabo este objetivo se plantean los siguientes **objetivos específicos**:

1. Realización de una revisión bibliográfica sobre la toxicidad y presencia de micotoxinas en alimentos, piensos y muestras biológicas, así como del efecto citoprotector de la quercetina.

2. Determinación de la presencia de multi-micotoxinas en muestras de orina humana, muestras biológicas y piensos de rata por cromatografía-espectrometría de masas.

3. Evaluación *in vitro* de la citotoxicidad, genotoxicidad, estrés oxidativo y alteración del ciclo celular inducido por Beauvericina y Eniatina B en células Jurkat (linfocitos T humanos).

4. Estudio de la toxicidad aguda *in vivo* de Eniatinas A, A1, B y B1 tras administración única oral en rata Wistar.

5. Estudio de toxicidad subaguda *in vivo* de Eniatina A en rata Wistar tras 28 días de exposición oral.

6. Estudio transcriptómico y de los mecanismos de acción de Beauvericina y Eniatina B en células Jurkat mediante secuenciación de RNA y análisis bioinformático.

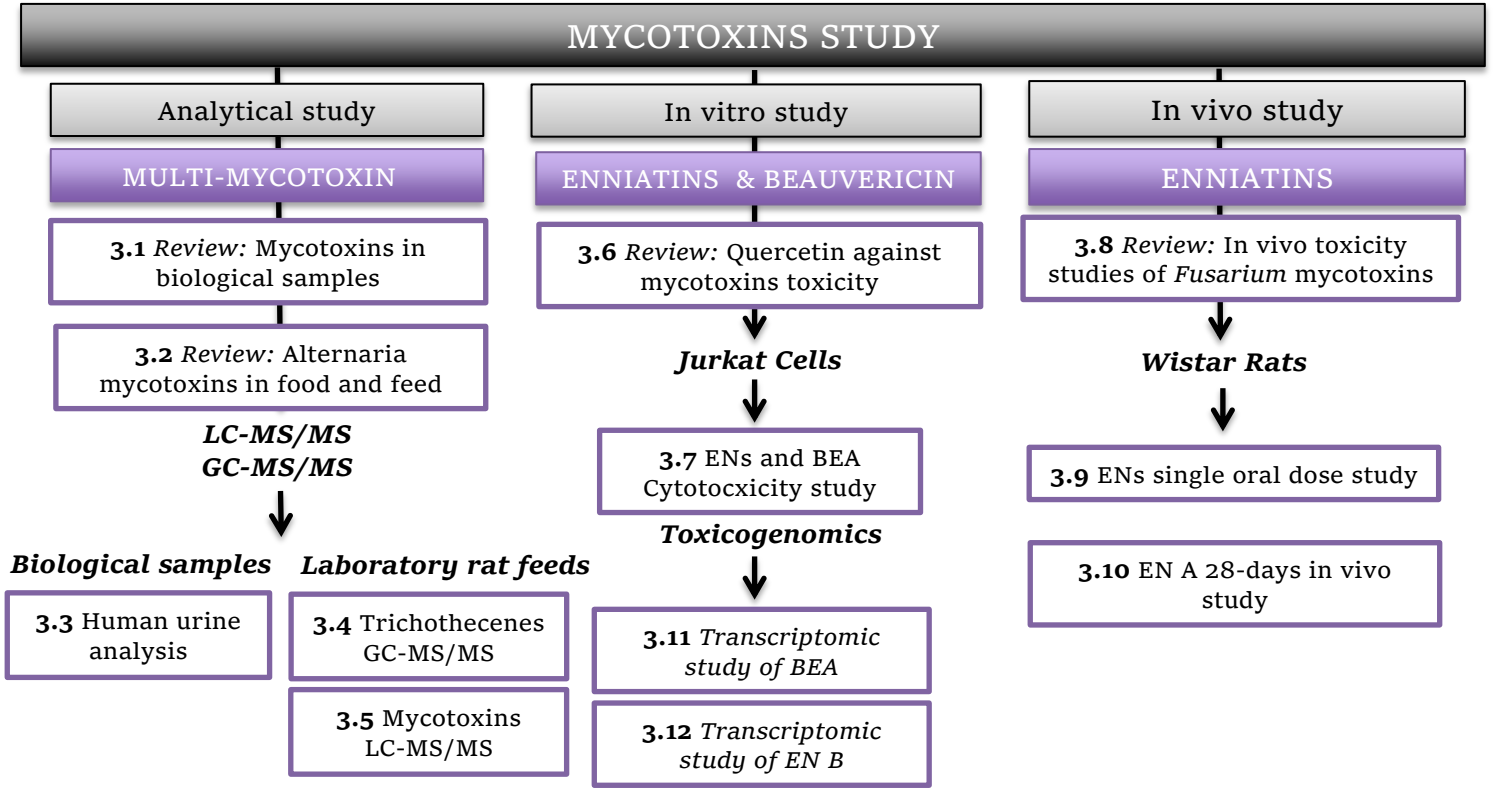
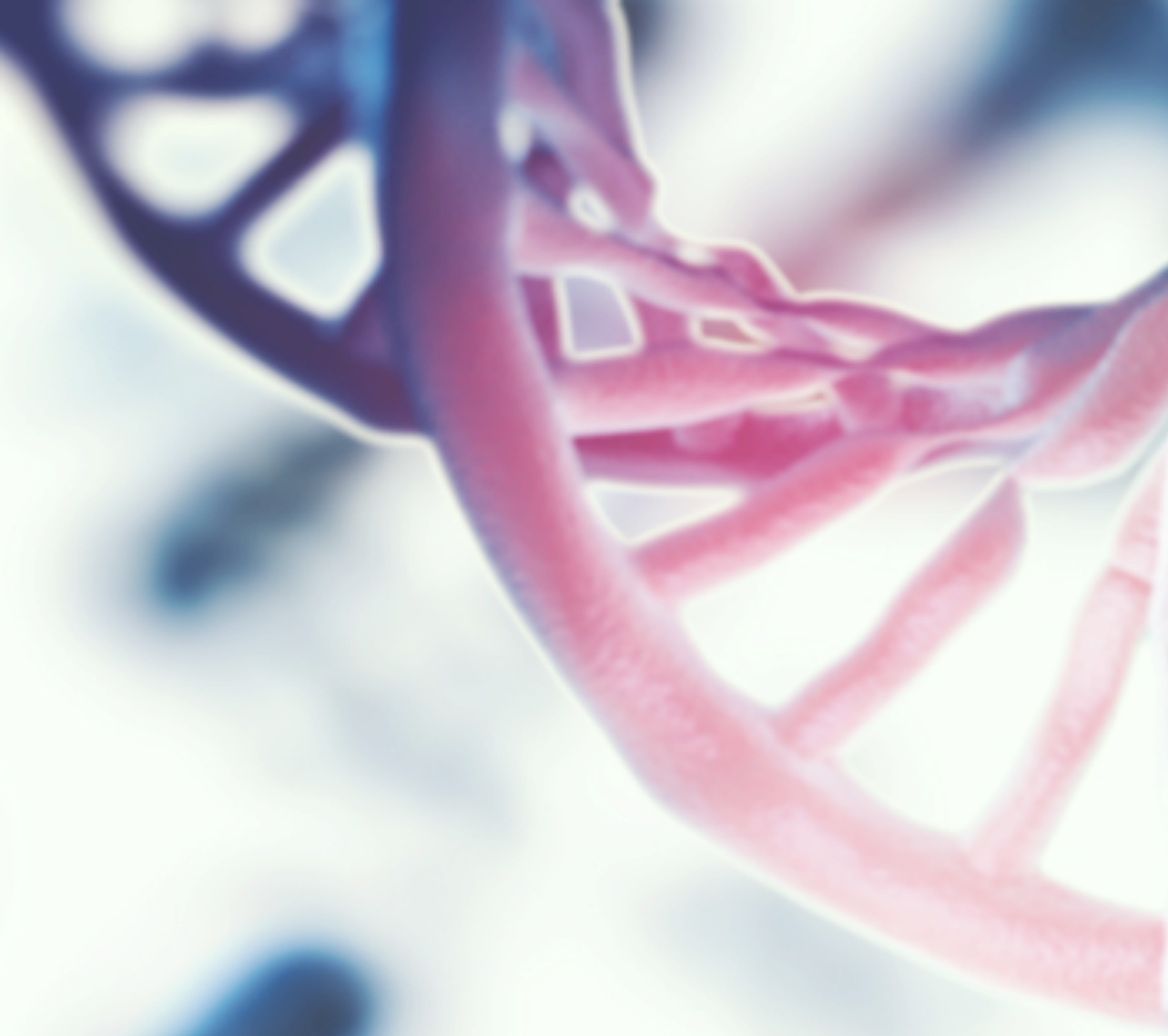


Figure 8. Work plan for *in vitro, in vivo,* and transcriptomic study of emerging mycotoxins.



3. RESULTS

3.1 Studies on the Presence of Mycotoxins in Biological Samples: an Overview

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Studies on the Presence of Mycotoxins in Biological Samples: an Overview

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Abstract

Mycotoxins are fungal secondary metabolites with bioaccumulation levels leading to their carry-over into animal fluids, organs, and tissues. As a consequence, mycotoxin determination in biological samples from humans and animals has been reported worldwide. Since most mycotoxins show toxic effects at low concentrations and considering the extremely low levels present in biological samples, the application of reliable detection methods is required. This review summarizes the information regarding the studies involving mycotoxin determination in biological samples over the last 10 years. Relevant data on extraction methodology, detection techniques, sample size, limits of detection, and quantitation are presented herein. Briefly, liquid-liquid extraction followed by LC-MS/MS determination was the most common technique. The most analyzed mycotoxin was ochratoxin A, followed by zearalenone and deoxynivalenol—including their metabolites, enniatins, fumonisins, aflatoxins, T-2 and HT-2 toxins. Moreover, the studies were classified by their purpose, mainly focused on the development of analytical methodologies, mycotoxin biomonitoring, and exposure assessment. The study of tissue distribution, bioaccumulation, carry-over, persistence and transference of mycotoxins, as well as, toxicokinetics and ADME (absorption, distribution, metabolism and excretion) were other proposed goals for biological sample analysis. Finally, an overview of risk assessment was discussed.

1. INTRODUCTION

Mycotoxins are secondary metabolites of low molecular weight, approximately of <1000 Da, produced both pre- and post-harvest by several fungus species [1]. From about 200 identified filamentous fungi, the most prevalent toxigenic species belong to the genera *Aspergillus, Fusarium, Penicillium*, and *Alternaria. Fusarium* and Alternaria usually represent a high mycotoxicological risk at pre-harvest level or in freshly harvested products on drying, whereas Aspergillus and Penicillium toxigenic species pose a higher risk for stored food and feed products or other sorts of processing [2]. It is difficult to reduce mycotoxin exposure risks because they occur naturally under certain temperature and moisture conditions, contaminating the food throughout the food chain, in process, transport or storage [3]. The reason for mycotoxins production is not yet known since they seem not to be necessary for growth nor the development of fungi. Moreover, it is genotypically specific but not limited to one species or one toxin per species [4]. Several factors such as environmental and ecological conditions -temperature, relative humidity, substrate and use of fungicides- contribute to mycotoxin presence or production in food and feed, however, the interrelations between all these factors are not yet well understood and toxin production cannot reasonably be predicted [2,5].

Fusarium genus includes over 90 described species and produces three of the most important classes of mycotoxins with respect to animal health and production; trichothecenes (TCTs), fumonisins (FBs), and zearalenones (ZENs), and the less studied emerging mycotoxins; fusaproliferin (FUS), beauvericin (BEA), enniatins (ENs), and moniliformin

(MON) [6]. The toxicity of fusariotoxins varies strongly depending on the toxin and the animal species [7].

TCTs are vastly cytotoxic to eukaryotic cells since they inhibit the synthesis of nucleic acids and proteins, cell division and mitochondrial function, as well as, destabilize cell membranes. Some acute toxic events have been reported, such as alimentary toxic aleukia, characterized by gastrointestinal tract irritation, vomiting, diarrhea, leukemia, anemia, and even death [8].

FBs toxicity is mainly due to their capacity of inhibiting ceramide synthase leading to sphingolipid biosynthesis disruption with disturbances of cellular processes, such as cell growth, differentiation, morphology, permeability, and apoptosis. In addition, FB1 promotes the development of cancer in animals and seems to increase the incidence of esophageal and hepatic cancer in humans, neural tube defects, as well as, multiple diseases in experimental animals such as leukoencephalomalacia in horses and pulmonary edema syndrome in pigs [9].

ZEN acute toxicity is relatively low but it strongly interferes with estrogen receptors and, as a consequence, affects the reproductive tract. Moreover, ZEN leads to decreased fertility, precocious puberty, changes in weight of the thyroid, adrenal, and pituitary glands; alteration of progesterone and estradiol levels in serum, fibrosis and hyperplasia in the uterus, breast cancer, endometrial carcinoma, and liver damages that may lead to liver cancer [8].

Other secondary metabolites of potential importance not exclusively produced by *Fusarium* strains include: acuminatum, butenolide, chlamydosporol, culmorin, cyclonerodiol, equisetin, fusaprolieferins,

fusarochromanones, fusaric acids, fusarins, napthoquinones, sambutoxin, and wortmannin [10].

Penicillium is a large genus with 150 recognized species of which 50 or more are of common occurrence. These fungi have been reported to produce several toxins namely citrinin (CIT), cyclopiazonic acid, ochratoxin A (OTA), patulin (PAT), penicillic acid, penitrem A, roquefortine, frequentin, palitantin, mycophenolic acid, viomellein, gliotoxin, citreoviridin, and rubratoxin B [11].

OTA is mainly known for its nephrotoxic properties and it is considered to be the possible etiological cause of some kidney diseases. Moreover, OTA is mutagenic, teratogenic, neurotoxic, hepatotoxic, and immunotoxic [12].

PAT exhibits a number of toxic effects in humans and other animals, whereas CIT has antibiotic properties against Gram-positive bacteria, but it has never been used as a drug due to its high nephrotoxicity. The kidney is the major target organ of CIT toxicity, however other body parts such as liver and bone marrow have also been reported [8,11].

Aspergillus genus contains significant mycotoxigenic species such as *A. flavus* and *A. parasiticus*, which make AFs; *A. ochraceus*, which makes OTA; and *A. versicolor*, which produces sterigmatocystin (STE). AFs have immunosuppressive properties and they are potent carcinogens particularly affecting the liver. They are related to hepatocellular carcinoma and several studies linked liver cancer with the presence of AFs in food [13]. Moreover, they are associated with occasional outbreaks of acute aflatoxicosis that lead to death shortly after exposure [9].

Alternaria species can produce around 70 toxic secondary metabolites which need for more information about their toxicity, being of relevance;

alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), tentoxin (TEN), and altenuene (ALT). *Alternaria* toxins are suspected mutagenic-carcinogens. TeA has been reported to be toxic to several animal species, such as mice, chicken, and dogs [14]. The acute toxicity of AOH, AME, ALT and TEN is low, although there are several reports on the mutagenic and genotoxic activities mainly of AOH and AME. These two mycotoxins are teratogenic and fetotoxic, they seem to be mutagenic, and to have estrogenic activity. AME provokes DNA strand breaks *in vitro* in consequence of topoisomerase poisoning, altertoxin I (ATX I) is cytotoxic and mutagenic, TeA and AME cause precancerous alterations in the esophageal mucosa of mice [15].

From the approximately 400 different compounds identified falling into the class of mycotoxins about 10-15 are considered to be of commercial interest. These are the major compounds in their families and those most commonly found. Based on the effects on human and animal health, AFs, FBs, TCTs, OTA, ZEN, and PAT are recognized as the most important food mycotoxins [4]. However, the severity of the effects that mycotoxins produce largely depends on the ingested amounts, exposure duration, and toxic synergisms that may result from the simultaneous ingestion of different mycotoxins [1,8].

The International Agency for Cancer Research (IARC) has formally classified a number of mycotoxins as agents that are proven, Group 1 (AFB1, AFB2, AFG1 and AFG2); and possibly, Group 2B (OTA, FB1 and FB2, AFM1) carcinogenic to humans [16].

Besides their notorious toxicity, some mycotoxins are thermally stable and demonstrate several levels of bioaccumulation [1]. Mycotoxins

occurrence in food and feed is either consequence of direct contamination of plant materials or products thereof, or by carry-over of mycotoxins and their metabolites into animal tissues, milk and eggs after contaminated feed intake [5]. The term carry-over is often used to describe mycotoxins transfer from feed to edible tissues in order to enable a risk evaluation for the consumer arising from feeding mycotoxin-contaminated diets to food producing animals. This carry-over is usually reported as carry-over factor (transfer factor, bio-concentration factor, etc.) or as carry-over rate (transfer rate, bio-concentration rate, etc.). Both expressions of carry-over are sometimes presented as percentage of concentration or intake, respectively. As a consequence of carry-over and bioaccumulation, mycotoxins contamination were reported not only in a number of agricultural commodities, foods and feedstuffs, but also in animal derived products and biological fluids and tissues from humans and animals at geographically diverse locations [4].

With regard to bioaccumulation, some studies reported that ZEN is accumulated in living organisms, being capable of contaminating all trophic levels of the food chain, from crop plants to human consumers [17]. Residues of ZEN, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL) and DON were detectable in pig liver, muscle, and bile after 28 days of mycotoxin feeding [18]. DON was accumulated in mice spleen, liver, lung, and kidney following similar kinetics to plasma, with maximum detectable concentrations at 15-30 min after oral exposure [19]. However, its rapid absorption, distribution and elimination may contribute to its generally low carry-over [20]. OTA was absorbed into the body and distributed at a high concentration in the kidney, which is considered the major target organ [12]. Several studies have demonstrated transplacental transfer of OTA in swine and humans, showing OTA

concentration in fetal serum to be twice the maternal concentration [21]. The ability to transform AFB1 in feed to AFM1 in milk has been examined in the past demostrating that the extent of carry-over (2.5-5.8%) was directly correlated to milk yield in cows [22]. Low AFBs carry-over (AFM1 0.02% and AFM2 0.31%) was reported in donkey milk after naturally contaminated feed administration (AFB1: 202 and AFB2: 11 μg/kg), being not detectable after 28 h from the last contaminated feeding [23]. Negligible carry-over rates (0.0075 and 0-0.0017%) were observed in cow milk after DON (2.62-5.24 mg/Kg) and ZEN (0.33-0.66 mg/Kg) contaminated feed administration [24]. Due to the lipophilic nature of some mycotoxins, such as ENs and BEA, detectable concentrations were recovered from broiler and mice organs and tissues [25-26].

Extensive analytical efforts have been made to enable fast and reliable analysis of a large number of mycotoxins in biological samples. Due to mycotoxins general prevention and control strategies involve very low concentrations limits the application of effective, sensitive and accurate methods for their detection is required. *Liquid-liquid extraction (LLE)* by aqueous and/or organic solvents is used largely depending on mycotoxin structure. Additional energy may increase the LLE efficiency in *ultrasound energy (UE) or microwave-assisted extraction (MAE)* [27], while solvent boiling point increasement by pressure retains the liquid phase in *pressurized liquid extraction (PLE),* also known as *accelerated solvent extraction (ASE)* [28]. *Dispersive liquid-liquid micro extraction (DLLME)* is based on a ternary component solvent system where dispersant and extractant solvents are combined enhancing the surface area between the organic and the aqueous phase and facilitating the achivement of equilibrium state [29]. In *salting out*

liquid-liquid extraction (SALLE) the addition of an inorganic salt into a miscible mixture forces the formation of a two-phase system [30], whereas *QuEChERS (Quick Easy Cheap Effective Rugged and Safe),* based on a modified solvent clean-up that uses extraction in ACN followed by a salting out step and a quick dispersive solid-phase extraction (d-SPE), expands the polarity range of the amenable compounds and allows the extracts purification by using small amounts of non-chlorinated organic solvents [1]. *Solid phase extraction (SPE)* is a more rapid alternative than LLE, which retains analytes on a special sorbent cartridge, often used for clean-up and extract pre-concentration after the selection of the most appropriate packing materials to reach high and stable recovery rates [31].

Extracts from biological samples can be complicated mixtures where trace amounts of a target molecule may be masked by interfering compounds, affecting the separation resolution and the sensitivity of the results. The liquid extracts could be charged onto a large variety of sorbent materials, mainly immunoaffinity columns (IAC) consisting on immobilized antibodies with excellent recovery and specificity but high costs [4]. SPE cartridges, MycoSep columns, and MIPs are cheaper alternatives for sample purification [32].

There are a variety of selective and sensitive techniques for mycotoxins determination. *High-Performance Liquid Chromatography* (HPLC) is widespread because of their superior performance and reliability compared with *thin-layer chromatography* (TLC) with high quality of separation and low limits of detection (LOD). *Gas chromatography-mass spectrometry (GC-MS)* is used for determination of organic compounds with thermal stability and volatility, as well as non-volatile mycotoxins chemically derivatized [33]. Multiple detection systems may be coupled to

chtomatography; fluorescence (FD), ultra-violet (UV), diode-array (DAD), electrochemical (EC), mass spectrometry (MS), and tandem-mass spectrometry (MS/MS), which has advanced in the last years to the status of the reference in the field of mycotoxins analysis. *Immunoaffinity methods* such as ELISA rely on antigen-antibody reaction, normally based on a competitive assay. Direct ELISA is quick and eliminates cross-reactivity, while indirect ELISA with higher immunoreactivity is generally more sensitive but cross-reactivity may lead to false positive results or overestimation, being required further confirmation by other analytical method [4-5]. Recently, the advances in nano-sensor technologies for mycotoxins determination have gained considerable importance, since aptamers offered themselves to be ideal candidates as biocomponents in biosensors (aptasensors) [34].

In this study, the analytical methods for determining the presence of mycotoxins and its metabolites in biological samples in the last 10 years were discussed. Relevant data on extraction methodolgy, detection techniques, sample size, limits of detection and quantitation, and most studied mycotoxins were evaluated herein. The studies were clasified by both the analyzed matix/matrices and by their main purposes for biological samples analysis. The origin of the analyzed biological samples (animal specie) was also investigated. Relevant information regarding mycotoxins bioavailability, bioaccumulation, and ADME was collected. Finally, an overview of human risk assessment based on available biomonitoring data was discussed.

2. RESULTS AND DISCUSSION

2.1 Mycotoxins Analysis in Biological Fluids

2.1.1 Serum

One of the most common techniques for mycotoxins analysis in serum is LLE with different solvents. The single compound OTA was commonly extracted from human serum using CHCl3 [35-36] in combination with SPE [37] or IAC [38], or with CH2Cl2 [39-40], often followed by IAC [21]. Direct SPE [41] or IAC [42-43] techniques were also performed, even combined between them [44] for OTA extraction from human serum. ACN was used for LLE of ENs B and B1 [45], and DON [46-47] from chicken serum samples. ZEN was extracted from rat serum using t-butyl methyl ether (TBME) [48-49], while AFB1 was extracted from human serum by direct IAC procedure [50].

When multi-mycotoxins were analyzed from serum, ACN was widely used for the extraction of several compounds including DON, 3-ADON, 15-ADON, DOM-1, T-2, HT-2, OTA, FB1, AFB1, ZEN, α-ZOL, β-ZOL, zearalanone (ZAN), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), and EN A, A1, B, B1 from pig [51-54], laying hens, chicken, and turkey poults serum [55-57]. OTA and OTα extraction from human serum was performed by the mixture CHCl3/isopropanol [58], while DLLME approach using the solvents mixture ACN/EtOAc was developed for the extraction of AFs, OTA, FUS-X, STG, FBs, ENs and BEA from fish serum [59].

Common serum sample size was 250 μl, ranging from 50 μl to 6 ml in some cases. Achieved LODs were between 0.000091-12 μg/L and and LOQs ranged from 0.025 to 17 μg/L.

Table 1 shows the latest studies of one single mycotoxin and multi-mycotoxins studies in serum, including sample size, studied mycotoxins, extraction and detection methods, and LODs-LOQs.

Table 1. Studies of one single mycotoxin and multi-mycotoxin determination in serum

Specie	Volume (µl)	Mycotoxin	Extraction procedure	Detection technique	LOD (µg/L)	LOQ (µg/L)	Ref.
			Single mycotoxin				
Human	50	OTA	LLE: CH2Cl2	ELISA vs. CE-LIF	0.5	-	[39]
Human	-	OTA	IAC	HPLC-FD	-	-	[42]
Human	-	OTA	SPE: Sep-Pak RP-18. IAC: Ochraprep	HPLC-FD	0.1	0.4	[44]
Human	-	OTA	LLE: CHCl3/HCl	HPLC-FD	0.05	-	[35]
Human	2000	OTA	LLE: CHCl3	HPLC-FD LC-ESI-MS/MS	0.01	0.07	[36]
Human	6000	OTA	SPE: C18	HPLC-FD	0.1	0.2	[41]
Human	1000	OTA	LLE: CHCl3, SPE	HPLC-FD	0.05	-	[37]
Human	2000-3000	OTA	LLE: CHCl3, IAC: Ochraprep	ELISA and HPLC-FD	-	0.050	[38]
Human		OTA	IAC: Ochraprep	HPLC-FD	-	0.1	[43]
Human	-	AFB1	IAC: Easi-Extract Aflatoxin	ELISA			[50]
Rat	100	ZEN	LLE: TBME	LC-MS/MS		0.5	[48]
Rat	100	ZEN	LLE: TBME	HPLC-FD		10	[49]
Swine	800	OTA	LLE: CH2Cl2	HPLC	0.1	-	[40]
Chicken	250	DON	LLE: ACN	LC-MS/MS	0.1-02	1	[46-47]
Horse	2000	OTA	LLE: CH2Cl2, IAC: Ochratest	ELISA, HPLC-FD	0.015	-	[21]

			Multi-mycotoxin				
Human	500	OTA, OTα	LLE: CHCl3/isopropanol	HPLC-FD	0.05	0.1	[58]
Chicken	20-250	EN B, EN B1	LLE: ACN	LC/MS/MS, UHPLC-HRMS	0.00009 1- 0.00017	0.025	[45]
Pig	250	EN A, A1, B, B1	LLE: ACN	LC-MS/MS	0.01- 0.001	0.1-0.2	[52-53]
Pig	250	DON, T-2, HT-2, OTA, FB1, AFB1, ZEN, ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL, DOM-1	LLE: ACN	LC-MS/MS	0.01- 0.52	0.5-10	[51,54]
Chicken, pig, laying hens, turkey poults	250	ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL,	LLE: ACN	LC–MS/MS (U)HPLC–HR-MS	0.004- 0.07	0.2-5	[55,57]
Chicken, Pig	250	DON, 3-ADON, 15-ADON, DOM-1	LLE: ACN	LC–MS/MS	0.01-0.7	0.1-2	[56]
Fish	250	AFB1, AFB2, AFG1, AFG2, OTA, FUS-X, STG, FB1, FB2, FB3, BEA, EN A, EN A1, EN B, EN B1	DLLME: ACN/EtOAc	LC/MS/MS	0.1-12.0	1.5-17.0	[59]

2.1.2 Urine

Urinary studies often encompassed a large number of mycotoxins and metabolites, where various extraction techniques were combined to achieve the highest variety of studied compounds. High method sensitivity is of most importance since the concentration of these analytes in urine samples is often present in very low concentration range. From the relatively few studies performed in one single mycotoxin in urine, OTA was the most common one, extracted from human urine by IAC [60-62], an automated multi-fiber SPME system [63], or the classical LLE with CHCl3-isopropanol [64].

Other compounds such as STE [65], DON-GlcA [66] and AFB1-N7-Gua [67] were individually analyzed in urine, by SPE, dilute-and-shoot, and SPE-IAC, respectively. Several authors have performed the so named "fast" sample preparation approaches such as filter-and-shoot; dilute-and-shoot; and dilute-evaporate-and-shoot techniques for multi-mycotoxins extraction in urine. Several mycotoxins including DON, DON-3-GlcA, DON-15-GlcA, DOM-1, NIV, T-2, HT-2, HT-2-4-GlcA, FB1, AFB1, AFB2, AFG1, AFG2, AFM1, FB1, FB2, ZEN, ZAN, α-ZOL, β-ZOL, ZEN-14-GlcA, ZAN-14-GlcA, α-ZOL-14-GlcA, β-ZOL-14-GlcA, OTA, OTα, EN B, DH-CIT, were extracted from human urine by direct dilution (1/10 factor) with H_2O/ACN/HCOOH (94:5:1) [68-69] or H_2O/ACN (90:10) [64,70-72]. The advantage of the simple sample preparation in these fast techniques needs to be compensated by latest MS instrumentation, and highlights a high request for equipment with heightened sensitivity [68] or methods involving SPE or IAC cleanup. Therefore, the complexity of urine matrix and the low analytes concentrations expected in urine, leads to the consideration of more elaborated extraction techniques, and makes sample clean-up often considered necessary. For instance, the combination of filter-

and-shoot methodology and EtOAc-LLE followed by SPE method was carried out for the extraction of 32 and 18 mycotoxins and metabolites, respectively, from human urine [73-74].

SPE technique has been also widely used for urine mycotoxin extraction in the last years, from a single compound extraction; STG from cattle urine [65], to multi-mycotoxin studies including several mycotoxins such as DON, DON-GlcAs, DOM-1, DOM-1-GlcA, AFM1, FB1, ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL, OTA extracted from human, rat, swine and bovine urine using C18 SPE cartridges [75-77]. The extraction of ZEN, α-ZOL, β-ZOL, ZAN from bovine urine was performed by TBME-LLE followed by hexane washing and C18 SPE [78].

Different sample preparation protocols were compared for the extraction of DON, OTA, FB1, AFB1, ZEN, T-2, HT-2, AFB1, CIT, DOM, DON-2-GlcA, ZEN-14-GlcA, α-ZOL, β-ZOL, 4-OH-OTA, OTα, AFM1, AFB1-N7-Gua from human urine, including fast dilute-and-shoot techniques, and methodologies based on LLE-SPE. Due to the low signal intensity reached by dilute-and-shoot methodologies they were considered not suitable for routine mycotoxin monitoring and SPE procedure was deemed necessary. Thus, EtOAc/formic acid (99:1) LLE followed by SAX-SPE procedure was selected after its comparison with LLE-Oasis HLB SPE cartridges clean-up [79].

In some cases, SPE extraction was combined with IAC procedure. For instance, AFM1, OTA, DON, DOM-1, α-ZOL, β-ZOL and FB1 were extracted from human and pig urine after sample pass throughout a Oasis HLB column followed by IAC clean-up [80]. Sample clean-up by a multiantibody IAC (Myco6in1) and Oasis HLB SPE connected in tandem was performed for the analysis of DON, DOM-1, OTA, AFB1, AFM1, FB1, ZEN and α-ZOL from pig

urine [81]. The extraction of AFB1-N7-Gua from human urine was performed using two SPE procedures; MCX SPE and Bond elute LRC C18 SPE, intercalated by IAC clean-up procedure [67].

Direct IAC procedures were carried out for the extraction of AFB1, AFB2, AFG1, AFG2, OTA, DON, ZEN, FB1, FB2, T-2, HT-2 [82-83], DON, DOM-1 [84-86], CIT, OH-CIT [87-88], FB1, FB2 [89], AFM1, OTA, FB1, FB2, OTA and OTα [82] from human urine; and for ZEN, ZAN, and their metabolites from bovine and swine urine [90].

QuEChERS procedure has been widely used for mycotoxins analysis in urine [91]. Similar aproach based on a salting-out assisted ACN extraction followed by a dispersive solid phase extraction (d-SPE) was used for the analysis of 15 mycotoxins and metabolites including DOM-1, DON, 3-ADON, FUS-X, DAS, NIV, NEO, HT-2, T-2, ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL from human urine [31,92-93]. SALLE methodology based on ACN/NaCl-C18 extraction was selected to analyze DON, DOM-1, 3-ADON, 15-ADON, ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL from human urine [94]. Similar SALLE procedure performed in two steps (EtOAc-ACN) was used for the extraction of DON, NEO, AFB1, AFM1, HT-2, T-2, OTA, OTα, ZEN, α-ZOL, β-ZOL and FB1 from human and pig urine [30].

Urine sample size ranged from 100 μl to 20 ml. Achieved LODs were between 0.000125-12 μg/L and LOQs ranged between 0.0005-40 μg/L.

Tables 2 and 3 show the latest studies of single and related mycotoxins analysis, and multi-mycotoxins determination in urine, respectively. Sample size, studied compounds, extraction and detection methods, and LODs-LOQs were included.

Table 2. Studies of one single mycotoxin and structurally related mycotoxins determination in urine.

Specie	Sample Volume	Mycotoxins	Extraction procedure	Detection technique	LOD (µg/L)	LOQ (µg/L)	Ref.
			Single mycotoxin				
Human	10 ml	OTA	IAC: OchraTest	HPLC-FD	-	0.02	[60]
Human	1 ml (0.5 ml)	OTA	SPME	HPLC –ESI-MS/MS	0.3	0.7	[63]
Human	10 ml	OTA	IAC: OchraTest	HPLC-FD	-	0.007	[61]
Human	5 ml	OTA, OTα	IAC	HPLC-FD	0.05	0.1	[62]
Human	20 ml	AFB1-N7-Gua	SPE: MCX, IAC: Bond elute LRC, SPE: C18	HPLC–ESI-MS/MS	-	-	[67]
Human	0.5 ml	DON-GlcA	Dilute-shoot: ACN/H2O	LC–MS/MS	3-6	10-20	[66]
Cattle	0.5 ml	STG	SPE: C18	LC-MS/MS	-	-	[65]
			Multi-mycotoxin				
Human	1 ml	DON, DOM-1	IAC: Wide Bore DON	LC–MS/MS	0.5	-	[84]
Human	0.1 ml	DON, DON-GlcAs	Dilute-shoot: ACN/H2O	LC–MS/MS	4-10	13-33	[72]
Human Rat	0.4	DON, DOM-1, DOM-1-G, DON-G1, DON-G2	SPE: Strata-X	HPLC–APCI–MS/MS	1-2	3-6	[76]
Human	10 ml	FB1, FB2	PBS, IAC	HPLC–ESI-MS/MS	5	10	[89]

Human Swine Bovine	10 ml	ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL	SPE: C18	HPLC-EC	1.3-1.4	4.2-4.8	[75]
Bovine, swine	5 ml	ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL,	IAC	HPLC–ESI-MS/MS	0.56-0.68 (CCa)	-	[90]
Bovine	5 ml	ZEN, α-ZOL, β-ZOL, ZAN	LLE: TBME, Hx, SPE: C18, SPE: -NH2, derivatization	GC-MS	CCa: 0.06-.35	CCb: 0.11-0.60	[78]
Human	5 ml	CIT, HO-CIT	IAC	LC-MS/MS	0.02-0.05	0.05-0.1	[87-88]
Human	5 ml	DON, DOM-1	IAC	LC-MS/MS	0.10-0.16	0.2-0.3	[85-86]
Human	5 ml	OTA, OTA-GlcA, OTA-sulfates	LLE: CHCl3-Isopropanol	LC-MS/MS	0.1-0.5	0.5-1	[64]

CCa: decision limit; CCb: detection capability.

Table 3. Studies of multi-mycotoxins determination in urine.

Specie	Vol. (ml)	Mycotoxins	Extraction procedure	Detection technique	LOD (µg/L)	LOQ (µg/L)	Ref.
Human	10	AFM1, FB1, FB2, OTA, OTα	IAC	HPLC-ESI-MS/MS	0.001-0.045	0.004-0.135	[82]
Human	10	AFB1, AFB2, AFG1, AFG2, OTA, DON, ZEN, FB1, FB2, T-2, HT-2	IAC	LC-QTRAP-MS/MS	0.4-10	1.2-35	[83]
Human Pig	6	AFM1, OTA, DON, DOM-1, α-ZOL, β-ZOL, FB1	SPE: Oasis HLB, IAC	HPLC-Qtrap-MS/MS	0.001-2.2	0.002-4.4	[80]
Human Pig	5	DON, NEO, AFB1, AFM1, HT-2,HT2, OTA, OTα, ZEN, α-ZOL, β-ZOL, FB1	SALLE: MgSO4, EtOAc-ACN	LC-MS/MS	0.01-0.5	0.07-3.3	[30]
Pig	6	DON, DOM-1, OTA, AFB1, AFM1, FB1, ZEN and α-ZOL	Myco6in1 IAC-Oasis HLB SPE	LC-MS/MS	-	-	[81]
Human	10	DON, OTA, FB1, AFB1, ZEN, T-2, HT-2, AFB1, CIT, DOM, DON-2Glu, ZEN-14-GlcA, α-ZOL, β-ZOL, 4-OH-OTA, OTα, AFM1, AFB1-N7-Gua	LLE: EtOAc/FA, SPE: SAX	LC-MS/MS	0.01-3.65	0.02-5.76	[79]
Human	0.1	DON, D3Glu, D15Glu, DOM-1, NIV, T-2, HT-2, ZEN, ZEN-14-O-GlcA, α-ZOL, b-ZOL, FB1, FB2, OTA, AFM1	Dilute-shoot: ACN/H2O	HPLC-ESI-MS/MS	0.005-2	0.017-6.7	[71]
Human	-	DON, DON-3-GlcA, DON-15-GlcA, ZEN, ZEN-14-GlcA.	Dilute-shoot: ACN/H2O	LC-MS/MS	0.2-4	0.3-6	[138]

Human	6	DON, DOM-1, AFM1, FB1, ZEN, α-ZOL, β-ZOL, OTA,	SPE: Myco6in1® and OASIS® HLB columns	UPLC-MS/MS LC-QTrap MS/MS UPLC-API 5000 MS/MS	-	0.02-4.4 0.006-9.9	[77]
Human	0.1	AFM1, OTA, FB1, DON, DON-GlcAs, FB2, DOM-1, ZEN, ZEN-14-GlcA, α-ZOL, β-ZOL, T-2, HT-2, NIV.	Dilute-shoot: ACN/H2O	LC-MS/MS	0.05-12	0.15-40	[70]
Human	10	*LLE, SPE:* AFB1, AFB2, AFG1, AFG2, AFB1-N7-gua, AFM1, CIT, DON, DON-3-GlcA, DOM-1, FB1, HFB1, OTA, OTα, 4-OH-OTA, T-2, HT-2, ZEN, ZEN-14-GlcA, α-ZOL, β-ZOL. *Filter-shoot:* AFB1, AFB2, AFG1, AFG2, AFM1, CIT, OH-CIT, DON, DON-3-GlcA , DON-15-GlcA, DOM-1, DOM-1-3-GlcA, 3-ADON, 3-ADON-15-GlcA, 15-ADON, 15-ADON-3-GlcA, DAS, FB1, FB2, FB3, FUS-X, OTA, OTα, T-2, HT-2, ZEN, ZEN-14-GlcA, α-ZOL, α-ZOL-7-GlcA, α-ZOL-14-GlcA, β-ZOL, β-ZOL-14-GlcA.	LLE: EtOAc/FA, SPE Filter-shoot	LC-MS/MS	-	-	[73-74]
Human	10	AFB1, DAS, FusX, 3-AcDON, 15-AcDON, β-ZEL, α-ZEL, CIT, OTα, DOM- 1, FB1, FB2, FB3, DON, ZEN, T2, HT2, DON-3-GlcA, DOM-GlcA, ZEN-14-GlcA, β-ZEL-7-GlcA, β-ZEL-14-	Filter-shoot IAC (OTA, CIT, AFM1)	LC-MS/MS	0.001-0.2	0.003-0.5	[140]

		GlcA, α-ZEL-7-GlcA, α-ZEL-14-GlcA, 15-AcDON-3- GlcA, 3-AcDON-15-GlcA, OTA, CIT and AFM1					
Human	0.1	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB1, AFB1, AFB2, AFG1, AFG2, AFM1, ZEN, ZAN, α-ZOL, β-ZOL, ZEN-14-GlcA, ZAN-14-GlcA, α-ZOL-14-GlcA, β-ZOL-14-GlcA, OTA, OTα, EN B, DH-CIT	Dilute-shoot: H2O/ACN/FA	LC-MS/MS	0.0005 - 0.3125	0.0013 - 0.3125	[68]
Human	0.1	DON, DON-3-GlcA, T-2, HT-2, HT-2-4-GlcA, FB1, AFB1, AFB2, AFG1, AFG2, AFM1, ZEN, ZAN, α-ZOL, β-ZOL, ZEN-14-GlcA, ZAN-14-GlcA, α-ZOL-14-GlcA, β-ZOL-14-GlcA, OTA, OTα, EN B, DH-CIT	Dilute-shoot: H2O/ACN/FA	LC-MS/MS	0.000125-0.45	0.0005-0.9	[69]
Human	10	DOM-1, DON, 3-ADON, FUS-X, DAS, NIV, NEO, HT-2, T-2, ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL	QuECHERS, d-SPE	GC-MS/MS	0.12-4	0.25-8	[31,92-93]
Human	1	DON, DOM-1, 3-ADON, 15-ADON, ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL	SALLE: ACN, NaCl-C18	GC-MS/MS	0.12-4	0.25-8	[94]

2.1.3. Minor biological fluids and fluids combinations

Major reported fluids, such as serum and urine, were often combined and analyzed together by a single methodology. OTA and OTα were extracted by CHCl3/isopropanol LLE from human serum and urine samples [95-96]. Oasis HLB SPE was used for the extraction of DON, ZEN and its metabolites from pig serum and urine [97], while graphitized carbon black cartridges were used for ENs analysis from human serum and urine samples after LLE with MeOH/H2O [98]. The extraction of CIT from human serum and urine was performed by IAC after the comparison of two clean-up methods based on C18 SPE and IAC procedures [96,99]. The combined analysis of mycotoxins from serum, urine and feces has been often performed may be due to the interest of these biological matrices in toxicokinetic and ADME (absorption, distribution, metabolism and excretion) studies. Thus, ENs extraction from rat samples (serum, urine, feces) was performed by LLE using ACN [100] and EtOAc [101]. DON, ZEN and its metabolites were analyzed by SPE-IAC in horse urine, serum and feces samples [102], and DON, DOM and their sufonates were extracted from excreta and intestinal content from broiler chickens, pullets, roosters and turkeys by LLE using the mixture MeOH/H_2O/formic acid (49.5:49.5:1) [103].

Some studies were focused on minor analyzed biological fluids such as breast milk and bile. OTA and AFM1 were analized in human breast milk by LLE in different stages using CHCl3, ACN and petroleum ether [106-107]. AFB1, AFB2, AFG1, AFG2, AFM1 and OTA were extracted by LLE (acidified ACN-EtOAc) with low temperature purification (LTP) after the evaluation of other procedures such as LLE ($CHCl_3$-NaCl, ACN) and SPE [108-109]. QuEChERS methodology was satisfactorily performed for the analysis of

several mycotoxins in human breast milk including DON, 3-ADON, NIV, FUX-N, DAS, NEO, T-2, HT-2, ZEN and metabolites, OTA, STG, ENs, BEA and AFs [110]. Direct IAC was used for ZEN, AFM1, and AFM2 extraction from cow [22], human [111] and donkey [23] breast milk samples. DON, DOM-1, T-2 and HT-2 were extracted from bile and serum samples from pig and chicken by LLE using MeOH/H2 O and EtOAc [112,113].

Other biological fluids such as saliva, nasal secretions, and amniotic fluid of pregnant women have been also analyzed, but data published so far, still do not allow their use as quantitative alternative tools for assessing environmental exposures and they are often included in larger studies comprising a wide range of organs and tissues [28,114].

Table 4 shows the latest studies of mycotoxin analysis in minor biological fluids, individually or combinations of fluids, including sample size, studied compounds, extraction and detection methods, and LODs-LOQs.

Table 4. Studies of multi-mycotoxins determination in minor biological fluids and fluids combination.

Specie	Sample	Volume	Mycotoxins	Extraction procedure	Detection technique	LOD (µg/L)	LOQ (µg/L)	Ref.
Human	Breast milk	1 ml	OTA	LLE: CHCl3	HPLC- FLD	0.01	-	[106]
Human	Breast milk	1 ml	AFM1, OTA	LLE: CHCl3, ACN	ELISA, HPLC-FD	-	-	[107]
Human	Breast milk	5 ml	ZEN	IAC	ELISA HPLC-FLD	0.06 0.02-0.05	-	[111]
Human	Breast milk	10 ml	DON, 3-ADON, NIV, FUSX, NEO, DAS, HT-2, T-2, ZEN, α-ZOL, β-ZOL, FB1, FB2, FB3, EN A, EN A1, EN B, EN B1, BEA, AFB1, AFB2, AFG1, AFG2, AFM1, STG, OTA, OTα,	QuEChERS	UHPLC–HRMS	-	1-50	[110]
Cow	Milk							[24]
Donkey	Milk		AFM1 and AFM2	IAC	HPLC-FLD, LC-MS/MS			[23]
Cow	Milk	50 ml	AFM1	IAC	LC-MS/MS	-	0.01	[22]

Human	Serum Urine	1 ml 5ml	OTA, OTα	LLE: CHCl3/isopropanol	HPLC-FLD HPLC–ESI-MS/MS	0.07 0.02	0.1 0.5	[95-96]
Human	Serum Urine	1 ml 5 ml	CIT	LLE: ACN, IAC: CitriTest	HPLC- FLD			[96-99]
Human	Serum Urine	250 µl 5 ml	ENs, BEA	LLE: MeOH/H2O, SPE: GCB	LC-MS/MS	0.01-0.0025-0.02	0.02-0.04 0.005-0.02	[98]
Chicken Pig	Serum Bile	250 µl 1 ml	DON, DOM-1, T-2, HT-2	LLE: MeOH, SPE LLE: MeOH/H2O, EtOAc	LC-MS/MS	0.01-0.63	1.0-2.5	[112-113]
Pig	Serum, urine, liquor	500 µl	ZEN, DON, ZAN, α-ZOL, β-ZOL,α-ZAL, β-ZAL	SPE: Oasis HLB	HPLC-ESI- MS/MS	0.005-0.71 0.03-0.16 0.02-0.21	0.08-2.37 0.1-0.52 0.07-0.70	[97]
Rat	Serum, urine, feces	200 µl 100 mg	EN A	LLE: EtOAc	LC-MS/MS	1.8-2.3	5.4-7	[101]

Rat	Serum, urine, feces	500 µl 500 mg	EN A, A1, B, B1	LLE: ACN	LC-MS/MS	0.2-1	2-10	[100]
Horse	Serum, urine, feces	1 ml 5 ml 2 g	ZEN, DON, ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL	IAC SPE: C18 , IAC SPE: C18 , IAC	HPLC–APCI–MS/MS	0.1-0.3 0.1-0.2 0.1-0.5	0.5-0.6 0.5-1 0.5-1	[102]
Human	Feces	1-2 g 1 ml	OTA, OTB	SPE: C18	HPLC- FLD	-	1.25-2.22 1.44-2.99	[108]
Human	Breast milk	2 ml	AFB1, AFB2, AFG1, AFG2, AFM1, OTA	LLE: ACN/EtOA, LTP (low temperature purification)	HPLC- FLD LC–MS/MS	-	0.005-0.03	[109]

2.2 Mycotoxins Analysis in Organs and Tissues

Many combinations of different methodologies have been carried out for mycotoxin extractionfrom solid biological samples namely tissues and organs. LLE techniques have been performed withseveral solvents; ACN and ACN-H2 O were used for BEA and ENs extraction from mice samplesincluding liver, kidney, colon, fat, brain, muscle, tumor, urine, and serum [26,115]. Masked andconjugated forms of DON and ZEN were extracted using ACN/formic acid (99/1) from several ratsamples including plasma, urine, liver, kidney, bladder, spleen, lung, stomach, small intestine, andlarge intestine [116]. AFB1 and OTA were extracted with the same solvent mixture from rat plasma, liver, and kidney [117]. LLE for ENs and type A trichothecenes (T-2, HT-2, DAS) was performed using EtOAc in several samples of rat and broiler, respectively [118,119].

Other solvents have been used in recent years for mycotoxin LLE from organs and tissues. For instance, OTA was extracted using CH_2Cl_2 [120] and by a solvent mixture of ice-cold absolute ethanol/trichloroacetic acid [121] from pig and rat samples; plasma, liver, and kidney, respectively.

TBME was used for ZEN extraction from several rat samples including serum, bile, urine, lung, liver, spleen, kidneys, heart, testes, brain, muscle, adipose tissue, stomach, and small intestine [122]. In some cases LLE was followed by other extraction or purification systems. After ACN-LLE of T-2, rat liver and kidney samples were passed through a purification column (activated charcoal: celite: aluminum trioxide) for sample purification [123]. The combination of LLE followed by SPE has beenwidely used for multi-mycotoxin extraction from several biological samples. OTA was extractedfrom hen kidney, liver, and bile by CH_2Cl_2 -LLE followed by SPE [27]. The type B

trichothecenesFUS-X and NIV were extracted from pig and chicken plasma, urine, feces, liver, kidney, spleen, muscle, intestine heart, screta, and bile by the mixture ACN/H2 O (3:1) followed by C18 Sep-pak silicacartridge [124,125], while type A trichothecenes T-2, HT-2, T-2 triol extraction from pig samples (plasma, fat, muscle, stomach, brain, small intestines, heart, lung, spleen, urine and feces) was performed byEtOAc-LLE combined with bond-elut mycotoxin SPE cartridge [126]. The emerging mycotoxins ENsand BEA were extracted using ACN followed by SPE silica column from broiler and poultry liver and tissues [127] or C18 cartridges from fish liver, viscera, tissue, and head [128]. Several trichothecenes; NIV, DON, DOM, NEO, 3-ADON, 15-ADON, T-2-triol, HT-2, and T-2 were extracted from chicken and pig muscle and liver combining ACN/EtOAc (1:3)-LLE and Oasis HLB cartridges [129].

In addition to LLE and SPE techniques, some authors included an hexane deffation step to ensure the removal of fat components present in the matrix, which could interfere in the detection process. The extraction of DON, 3-ADON, 15-ADON and DOM-1 from chicken samples including muscle, liver, kidney and fat, was performed by EtOAc-LLE followed by hexane deffation and Oasis HLB cartridge [130]. The extraction of ZEN, ZAN and their metabolites α-ZOL, β-ZOL, α-ZAL and β-ZAL from bovine liver and muscle was performed by several steps including MeOH and EtOAc extraction, intercalated by repeated hexane defattion steps [131]. FB1 and its derivated aminopentol-1 (AP-1) were extracted from swine liver by MeOH/H_2O (80:20)-LLE followed by hexane deffation and Oasis HLB cartridge [132]. The extraction of 28 mycotoxins and metabolites from several animal species (dog, rabbit, rat) and human samples including urine, blood, faeces, saliva, nasal secretions, breast milk, amniotic fluid of pregnant women, liver, spleen,

lung, kidney, stomach, colon, brain, urine, blood and faeces was studied throughout the comparison of three extraction methods based on LLE, QuEChERS and PLE. Although the three methodologies showed satisfactory extraction efficiency, PLE was selected using the mixture ACN/H2O/acetic acid (80:19:1) for biological fluids and ACN/H2O/hexane/acetic acid (60:14:25:1) for organs and tissues [28]. OTA was extracted by ACN-LLE with hexane defattion followed by IAC from cow serum, liver, kidney, muscles, fat, intestine and milk [133], while CHCl3/phosphoric acid (10:1)-LLE followed by IAC OchraTest WB was used for OTA extraction from muscles, liver and kidneys from swine, cattle, sheep, horses, fish, chickens, turkeys, geese, and ducks [134]. The same solvent mixture was used to extract ZEN and its metabolites from goat plasma, urine, feces, and liver followed by IAC cleanup procedure [135]. Type A trichothecenes T-2, HT-2 and T-2 triol were extracted from boar liver, kidney spleen, hearth, muscle, lung, ovary and uterus by MeOH-LLE followed by IAC [136].

IAC technique has been also combined with previous SPE using EtOAc in ChemElut columns for the extraction of DON, DOM-1 from pig plasma, bile, urine, liver, kidney and muscle [137]. FB1 was extracted from turkey poult plasma by ACN extraction in C18 supelclean column followed by SAX cartridge, and from muscle, liver and kidney using ACN/MeOH (50:50) extraction, followed by hexane defattion and passed through fumoniprep cartridge [138]. Since a wide variety of different tissues and organs were analyzed, sample size used for the analysis ranged from 25 mg to 20 g in solid samples and between 50 μl and 5 ml for liquid biological samples. Achieved LODs were in the range of 0.015-200 μg/Kg-μg/L, and LOQs ranged from 0.05 to 600 μg/Kg-μg/L.

Table 5 shows the latest studies of on single mycotoxin analyzed in organs and tissues, where OTA, followed by DON were the most common ones. Singles studies of ZEN, NIV, FB1, T-2 and HT-2 were also perfomed. In Table 6 the latest studies focused on structurally related compounds carried out in organs and tissues are shown, while multi-mycotoxins studies are summarized in Table 7.

The sample size, studied compounds, extraction and detection methods, and LODs-LOQs are indicated.

Table 5. Studies of single mycotoxin determination in organs and tissues.

Specie	Biological sample	Sample Size	Mycotoxins	Extraction procedure	Detection technique	LOD (µg/kg)	LOQ (µg/kg)	Ref.
Mouse	Plasma, spleen, liver, lung and kidney	40-200 mg	DON	PBS	ELISA	-	-	[19]
Mouse	Plasma, liver, kidney, heart, spleen and brain	100 µl (extract)	DON	ice-cold ethanol/trichloroacetic ac.	ELISA	-	-	[142]
Rat	Plasma, liver, kidney	250 µl 200-400 mg	OTA	LLE: ACN, SPE	HPLC-FLD	1-14.3	8.4-52.8	[119]
Pig	Plasma, liver, kidney	800 µl 20 g	OTA	LLE: ACN, SPE	HPLC-FD and LC-MS/MS	0.14 0.25	0.25 0.5	[118]
Swine, cattle, sheep, horse, fish, chicken, turkey, geese, duck	Muscles, liver and kidneys	10 g	OTA	LLE: $CHCl_3$, IAC: OchraTest	LC–FD	-	0.2	[133]
Laying Hens	Kidneys, liver, bile (eggs)	2.5 g 200 µl	OTA	LLE: $CHCl_3$, SPE	HPLC-FD	0.3-0.5	1	[27]

Turkey poults	Plasma, muscle, liver and kidney	250 µl 1g	FB1	SPE: SAX IAC: FumoniPrep	HPLC-FLD	13	25	[137]
Rat	Serum, bile, and urine. Lung, liver, spleen, kidneys, heart, testes, brain, muscle, adipose tissue, stomach, and small intestine		ZAN	LLE, IAC	LC-MS/MS HPLC-FD	-	0.5 10	[120]
Rat	Liver and kidney	1 g	T-2	LLE: ACN, SPE: charcoal	HPTLC	-	100	[121]
Rat	Serum, stomach, duodenum, jejunum, ileum, colon, liver	0.5 g 0.5 ml	EN A	LLE: EtOAc	LC-MS/MS	200	600	[116]
Chicken	Serum, liver, kidney, heart, muscle, small intestine and excreta	1 ml 5 g	NIV	LLE: ACN/H2O (NH4)2SO4, SPE C18	LC-MS/MS	-	2-2.5	[123]
Cow	Serum, milk, liver, kidney, muscles, fat, jejuno, ileum	2-5 ml 10 g	OTA	LLE: ACN/Hex, IAC	HPLC-FD	-	-	[132]

Table 6. Studies of structurally related mycotoxins determination in organs and tissues.

Specie	Biological sample	Sample Size	Mycotoxins	Extraction procedure	Detection technique	LOD (µg/kg)	LOQ (µg/kg)	Ref.
Swine	Plasma, liver	10 g	FB1, AP-1	LLE: ACN, SPE: C18, SAX, Oasis HLB. LLE: ACN/MeOH, Hx, IAC: FumoniPrep	HPLC-FD	10-20	42-75	[131]
Pig	Plasma, bile, urine, liver, kidney, muscle	1.5 ml 1ml 1ml 2g 2.4g	DON, DOM-1	LLE:Cl3, IAC: DON-test	LC-MS/MS	1.5-10	2-10	[136]
Goat	Plasma, urine, feces, liver	5 ml 5g	ZAN and metabolites	LLE: EtOAc, IAC: Easi-Extract ZAN	HPLC	-	2.1-46.6	[134]
Bovine	Mucle, liver	5 g	ZEN, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL	LLE: MeOH-Hx, SPE: Sep-pak amino	UPLC-MS/MS; CLEIA	0.5	0.5-0.7	[130]
Boar	Muscle, liver, kidney, spleen, cardiac muscle, lung, ovary, uterus	3g	ZEN, α-ZOL, β-ZOL	LLE: MeOH, IAC	LC-MS	1	-	[135]

Pig	Plasma, fat, muscle, stomach, brain, small intestines, heart, lung, spleen, urine, feces	0.5 ml 2g	T-2, HT-2, T-2 triol	LLE: ACN. LLE: EtOAc, SPE: Varian Bond-Elut	LC-MS/MS	0.3-2	1-5	[124]
Broiler, poultry	Liver and meat	5 g	ENs, BEA	LLE, SPE	LC-MS/MS	0.015-0.56	0.03-1.12	[125
Fish	Liver, viscera, tissue, head	5-10 g	ENs	LLE: ACN, SPE: C18	LC-MS/MS	0.3-3	1-10	[126]
Mice	Liver, kidney, colon, fat, brain, muscle, tumor urine, serum	0.2 g 50 µl	BEA, EN B	LLE: ACN	LC-MS/MS	-	0.05–0.15	[26]
Mice	Serum, Brain	50 µl	BEA, ENs	LLE: ACN-H2O	UPLC-MS/MS	0.3	-	[113]

Table 7. Studies of multi-mycotoxins determination in organs and tissues.

Specie	Biological sample	Sample Size	Mycotoxins	Extraction procedure	Detection technique	LOD (µg/kg)	LOQ (µg/kg)	Ref.
Human	Urine and nasal secretions (nasal washes, sputa), heart, liver, urine		TCT, AFs, OTA	PBS, formalin	ELISA, Fluorometry	0.2-2	-	[112]
Human Rat Dogs Rabbit	*Human:* urine, blood, faeces, saliva, nasal secretions, breast milk, amniotic fluid of pregnant women. *Animal:* liver, spleen, lung, kidney, stomach, colon, brain, urine, blood, feces.	200 µl 200 µg	AFB1, AFB2, AFG1, AF2, AFM1, AFM2, OTA, DON, NIV, T-2, HT-2, 3-ADON, 15-ADON, NEO, FUS-X, DAS, MAS, ZEN, ZAN, α-ZOL, β-ZOL, α-ZAL, β-ZAL, T-2 triol, T-2 tertraol, DOM-1, FB1, FB2	PLE: ACN/ H2O/hx/acetic acid	HPLC–MS/MS	CCa: 0.01-0.69	0.2-0.5 CCb: 0.15-1.26	[28]
Chicken Pig	Muscle, liver	1g	T-2, HT-2, T-2-triol, NEO, DON, 3-Ac-DON, 5-Ac-DON, DOM, NIV	LLE: ACN/EtOAc SPE: Oasis HLB	UPLC-MS/MS	3-15	10-50	[128]

Rat	Plasma, liver, kidney	100 µl (25 mg tissue)	ABF1, OTA	LLE: CHCl3, IAC	UHPLC-FLD	0.01-0.3	2-8	[115]
Rat	Plasma, urine, liver, kidney, bladder, spleen, lung, stomach, small intestine, large intestine	-	DON-3G, ZEN-14-G, 3-ADON, 15-ADON	LLE: ACN	UPLC-MS/MS	0.3-16.3	0.6-54.4	[114]
Chicken	Muscle, liver, kidney, fat, tissues	2 g	DON, 3-ADON, 15-ADON, DOM-1	LLE: EtOAc, SPE: Oasis HLB	LC–MS/MS	CCa: 0.16-0.92	CCb: 0.68-2.07	[129]
Chickens	Muscle and liver	1 g	NIV, DON, DOM, NEO, 3-ADON, 15-ADON, T-2-triol, HT-2, T-2	LLE: ACN/H2O, SPE: Oasis HLB, IAC: charcoal/alumina/celite	UPLC–MS/MS	1-5	3-15	[127]
Broiler	Heart, liver, spleen, lung, kidney, stomach, small intestine, muscle, bone, brain	1 g	T-2, HT-2, DAS	LLE: EtOAc	LC–MS/MS	0.02-0.05	0.08-0.17	[117]

Pig	Plasma, urine feces, liver, kidney, spleen, muscle, intestine, bile	1 ml 5 g	FUS-X, NIV	LLE: ACN/H2O, SPE: C18	LC-MS/MS	1.0-1.8	1.11-2.4	[122]
Pig	Bile, liver and muscle	-	ZEN, α-ZOL, β-ZOL, DON	LLE: MeOH/H2O, Hx, SPE: Oasis HLB	HPLC and EIA	-	-	[18]

2.3 Most common methodologies

From the analyzed studies it was shown that LLE (24%) follow by LLE-SPE (19%) and IAC (17%) was often prefered to extract mycotoxins from biological samples in the last years. Other less used extraction techniques were dilute-and-shoot (10%), LLE-IAC (9%), SPE-IAC (9%), and SPE (6%).

In serum and other fluid samples more than 50% of the studies performed mycotoxins extraction by LLE procedures. However, mycotoxins analysis in urine included a wide variety of methods, with fast techiques such as dilute-and-shoot (31%), followed by IAC (28%), SPE-IAC (11%) and LLE-SPE (11%) being the most representative. With regard to organs and tissues analysis almost half of the studies were based on LLE-SPE (45%) followed by LLE-IAC (26%), and LLE (19%).

Regarding mycotoxin determination the great majority (55%) was performed by LC-MS/MS-including HRMS. Other detection systems such as LC-FD (23%), ELISA (8%), and GC-MS/MS (4%) were also used. It should be noted that this detection system trend remains similar when analyzing serum individually, other biological fluids, and even organs and tissues. However, the LC-MS/MS proportion considerably increases (79%) in the case of urine sample analysis. This preference by MS/MS detectors could be explained by the very low mycotoxin levels generally found in urine samples, along with the clear trend towards multi-analyte method development and application in urine mycotoxin biomarker research [104].

2.4 Most studied mycotoxins

The most analyzed mycotoxin considering all biological samples was OTA, either alone or in combination with other mycotoxin determination.

Indeed, the studies focused in one single compound (or structurally related comopounds) were mainly about OTA, and in minor proportion ZEN and its metabolites, DON and its metabolites, ENs and BEA, FBs, AFs, T-2 and HT-2.

With regard to serum samples, this predominance of OTA was even higher, becoming the main compound in almost half of the studies, followed by AFs, DON-ZEN, and their metabolites, and the minor Fusarium mycotoxins ENs and BEA. As it was reported below, urine samples included the largest number of compounds in a single analysis, however, the same tendency followed in serum was shown (OTA > ZEN-DON > AFs), including other commonly studied mycotoxins; FBs, T-2, and HT-2. In minor biological fluids OTA, DON, and AFs were the most analyzed mycotoxins, followed by the emerging fusarotoxins. In the case of organs and tissue analysis, the most representative mycotoxins were OTA, type A and B TCTs, and ZEN including its metabolites.

2.5 Biological samples origin

The animal species of origin for the studied biological samples were analyzed. As it was expected, when non-invasive collection samples were used, human samples were interesting goals for mycotoxin determination. Thus, as it is shown in Figure 1, half of the serum samples studied as individually matrix (only serum analysis), were from human provenance (50%), followed by pig (17%), chicken (13%), rat (9%), and in minor proportion fish (5%), horse (5%), hens (1%), and turkey (1%). Similarly, in the case of fluid combination studies, including feces analysis, half of the samples were also from human (53%), followed by rat (12%), cow (12%), pig (9%), and minor proportion horse (6%), donkey (6%), and chicken (3%).

On the other hand, in studies involving mycotoxin urine analysis the great majority were from human (80%) and to a lesser extent from pig (9%), bovine (6%), cattle (3%), and rat (2%). Finally, the studies involving mycotoxins analysis in organs and tissues were generally focused on laboratory animal samples, mainly rat (22%), pig (18%), chicken (16%), mice (10%), hens (5%), and in minor proportion other animal species such as fish (4%), bovine (3%), goat (3%), boar (3%), cow (3%), dog (1%), and rabbit (1%). Due to these studies being relatively complex and which often included a wide number of different matrices, biological samples from human (4%) were also found (i.e., saliva, nasal secretions, amniotic fluid, breast milk, etc.).

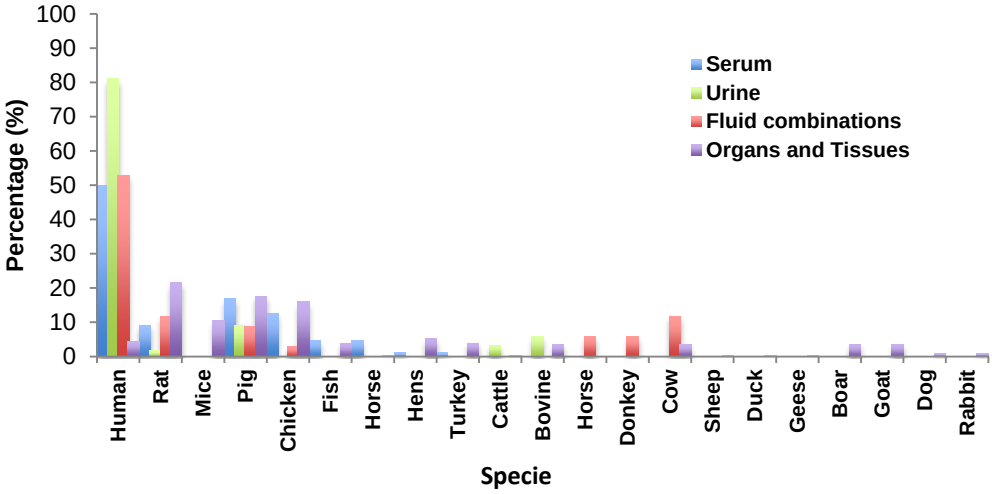

Figure 1. Animal species of biological sample origin: percentage of studies in serum, urine, fluids, and tissues.

2.6 Expected Purposes of Biological Samples Analysis

There has been shown wide importance of mycotoxins analysis in biological samples in recent years since large and varied information can be obtained from them. Thus, the studies of mycotoxins in biological samples

performed in recent years had different purposes, from analytical method development -including a small method demonstration/application by analyzing a few number of samples, sometimes part of a larger pilot study- or determination of mycotoxin content and its relation with some diseases (eg. nephropathy), to toxicokinetics, ADME (absorption, distribution, metabolism, elimination) and bioavaliability studies, tissue persistence data in different animal species, and human biomonitoring and expossure assessment. As Figure 2 shows, the most common studies were focused on method developmet, due to the high sensitivity requirements for mycotoxin determination in biological samples considering the low levels generally present in them. On the other hand, human biomonitoring is increasingly being recognized as an efficient and cost-effective way of assessing human exposure to food contaminants, including mycotoxins.

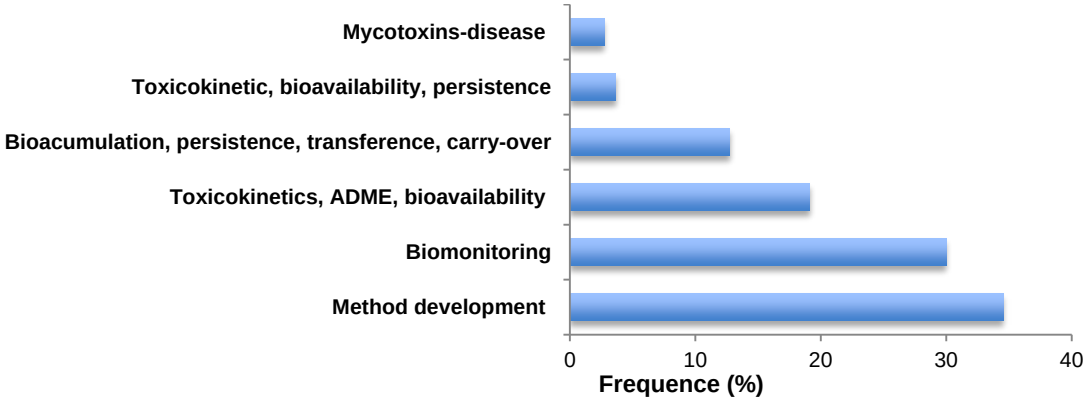

Figure 2. Study classification depending on the main purpose of mycotoxin determination in biological samples.

Interestingly, using validated biomarkers of exposure is possible to cover exposure from all sources, decreasing uncertainties related to occurrence and consumption rates. Moreover it can be used to establish

population reference ranges and identify vulnerable consumer groups and individuals with higher exposures [140]. For human biomonitoring easily accessible biological matrices such as urine or blood were used, with urine being preferred for several reasons including the non-invasive sampling and higher acceptance by study participants. Consequently, biomonitoring studies have been frequently performed almost worldwide (Figure 3), including Nigeria [70], Bangladesh [58,69,85,86,88,96], Haiti [69], Turkey [64,106], Belgium [73,74,105], Portugal [60,61,89], Spain [36,61,93], Germany [35,62,64,68,69,86,87], Italy [77,111], Austria [72], Czech Republic [38,43], Tunisia [41], Brazil [40,109], Chile [44], Cameroon [141], Egypt [50], Pakistan [37], Iran [107],and China [84].

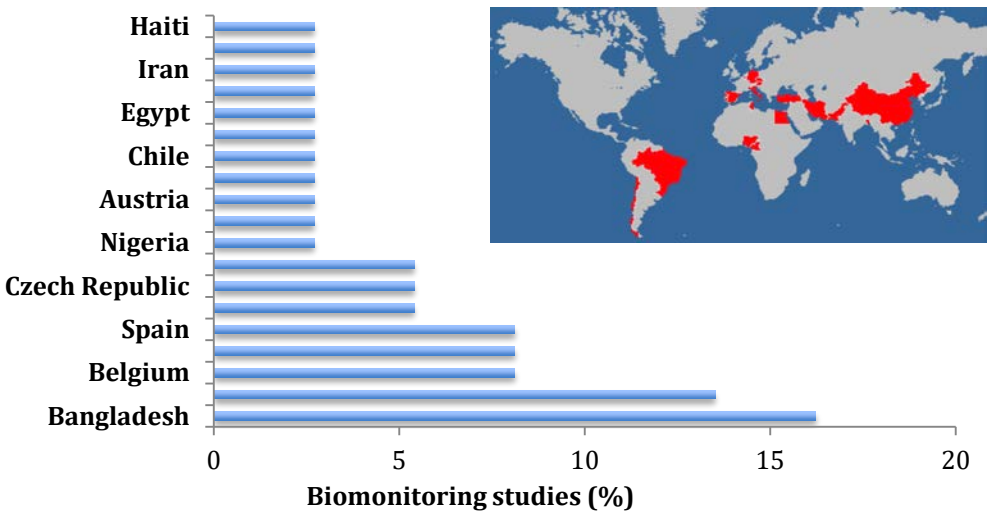

Figure 3. Percentage of biomonitoring and exposure assessment studies performed through mycotoxin analysis in biological samples worldwide.

In these population studies, most often performed on human urine, samples from volunteers, from 27 up to 418, were collected and analyzed.

Some of these studies reported additional information to that of the sample analysis. Thus, the correlation between mycotoxin content and different parameters such as the ingested diet—thought-out food questionnaires completed by the volunteers [36,68], estimation of the probably daily intake (PDI) [44,69], correlation with socio-demographic factors and anthropometric characteristics [141], or exposure to airborne molds [50] were evaluated.

With regard to studies focused on toxicokinetics, absorption, metabolism, and bioavailability, mycotoxin concentration was generally determined after mycotoxin feeding or administration; oral, per os, intravenous (IV), intraperitoneal (IP). Other studies were focused on mycotoxin tissue distribution and persistence in several animals such as chicken [125], mice [19,26,139], rat [122], pig [126]; bioaccumulation, and persistence [7,138], carry-over [20,22,24,32,133], and tissue residues [18,137].

It must be borne in mind that all this information about mycotoxins toxicokinetics, metabolism and bioavailability is highly necessary to both be able to calculate PDIs in individuals or population and to establish the TDIs by the regulatory authorities, and thus to allow the exposure assessment to these toxic and ubiquitous compounds.

2.7 Mycotoxins Bioaccumulation Findings

Broad information has been obtained in the last years with regard to mycotoxins bioavaliability, toxicokinetics, ADME, bioaccumulation and tissue persistence by mycotoxins analysis in biological samples, with the main focus on DON, ZEN, OTA, ENs and BEA, NIV, T-2 and FBs.

2.7.1 DON and Metabolites

DON was detected in plasma (12 µg/L), liver, kidney, spleen, heart, and brain up to 19.5 µg/g after oral administation (25 mg/kg bw) in mice with highest plasma concentrations within 5-15 min after dosing [139]. Similary, DON was reported in plasma, spleen, liver, lung and kidney after oral and intranasal administration (5 mg/kg bw) in mice with maximal concetrations within 15-30 min, declining to 75-90% after 120 min. Moreover, plasma and tissue DON concentrations were 1.5-3 times higher after intranasal exposure than following oral exposure suggesting that DON was more toxic nasally administered than orally in mice [19]. Also in pig, DON was detected in serum (5-17 µg/L), kidney, urine, bile, liver, muscle at low concentrations after 28 days feed supplementation (DON: 0.28-2.31, DON-sulfonate: 1.85 mg/kg). DON-sulfonate was stable under porcine digestive tract conditions and probably absorbed to the same extent as DON [137]. DON-3-sulfate was the major DON metabolite in chicken, pullet, rooster, and turkey after oral administration of DON by naturally contaminated feed (0.2–11 mg/kg). Fast and efficient absorption of DON between crop and jejunum was observed, followed by the conversion to DON-3 sulfate in intestinal mucosa, liver, and possibly kidney, and the rapid elimination into excreta via bile and urine [106]. DON showed low absolute oral bioavailability (19.3%) after oral administration (0.75 mg/kg bw) in broiler chickens. Volumes of distribution, total body clearance, and elimination half-life were 4.99 L/kg, 0.12 L/min kg, and 27.9 min, respectively, after IV administration [113]. Rapid clearance ($t_{1/2\alpha} = 20.4$ min, $t_{1/2\beta} = 11.8$ h) was observed in mice with 5% and 2% maximum plasma DON concentrations remaining after 8 and 24 h, respectively, with DON distribution and clearance kinetics in other tissues

similar to that of plasma [139]. Differences in the urinary metabolite profile of DON in human and rat were observed. DON and DON glucuronide were found in both human and rat urines, whereas DOM-1 and its glucuronide conjugate were only detected in rat urine. Human DON urinary levels ranged 0.003 and 0.008 μ g/mL whereas rat DON and DOM-1 urinary levels were between 1.9 and 4.9 μg/mL and 1.6 and 5.9 μg/mL, respectively, after oral administration (3.6 mg/kw bw/day over 4 days) [76]. DON urinary daily excretion of 35.2 μg was determined in humans after 49.2 g DON daily intake, representing 68.3% of the established DON provisional maximum tolerable daily intake (PMTDI) [31].

27.2 ZEN and Metabolites

ZEN absolute oral bioavailability was 10.3% after oral administration (16 mg/kg) in rats with elimination half-life of 8.5 h. The systemic clearance, volume o distribution, and elimination half-life after IV administration (2 mg/kg) were 6.5 L/h/kg, 4.7 L/kg, and 1.9 h, respectively [48] while in broiler chickens ZEN volumes of distribution, total body clearance, and elimination half-life were 22.26 L/kg, 0.48 L/min kg, and 3.9 min, respectively, after oral administration (0.3 mg/kg bw) [113]. ZEN was rapidly absorbed (Tmax = 0.32–0.97 h) and eliminated ($t_{1/2el}$ = 0.29–0.46 h) after oral and IV administration (3 mg/kg bw) in poultry, showing absolute oral bioavailability of 7-10% [55]. Accordingly, rapid absorption and low absolute oral bioavailability (2.7%) was shown in rats after oral administration (8 mg/kg). ZEN was excreted unchanged in rat urine (0.5%) and bile (0.91%), showing average clearance and volume of distribution of 5.0–6.6 L/h/kg, and 2-4.7 L/kg, respectively, after IV infusion over 6 h (1.12-2.25 mg/h/kg). The highest ZEN concentrations were found in small intestine, kidneys, liver, adipose

tissue, and lung [122]. ZEN wa distributed ($t_{1/2\alpha}$ = 3.15 h) and eliminated ($t_{1/2el\beta}$ = 3.15 h) after single IV injection (1.2–2.4 mg/kg bw) i goat. Only α-ZOL and β-ZOL were detected in liver tissues at 48 h after IV administration. ZEN, α-ZOL and β-ZOL were excreted in urine and feces, β-ZOL being the predominant metabolite. The ZEN and ZOL in urine were mostly in their glucuronide and/or sulfate conjugated forms, while those in feces were largely in their free forms [135]. ZEN glucoronidation degree was 27% in pig urine (α-ZOL 88%, β-ZOL 94%) and 62% in liver (α-ZOL 77%, β-ZOL 29%). High amounts of ZEN and non-glucuronidated ZOL and α-ZOL were found in muscles, indicating that ZEN metabolism is not restricted to hepatic and gastrointestinal metabolic pathways [142]. ZEN biotransformation to α-ZOL and β-ZOL were equally reported after IV administration in poultry, but increased for β-ZOL after oral administration indicating presystemic biotransformation [55]. Highest values of ZEN carry-over factor were identified in the same tissues after oral administration bolus (150 μg/kg) and diet supplementation (50 μg/kg) in boar, showing ZEN residues in spleen (20 ng/g), cardiac muscle (18 ng/g), kidneys (15 ng/g), muscle (12 ng/g), uterus (11 ng/g), and kidneys (10 ng/g) [136].

Serum and urine concentrations of DON, ZEN and its metabolites increased with diet concentrations increase in pig (ZEN 0.01-0.29 mg/kg and DON 0.03-4.52 mg/kg; over 29 days), showing high correlation between the dietary DON intake and the sum of DON and DOM-1 concentration in serum, but accumulation was not shown. ZEN, α-ZEN, DON and DOM-1 were detected in serum, urine, and liquor at lower concentrations [97]. ZEN was clearly formed from ZEN-14G, while the acetylated forms of 3-ADON and 15-ADON were hydrolysed in the stomach after oral administration in rats, in contrast

to DON-3G. Rats can directly glucuronidate ADONs without deacetylation, showing DON-3GlcA accumulation in the small intestines [116]. DON and ZEN residues were found in pig bile, liver and muscle with highest residues in bile after both organically and conventionally wheat feeding [18]. Non quantification plasma level were found for ZEN and T-2 after oral administration (T-2 0.02 mg/kg bw and ZEN 0.3 mg/kg bw) in broiler chickens [113]. Good correlation was observed between the amount of mycotoxins ingestion and the amount of excreted biomarkers in urine 24 h after administration in pig, showing linear dose-response (r^2: 0.68-0.78) for the mycotoxin and its biomarker (DON-DOM-1, OTA, AFB1-AFM1, FB1, ZEN-α-ZOL). Mean percentages of dietary mycotoxins excreted as biomarkers for ZEN (0.6-5.7 μg/kg bw), DON (7.2-77.4 μg/kg bw), FB1 (3.7-150.2 μg/kg bw), OTA (0.2-1.3 μg/kg bw), and AFB1 (0.2-1.3 μg/kg bw) were 36.8, 28.5, 2.6, 2.6 and 2.5%, respectively [81].

2.7.3 OTA

OTA was detected in 28.8% of the analyzed swine kidney samples (n=1092) in concentrations ranging 0.2 to 29.2 μg/kg, but non-quiantificable OTA levels were found in muscle, liver and kidney of cattle, sheep, horse, fish, chicken, turkey, geese, and duck [134]. Relevant OTA biliary excretion after dietary suplementation (10 and 200 μg/kg) during 6 weeks was shown in laying hens with constant ratio between OTA bile concentration and ingested OTA. Higher levels of OTA were reported in bile than in kidney and liver [27]. However, OTA residues were not detected in cow tissues and milk, but small amount of OTA (0.1 μg/kg) was detected in plasma after dietary OTA

suplementation (>100 μg/kg) during 28 days, indicating OTA non carry-over into milk and tissues [133].

2.7.4 ENs and BEA

EN B1 was rapidly absorbed ($t_{1/2\alpha}$ = 0.15 h, Tmax = 0.24 h), distributed and eliminated ($t_{1/2el\alpha}$ = 0.15 h; $t_{1/2el\beta}$ = 1.57 h) after oral administration (0.05 mg/kg/bw) in pigs with absolute oral bioavailability of 90.9% indicating clear systemic exposure, and rapidly distributed and eliminated ($t_{1/2el\alpha}$=0.15 h; $t_{1/2el\beta}$ = 1.13 h) after IV administration (0.05 mg/kg/bw) [53]. EN B1 and EN B were poorly absorbed after oral and IV administation (0.2 mg/kg/bw) in chicken, with absolute oral bioavailabilities of 0.05 and 0.11%, respectively. Both were readily distributed to the tissues, with mean volumes of distribution of 33.91 and 25.09 L/kg, respectively, and mean total body clearance of 7.10 and 6.63 L/h/kg. Oxidation was the major phase I biotransformation pathway for both ENs, but neither glucuronide nor sulfate phase II metabolites were detected [45]. EN A was detected in rat serum after EN A dietary supplementation during 28 days (465 mg/kg) in an exposure time-dependent manner, reaching serum concentration of 4.76 mg/ml in the fourth week. However, EN A was not detected in feces and urine samples [101]. EN A was detected in rat tissues and fluids after 28 days feed suplementation (20.91 mg/kg bw/day) with highest concentrations in liver (23 mg/kg), and contents of jejunum (9.6 mg/kg), colon (7.3 mg/kg) and stomach (4.6 mg/kg), as well as in serum (5 mg/kg) [118]. BEA and ENs traces levels were detected in poultry tissues with concentrations lower than 2 μg/kg [127], and both emerging mycotoxins were found in mice tissues and serum after IP administration (5 mg/kg bw, 2-3 days), with higher amounts in

liver (EN B: 2.9 μg/kg and BEA: 41.7 μg/kg), and fat (EN B: 2.5 μg/kg and BEA: 33 μg/kg), indicating their tendency to bioaccumulate in lipophilic tissues [26]. Moreover, BEA and ENs crossed the blood-brain barrier in mice exerting a high initial brain influx rate and reaching mainly the brain parenchyma (95%) after their penetration, with negligible efflux after 15 min of intra-cerebroventricular injection. Therefore, BEA and ENs are able of reach the systemic circulation through various routes of exposure and may exert central nervous system (CNS) effects passing the blood-brain barrier (BBB) [115].

2.7.5 NIV and FUS-X

NIV was poorly absorbed orally with low bioavailability (4%) and rapidly eliminated via feces in chicken after oral administration (0.8 mg/kg bw). Elimination half-life was 2.51 h and 5.27 h and after oral and IV administration, respectively. NIV was detected in small intestine, kidney, heart, liver and muscle suggesting that it is absorbed from the gastrointestinal tract diffusing into various broiler tissues [125]. FUS-X and NIV were detected in pig plasma, urine, feces and tissues after a single IV and oral administration (1 mg/kg bw), and in vital organs (24 h after oral administration), with highest FUS-X concentration in liver (166 ng/g), kidney, (66.3 ng/g), and spleen (7.4 ng/g) 3 h after oral administration [124].

2.7.6 T-2 and HT-2

T-2 toxin was rapidly absorbed and metabolized into HT-2 and T-2 triol after oral and IV administration (1 mg/kg bw) in pig. HT-2 and T-2 triol metabolites were rapidly distributed into tissues, mainly liver (216.3 μg /kg), kidney (206. μg /kg), and the small intestines (140.5 μg /kg), still detected at

6 h after administration. The highest T-2 concentration were detected in fat tissues (58.6 µg g/kg), lungs (54.0 µg/kg) and spleen (47.8 µg /kg). T-2 was quickly eliminated in plasma after IV administration, and low urine excretion (<7%) was shown for T-2, HT-2, and T-2 triol, with only HT-2 (0.25%) being detected in feces [126]. T-2 Volumes of distribution, total body clearance and elimination half-life was 0.14 L/kg, 0.03 L/min kg, and 31.8 min after oral administration (0.02 mg/kg bw) in broiler chickens [113]. Trace concentrations of T-2, NEO and T-2-triol, as well as large amount of HT-2 were detected chicken muscle and liver after oral administration (3 mg/kg of bw) indicating that T-2 toxin was rapidly metabolized to mainly HT-2 as the main metabolite, which was even detected in liver 48h after administration [129]. Contrary, non-residual T-2 toxin was detected in any rat organ nor tissues even at high exposure level (20 mg/kg) in rat [123].

2.7.7 FBs

FB1 bioavailability was 3.2% after single-dose oral administration (100 mg/kg bw), with elimination half-life, mean residence time, and clearance of 214 min, 408 min and 7.5 ml/min/kg after oral bolus, respectively, and 85 and 52 min, and 7.5 ml/min/kg after IV administration (10 mg/kg bw). Liver and kidney contained the highest levels of FB1 after 24 h IV (liver: 46, kidney: 50 µg/kg) and oral administration (liver: 5458, kidney: 5785 µg/kg), being non detectable in muscle. Persistence of FB1 was observed after 9 weeks of FB1/FB2 feed suplementation (5-20 mg/kg) showing liver (11,922 µg/kg) and kidney (22 µg/kg) residues 8 h after the last intake [138].

2.8 Risk assessment

Since the consumption of contaminated food is considered the major source of human mycotoxin exposure, accurate estimation of mycotoxin exposure is compulsory to facilitate weighty risk assessment. The measurement of specific urinary mycotoxin biomarkers -both the metabolite generated by human metabolism or the parent toxin itself- is a valid alternative to measure mycotoxin exposure whenever biomarkers excretion correlates well with mycotoxin intake [77]. Thus, mycotoxin exposure assessment throughout biomonitoring studies based on mycotoxin analysis in human biological samples such as urine, serum and breast milk, have provided useful information over recent years, OTA, DON, and CIT being the most reported mycotoxins.

2.8.1 OTA

Analyzing swine serum samples was presented as an alternative approach to monitor the presence of OTA in feed to prevent the occurrence of ochratoxicosis in animal production. A direct relationship between OTA exposure levels and serum concentrations in slaughtered swine (n = 400) was reported in Brazil [40]. OTA exposure was also estimated based on human serum OTA levels carried out in several studies worldwide. OTA and OT-α were detected in 100% an 95% of the analyzed plasma samples in Bangladesh (n = 64) at ranges of 0.20-6.63 μg/L, and 0.10-0.79 μg/L, respectively. The calculated OTA intake on the basis of plasma concentration in the population was lower than the tolerable weekly OTA intake (120 ng/kg b.w/wk) set by EFSA [143]. Moreover, non-significant association was observed between OTA serum levels with the intake of typical staple foods in

Bangladesh [58]. OTA was detected in 77% of the analyzed serum samples in Chile (n = 88) at concentrations lower than 1 μg/L. The OTA continuous dietary intake was in all cases lower than the TDI defined by the International Scientific Committee on Ochratoxin A in 2002 [144]. Correlations between OTA levels in plasma and food consumption were not significant [44]. One hundred percent of OTA frequency was reported in serum samples from Spain (n = 168) in a range of 0.15–5.71 μg/L. OTA intake did not exceed the tolerable weekly OTA intake. Non-correlation was observed between the OTA serum levels and the individual consumption of 26 food groups described as possibly contaminated with OTA in the Spanish population [36]. In the German cohort OTA and OT-α were detected in 100% and 78% of all analyzed urines (n = 50) ranging between 0.02-1.82 μg/L, and 0.01-14.25 μg/L, respectively, indicating that the unmetabolized OTA excretion in urine represents only a small fraction (<3%) of the ingested dose [62]. OTA was detectable in 80% and 50% of infant urine samples from Germany (n = 10) and Turkey (n = 28) with concentrations ranging from 30 to 1360 ng/L confirming its frequent exposure in this group of under 2 year olds [64]. In Czech Republic OTA was found in almost all analyzed serum samples from pregnant women (n = 115) in concentrations up to 1.13 μg/L [43], and in women of the child rearing age (n = 100) up to 0.35 μg/L [38]. Czech Republic data from serum correlate with OTA dietary exposure assessment. OTA levels in pregnant women serum did not show significant difference from normal population data. OTA plasma levels detected in an assessment study carried out on German grain workers (n = 61) ranged between 0.07 and 0.75 μg/L. Evidence of a significant inhalatory burden of OTA was not found in grain workers [35]. OTA was investigated in the etiology of bladder cancer in

Pakistan patients (n = 87) and healthy individuals (n = 30). Ninety two percent of the analyzed serum samples showed concentrations up to 3.4 μg/mL and 1.2 μg/mL, respectively, and non-association was evidenced [37]. Data found in Tunisia seemed to relate chronic interstitial nephropathy and OTA. Food and serum OTA levels were significantly different from the healthy and nephropathy groups [41].

The analysis of OTA in urine is an appropriate biomarker and a very useful tool to monitor OTA exposure of populations. As urine collection is less invasive than blood, urinary studies for population exposure assessment have been widely performed. Ninety three percent of the analyzed pregnant women urine samples from Bangladesh (n = 54) were positive for OTA in concentrations lower than 0.84 μg/L, similar levels to those determined recently in the general population of this country [96]. OTA was found in 42 (n = 60) and 27 (n = 120) urine samples analyzed in Portugal at concentrations lower than 0.105 μg/L [60] and 0.208 μg/L [61], respectively, whereas 51 (n = 122) urine samples from Spain were positive at concentrations lower than and 0.124 μg/L. OTA urinary ranges in both populations were comparable to those found in other countries in Europe such as Italy and UK. However, for most countries a great variation in the range of OTA levels was observed [61]. OTA was present in 35% (n = 239) and 70% (n = 32) of adult urine samples from Belgium at lower concentrations (pg/mL). Estimated OTA PDI exceeded the TDI for OTA in 1% of the studied Belgium population [74,105].

Detecting OTA levels in breast milk provides very important information about the degree of exposure of both mother and baby, and it is used in estimation of the overall risk characterization. In this way, breast milk

samples from Turkey (n=75) were analyzed indicating a high exposure level of mothers to OTA. 100% of samples showed contamination in the range of 0.6-13.1 µg/L, representing a potential hazard of OTA to infants as well as their mothers [106].

2.8.2 DON and Metabolites

DON and its metabolites were detected in 58 analyzed urine samples from China (n=60) in concentrations up to 30.5 µg/g creatinine. Urinary DON was not significantly associated with rice intake [84]. DON was reported in 52% pregnant women urine samples from Bangledesh (n=54) in levels ranging 0.18-7.16 µg/L. No individual had an estimated daily DON intake above the provisional maximum tolerable daily intake (PMTDI) of 1 µg/kg b.w. set by the WHO/JECFA (2011). Moreover, DON exposure in pregnant women in Bangladesh appears to be modest and lower than observed in biomonitoring studies performed in Europe and Africa [85]. On the other hand 58% of Bangladeshi adult urine samples (n=164) were positive for DON in the rage of 0.16-1.78 µg/L., while German analyzed urines (n=50) contained DON (100%) and DOM-1 (40%) in concentrations up to 38.44 µg/L and 0.73 µg/L, respectively [86]. The mean DON intake in idividuals from both Bangladesh and Germany was lower than the PMTDI. However, the mean DON level in German urines was about 53-fold higher than that found in Bangladeshi samples indicating a low and high dietary DON exposure among the adult population in Bangladesh and Germany, respectively. Moreover, DON (29%) and DON-3-GlcA (82%) were detected in the analyzed urine samples from Germany (n=101) at concentrations up to 31 and 139 mg/g creatinine, respectively. The mean DON PDI of 12% samples exceeded the

established value [68]. DON (22%) and DON-GlcA's (96%) were detected in urine samples from Austria (n=27) with an average concentration (DON + DON-GlcA's) of 20.4 µg/L. 33% of the individuals exceeded the DON PMTDI value according to their DON urinary levels [72]. 37 urine samples from Spain (n=54) showed DON concentrations up to 69.1 µg/g creatinine. Based on DON urinary levels 8.1% of the volunteers from Spanish volunteers, as well as, 2 out of 9 exposed children, were estimated to exceed the DON PMTDI [93]. The overall DON incidence in Belgium urine samples was 70% for children (n=155) and 37% for adults (n=239) in concentrations up to 27 and 327 ng/mg creatinine, respectively. The calculated DON PDI possibly exceeded the PMTDI in 16-69% of the population [74]. All analyzed urine samples (n=32) from Belgium contained concentrations of DON (60%) at ng/L or its metabolites DON-15-GlcA (100%), DON-3-GlcA (90%) and DOM-1-GlcA (25%) This emphasizes the importance of glucuronidation for detoxification of DON in humans [105].

2.8.3 CIT

CIT and HO-CIT were detected in 94 and 71% of the analyzed urine samples from Bangladesh (n=69) in concentrations up to 1.22 and 7.47 µg/L, with significantly higher levels in the rural cohort compared to the urban cohort. However, it is unclear, whether this biomarker result reflects a difference in food habits and/or an additional occupational exposure [88]. 87% of the analyzed pregnant women urines from Bangladesh (n=54) were positive for CIT in concentrations lower than 6.93 µg/L. Based on urinary concentrations the calculated CIT PDI of 9% of the Bangladeshi pregnant women exceeded the preliminary tolerable value set by European Food Safety

Authority (0.2 µg/kg/day) [96,145]. In German population 82 and 84% of the analyzed urine samples (n=50) contained these mycotoxins with maximum concentrations of 0.1 and 0.5 µg/L indicating a widespread and variable CIT exposure [87]. With regard to Belgium, CIT and/or OH-CIT were detected in lower concentrations (pg/mL) in 90% of the analyzed urine samples (n=32) indicating that humans are much more exposed to CIT than realized before [105]. Moreover CIT was present in 72% and 59% Belgium urine samples from adults (n= 239) and children (n=155), respectively, with low average concentrations (< 73.3 pg/mg creatinine). Despite the mean detected concentration of HO-CIT was tenfold higher than CIT, a lower prevalence (6% and 12% for children and adults, respectively) was reported [74].

2.8.4 Multi-Mycotoxins

Apart from these most studied compounds, other relevent mycotoxins have been the focus of several biomonitoring studies, including AFs, FBs, ZEN and ENs. Eight mycotoxins including AFM1, DH-CIT, DON, DON-GLcA, EN B, FB1, OTA, and α-ZEL were detected in urine samples from Bangladesh (n=95), Germany (n=50), and Haiti (n=142). DON and DON-GlcA were exclusively detected in urines from Germany and Haiti whereas urinary OTA and DH-CIT concentrations were significantly higher in Bangladeshi samples. AFM1 was present in samples from Bangladesh and Haiti only. The mean PDI was below the TDI for FB1, AFB1, and ZEN, however calculated DON PDI exceeded the PMTDI in 6% of the samples from Germany (2/50) and Haiti (4/142) [69]. 73% of the analyzed children urine samples from Cameroon (n=220) were positive for OTA (32%), DON (17%), AFM1 (14%), FB1 (11%), β-ZOL (8%), ZEN (4%), α-ZOL (4%) and DON-3Glu (1%) in concentrations up

to 77 μg/L, indicating that children in Cameroon under the age 5 are exposed to high levels of carcinogenic substances such as FB1, AFM1 and OTA through breastfeeding [141]. A total of eight mycotoxins were detected in 51% or the analyzed urine samples from Nigeria (n= 120), with OTA (28.3%), AFM1 (14.2%), and FB1 (13.3%) being the most frequently ones. The mean estimated OTA daily intake (0.01 μg/kg bw/day) in Nigeria population was close to the suggested TDI of 0.017 μg/kg bw/day derived from the tolerable weekly intake recommended by EFSA (2006). The estimated mean AFB1 intake was 0.67 μg/kg bw/day (max=2.5 μg/kg bw/day), whereas the mean estimated FB1 intake was 35 μg/kg bw/day (max=76 μg/kg bw/day), a level significantly greater than the recommended TDI of 2 μg/kg bw/day [70,146]. Non detectable levels of FB1 and FB2 were observed in the analyzed human urine samples obtained from Portugal (n=68) [89]. The presence of ZEN+ ZOLs (100%), OTA (100%), DON (96%), FB1 (56%) and AFM1 (6%) were reported in urine samples from Italy (n=52) in concentrations up to 67 μg/L. The estimated human exposure to FB1 and ZEN was largely below the TDI, however 94% and 40% of the Italian volunteers exceeded the TDI for OTA and DON, respectively [77]. The duration of AFB1 exposure in bakers from Egypt (n=290) was significantly correlated with serum concentrations [50].

All analyzed breast milk samples from Italy (n=47) were positive for ZEN (0.26-1.78 μg/L) [111]. However, from the analyzed breast milk samples from Iran (n=136) only one sample showed contamination with AFM1 and two with OTA at low concentrations (ng/L) [107]. Only two breast milk samples from Brazil (n=224) were positive for AFB2 (0.005 μg/L), indicating non-infant risk derived from AFs and OTA exposure [109]. Although mycotoxins may be transferred from maternal blood to milk, breast milk is

comparably rarely evaluated even though the limits of mycotoxins in infant food are very restrictive and controlled by surveillance programs. Warth et al. [147] reviewed the current situation of mycotoxins in human breast milk reporting studies mainly focused on AFs and OTA in different locations such as Iran, Turkey, Egypt, Chile, Nigeria, Brazil, Tanzania, Cameroon, Germany, Italy, Poland, etc. However, little is still known about the pattern of mycotoxins and their metabolites in breast milk as well as lactational transfer rates or potential combinatory effects.

2.8.5 Mycotoxin Binders

The prevention of fungal infections is the most rational and efficient way to avoid mycotoxins in agricultural commodities, however, under certain environmental conditions mycotoxin contamination is unavoidable. Several studies have shown adsorbent materials (mycotoxin binders) with large affinity for mycotoxins by the formation of stable linkages, but most of them seem to only bind a small group of mycotoxins while demonstrating very little or no binding to others [148]. Some mycotoxin bindings can efficiently adsorb mycotoxins and they have already shown their efficacy in in vivo studies. For instance, activated carbon (1 g/kg bw) significantly reduced the absorption and oral availability of DON after oral bolus (0.750 mg DON/kg bw) in chicken [46] and even lower doses (0.1 g/kg bw) completely prevented DON absorption in pig after single oral bolus (0.05 mg/kg bw) [54].

Conversely, glucomannan mycotoxin adsorbent (2 kg/ton diet) did not prevent DON absorption (no significant differences of DON and DOM-1 plasma concentrations) at the dietary inclusion level (4-6.5 mg/kg) in turkey poults [47] and comercial mycotoxin adsorbent lack of protective effect against STE

adsorption in cattle after 72 days STE diet suplementation (0.01-0.24 mg/kg) [65]. In this way, the use of mycotoxin binders may be valuable when other preventive measures against molds and mycotoxins have failed. However, the selection of the appropriate adsorbent substance must be done considering its mycotoxin adsorption efficacy or mold inhibition, the safety to animals and humans, having high stability and ability to face diverse conditions during feed/food mixing, as well as cost effectiveness [149].

3. CONCLUSIONS

The latest studies of mycotoxin determination in biological samples -fluids, tissues and organs -were collected, studied, and summarized. Considering the great majority of biological samples the most common extraction technique used for mycotoxins extraction was LLE in a single step or followed by a SPE clean-up. Nevertheless, reported mycotoxin analyses in urine were mainly based on dilute-and-shoot, IAC and combinations of SPE-IAC and LLE-SPE techniques. Acetonitrile, ethyl acetate, dichlorometane, and methanol were the most common organic solvents employed for mycotoxin extraction. With regard to detection techniques, LC-MS/MS systems were the most commonly used -among other alternatives such as LC-FD, GC-MS/MS, and ELISA -especially in the case of urine samples where a higher number of compounds was simultaneously determined. The species from which the biological samples were collected were mainly human (non-invasive collection samples), followed by pig, rat, and chicken. The most analyzed mycotoxin was OTA, followed by DON-ZEN and their metabolites, AFs, FBs, ENs-BEA, T-2, and HT-2. The most common studies of mycotoxin analysis in biological samples were focused on method development and human

biomonitoring, followed by toxicokinetics, absorption, metabolism, and bioavailability studies. New insights of mycotoxin bioavailability, toxicokinetics, ADME, bioaccumulation, and tissue persistence have been obtained through the analysis of biological samples, mainly focused on DON, ZEN, OTA, ENs and BEA, T-2, NIV, and FB1. Despite the relatively high frequency of mycotoxins detected in biological samples from biomonitoring studies data, calculated PDIs were generally below the established TDIs. However, 24% of the studies reported TDIs above the established values in a variable percentage of individuals, with DON as the most representative. DON PDI raised PMTDI in regions such as Haiti, Germany, Spain, Austria, Italy, and Belgium. Also the children population showed DON PDI exceeding the established level in Spain and Belgium. On the other hand, OTA PDI exceeded TDI in Belgian adults and children, and in Italian adults. Finally, CIT PDI was higher than TDI for pregnant women from Bangladesh, while TDI for FB1 was exceeded in some of the individuals from Nigeria. Finally, it is worth noting that the future inmycotoxin detection from biological samples seems to be pointing towards aptasensors because of their specificity, sensitivity, and easy use. The difficulty of biomonitoring studies -samples of reduced volume or size with very low concentrations of mycotoxins -may be solved with this new fangled experimental approach. These results evidence the importance of biological sample analysis as a useful tool for human and animal exposure assessment to mycotoxins.

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3.2 *Alternaria* Mycotoxins in Food and Feed: An Overview

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Alternaria Mycotoxins in Food and Feed: An Overview

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Abstract

Alternaria is one of the major mycotoxigenic fungal genera with more than 70 reported metabolites. *Alternaria* mycotoxins showed notably toxicity, such as mutagenicity, carcinogenicity, induction of DNA strand break, sphingolipid metabolism disruption, or inhibition of enzymes activity and photophosphorylation. This review reports on the toxicity, stability, metabolism, current analytical methods, and prevalence of *Alternaria* mycotoxins in food and feed through the most recent published research. Half of the publications were focused on fruits, vegetables, and derived products—mainly tomato and apples—while cereals and cereal by-products represented 38%. The most studied compounds were alternariol, alternariol methyl ether, tentoxin, and tenuazonic acid, but altenuene, altertoxins (I, II, and III), and macrosporin have been gaining importance in recent years. Solid-liquid extraction (50%) with acetonitrile or ethyl acetate was the most common extraction methodology, followed by QuEChERS and dilution-direct injection (both 14%). High- and ultraperformance liquid chromatography coupled with tandem mass spectrometry was the predominant determination technique (80%). The highest levels of alternariol and alternariol methyl ether were found in lentils, oilseeds, tomatoes, carrots, juices, wines, and cereals. Tenuazonic acid highest levels were detected in cereals followed by beer, while alternariol, alternariol methyl ether, tenuazonic acid, and tentoxin were found in legumes, nuts, and oilseeds.

1.1. Introduction

Alternaria is a fungal genus that includes saprophytic and pathogenic species and that is widespread in nature. They can infect a wide variety of crops in the field and in the postharvest stage causing considerable losses due to fruit and vegetable decay [1]. The most common *Alternaria* species include *A. alternata, A. tenuissima, A. arborescens, A. radicina, A. brassicae, A. brassicicola,and A.* infectoria. They colonize a range of plants including cereals, tomatoes, apples, grapes, oil crops, oilseeds, sunflower seeds, oranges, lemons, melons, cucumbers, cauliflowers, peppers, and tangerines [2].

The optimum growth temperatures for *Alternaria* range from 22 to 30◦C; however, minimum growth temperatures ranging from 2.5 to 6.5ºC and, even lower, from 0 to-5ºC in cooler regions were reported [3]. Their ubiquitous occurrence and ability to grow and produce toxins even under unfavorable conditions (low temperatures and low water activity) make the *Alternaria* genus responsible for the spoilage of several commodities during transport and storage, even if they are refrigerated [1]. Therefore, *Alternaria* species has been shown to be a significant contaminant of refrigerated fruits, vegetables, and stored feedstuffs [4]. *A. alternata* is the most important within the genus as regards to mycotoxin contamination of fruits and vegetables.

However, the production of host specific toxins from pathogenic *A. alternata* strains seems not to be a real problem in terms of food safety while a much more important problem is its saprotrophic strains, which colonizes harvested plant products and can produce reasonable amounts of certain mycotoxins, which exert poisonous effects after consumption by humans [5].

Alternaria species have the ability to produce more than 70 toxins, which play important roles in fungal pathogenic-ity and food safety, since

some of them are harmful to humans and animals [6]. The studied *Alternaria* secondary metabolites belong to diverse chemical groups such as nitrogen-containing compounds (amide, cyclopeptides, etc.), steroids, terpenoids, pyranones, quinines, and phenolics [7]. The major *Alternaria* toxins belong to the chemical groups dibenzo-*α*-pyrones, which include alternariol (AOH) and alternariol monomethyl ether (AME) and cyclic tetrapeptides represented by tentoxin (TEN). These mycotoxins were the most commonly studied metabolites produced by *Alternaria* strains on different substrates (tomato, wheat, blueberries, walnuts, etc.) and some of the main *Alternaria* compounds thought to pose a risk to human and animal health because of their known toxicity and their frequent presence as natural contaminants in food [8].

In the last decade, Ostry [1], Scott et al. [9], and Fernández-Cruz et al. [10] have studied the occurrence of the major *Alternaria* mycotoxins: AOH, AME, and TEN. However, food-relevant *Alternaria* species are able to produce many more metabolites including that known as emerging *Alternaria* mycotoxins described as potentially hazardous.

In this sense, mycotoxin research has focused in recent years on the emerging group, along with the major *Alternaria* toxins [11]. Emerging *Alternaria* mycotoxins mainly belong to five chemical classes: pyranones or benzopyrones (altenuene (ALT); altenuisol (AS); altenusin (ALN)), amine/amide metabolites (tenuazonic acid (TeA); altersetin (ALS)), perylenequinones (altertoxins (ATXs), alterperylenol or alteichin (ALTCH), and stemphyltoxin (STE)), and anthraquinones (Macrosporin A, Altersolanol (As-A)). Table 1 summarizes the main *Alternaria* toxins reported in the scientific literature, for both major and emerging compounds, including the chemical name, molecular formula and weight, and CAS number.

Group and chemical class	Mycotoxin	Chemical name	Molecular formula	Molecular weight (g/mol)	CAS No
Major mycotoxins					
Benzopyrones/ Pyranones	Alternariol (AOH)	*3,7,9-Trihydroxy-1-methyl-6H-dibenzo[b,d]pyran-6-one*	$C_{14}H_{10}O_5$	258.226	641-38-3
	Alternariol monomethyl ether (AME)	*3,7-Dihydroxy-9-methoxy-1-methyl-6H-dibenzo[b,d]pyran-6-one*	$C_{15}H_{12}O_5$	272.253	23452-05-3
Cyclic tetrapeptides	Tentoxin (TEN)	*Cyclo[N-methyl-L-alanyl-L-leucyl-(αZ)-α,β-didehydro-Nmethylphenylalanylglycyl]*	$C_{22}H_{30}N_4O_4$	414.498	28540-82-1
	Dihidro-tentoxin (DH-TEN)	*Cyclo-(L-leucyl-N-methyl-(E)-dehydrophenylalanyl-glycyl-N-methyl-L-alanyl*	-	-	-
	Isotentoxin (isoTEN)	*Cyclo-(L-leucyl-N-methyl-L-phenylalanyl-glycyl-N-methyl-L-alanyl*	-	-	65452-16-6
Emerging mycotoxins					
Benzopyrones/ Pyranones	Altenuene (ALT)	*(2S,3S,4aS)-2,3,7-trihydroxy-9-methoxy-4a-methyl-2,3,4,4a-tetrahydro-6H-benzo[c]chromen-6-one*	$C_{15}H_{16}O_6$	292.284	889101-41-1
	Altenuisol (AS)	*2,7,9-trihydroxy-3-methoxybenzo[c]chromen-6-one*	$C_{14}H_{10}O_6$	2.742.256	42719-66-4
	Altenusin (ALN)	*2-(4,5-Dihydroxy-2-methylphenyl)-6-hydroxy-4-methoxybenzoic acid*	$C_{15}H_{14}O_6$	29.026.806	31186-12-6
	Infectopyrone	*(2E,4E)-5-(4-methoxy-5-methyl-6-oxopyran-2-yl)-3-methylhexa-2,4-dienoic acid*	$C_{14}H_{16}O_5$	2.642.738	-

Amine/Amide metabolites	Tenuazonic acid (TeA)	*(5S)-3-Acetyl-1,5-dihydro-4-hydroxy-5-[(1S)-1-methylpropyl]-2Hpyrrol-2-one*	$C_{10}H_{15}NO_3$	197.231	27778-66-1
	Altersetin (ALS)	*2H-Pyrrol-2-one,1,5-dihydro-4-hydroxy-5-(1-hydroxyethyl)-3-[[(1S,2R,4aS,6R,8aR)-1,2,4a,5,6,7,8,8a-*	$C_{24}H_{33}NO_4$	399.241	485815-64-3
Perylenequinone	Altertoxin I (ATX I)	*(1S,12aR,12bS)-1,2,11,12,12a,12b-Hexahydro-1,4,9,12atetrahydroxy-3,10-perylenedione*	$C_{20}H_{16}O_6$	352.337	56258-32-3
	Altertoxin II (ATX II)	*(7aR,8aR,8bS,8cR)-7a,8a,8b,8c,9,10-Hexahydro-1,6,8c-trihydroxyperylo-1,2-b]oxirene-7,11-dione*	$C_{20}H_{14}O_6$	350.321	56257-59-1
	Altertoxin III (ATX III)	*(1aR,1bS,5aR,6aR,6bS,10aR)-1a,1b,5a,6a,6b,10a-Hexahydro-4,9-dihydroxy-perylo[1,2-b:7,8-b']bisoxirene-5,10-dione*	$C_{20}H_{12}O_6$	348.306	105579-74-6
	Alterperylenol/ Alteichin (ALTCH)	*(1S,12aR,12bS)-1,4,9,12a-tetrahydroxy-2,12b-dihydro-1H-perylene-3,10-dione*	$C_{20}H_{14}O_6$	350.32	88899-62-1
	Stemphyltoxin I (STE I)	*Perylo(1,2-b)oxirene-7,11-dione, 7a,8a,8b,8c,9,10-hexahydro-1,6,8c,9-tetrahydroxy-, (7aR-(7aalpha,8aalpha,8bbeta,8calpha,9alpha))-*	$C_{20}H_{14}O_7$	366.324	102694-30-4
	Stemphyltoxin III (STE III)	*(7aR,8aR,8bS,8cR)-7a,8a,8b,8c-Tetrahydro-1,6,8c-trihydroxyperyleno-1,2-b]oxirene-7,11-dione*	$C_{20}H_{12}O_6$	348.309	102694-32-6

Anthraquinone	Macrosporin A	*1,7-dihydroxy-3-methoxy-6-methyl-9,10-anthraquinone*	$C_{16}H_{12}O_5$	28.426.348	22225-67-8
	Altersolanol A (As-A)	*(1R)-1,2,3,4-Tetrahydro-1,2α,3α,4β,5-pentahydroxy-7-methoxy-2-methyl-9,10-anthracenedione*	$C_{11}H_{16}O_8$	336.293	22268-16-2
Dihydro-isocoumarin	Monocerin	*(2S,3aR,9bR)-6-hydroxy-7,8-dimethoxy-2-propyl-2,3,3a,9b-tetrahydro-5H-furo[3,2-c]isochromen-5-one*	$C_{16}H_{20}O_6$	3.083.264	30270-60-1
	Altenuic acids I, II, III	-	$C_{15}H_{14}O_8$	-	-

Table 1. Chemical name, molecular formula, molecular weight, and CAS number for the main *Alternaria* mycotoxins.

1.2. Toxicity of *Alternaria* Mycotoxins

Toxicological data are limited to the above-mentioned major mycotoxins, and even these data are incomplete, with neither good bioavailability studies nor long-term clinical studies available [12]. Although little is known so far about their properties and toxicological mechanisms, bioavailability, and stability in the digestive tract, *Alternaria* toxins have been shown to have harmful effects in animals, including cytotoxicity, fetotoxicity, and ter-atogenicity. They have been related to a range of pathologies from hematological disorders to esophageal cancer. They are also mutagenic, clastogenic, and estrogenic in microbial and mammalian cell systems and tumorigenic in rats [1, 13].

The benzopyrone group is the most studied among all the *Alternaria* mycotoxins, and it was the first to be analyzed. This group encompasses the two major toxins AOH and AME, as well as ALT and AS. Although their toxicity is not fully understood and varies from one cell system to another, AOH and AME toxicity have been identified in various in vitro and in vivo systems [14, 15]. Lehmann et al. [16] reported the AOH estrogenic potential and its inhibitory effects on cell proliferation. Furthermore, AOH induced marked phe-notypic changes in mice macrophages, which could not be directly linked to an initial AOH-induced ROS production, cell cycle arrest or autophagy as seen as a consequence of AOH-induced double-stranded DNA breaks [17]. AME and AOH were not very acutely toxic; however, they do exert genotoxic, mutagenic, and carcinogenic properties and show remarkable cytotoxicity in microbial and mammalian cell culture. Moreover, AOH and AME were able to induce DNA strand break and gene mutations in

cultured human and animal cells [18, 19]. They interfered with the activity of human topoisomerases by affecting the stabilization of topoisomerase II-DNA-intermediates and the modulation of the redox balance in human colon carcinoma cells yet without any apparent negative impact on DNA integrity [20]. AOH and AME are frequently found in combination [21] as they share a common biosynthesis pathway [22]. Exposure of HCT116 cells to low AOH and AME concentrations resulted in loss in cell viability throughout the activation of the mitochondrial apoptotic process associated with the opening of the mitochondrial permeability transition pore and the loss of mitochondrial transmembrane potential. Higher toxic potential indicated synergetic effects when applied together [23]. Thus, AOH and AME levels decreased cell viability in Caco-2 cells through being more cytotoxic the binary combination [24]. AOH cytotoxicity decreased in Caco-2 cells in simultaneous combination with Soyasaponin I [25].

Although there have been no in vivo genotoxicity or carcinogenicity studies on experimental animals or humans for *Alternaria* toxins, some indications of precancerous changes have been reported in esophageal mice mucosa. Their presence in cereal grain has been suggested to be associated with high levels of human esophageal cancer in China, as well as in areas of Africa where high levels of *Alternaria alternata* contamination have been found [26]. The underlying mechanisms of action have not yet been fully clarified. Limited data are available for long-term toxicity effects of low concentrations of *Alternaria* toxins and their synergistic effect on other contaminants [13]. ALT has shown high acute toxicity with a LD50 value of 50 mg/kg bw in mice, whereas TeA is considered to be the most acutely toxic among the *Alternaria* mycotoxins [27]. TeA is known to occur in high

concentrations in commodities and has attracted increasing attention in recent years [11]. It inhibits protein biosynthesis at the ribosomal level in mammalian cells by suppressing the release of newly formed proteins from the ribosomes and is biologically active as it exerts cytotoxic, phytotoxic, antitumor, antiviral, antibiotic, and antibacterial effects [28]. TeA toxicity has been reported in chick embryos and several animal species, including guinea pigs, mice, rabbits, dogs, and rhesus monkeys [29]. TeA is acutely toxic in living organisms and its LD50 value in mice is similar to that of the Fusarium mycotoxin deoxynivalenol (DON) [30]. TeA showed acute toxic effects in rodents (oral LD50 for mice, 81–186 mg/kg bw; and for rats, 168–180 mg/kg bw) and chicken embryos (LD50 0.55 mg/egg). A short-term animal trial (33 days) on monkeys led to vomiting, bloody diarrhea, and hemorrhagic lesions in the intestinal tract after a treatment with 89.6 mg TeA/kg bw per day [31]. Additionally, TeA has been made responsible for the outbreak of onyalay, a human hematologic disorder disease occurring in Africa, and esophageal cancer in Linxian Province, China [29]. There are also reports of the interacting effects of binary combinations of TeA with *Fusarium* toxins (DON, nivalenol, zearalenone, enniatin B, and aurofusarin) in Caco-2 cells, leading to a decrease in cytotoxicity, compared to the expected synergistic effects. Especially when combined with DON, TeA was found to significantly reduce the cytotoxicity of this mycotoxin [32]. TeA epimerizes to a mixture of TeA and allo-TeA when treated with bases and under acidic conditions. The presence of allo-TeA in fungal culture extracts (*Alternaria brassicicola, Alternaria raphani,* and *Phoma sorghina*) has been shown. TeA and allo-TeA phytotoxic effects can be explained by the inhibition of photosynthesis by blocking the electron flow, although there are no further toxicological data

regarding allo-TeA in the published literature [31]. Different tautomers of 3-acetyl tetramic acid have been reported but it was not possible to distinguish between them. The TeA structure is still not clear, especially in an aqueous solution. Varying TeA structures are still widely used, resulting in different CAS registry numbers [33].

The perylenquinone derivatives, such as ATX I, ATX II, ATX III, Alterperylenol (ALTCH; synonym Alteichin), and stemphyltoxins (STE) are minor metabolites of *Alternaria* s but are considered to be very critical because of their mutagenic properties [34]. Because of the lack of available reference compounds, in particular for ATXs, analytical studies remain less common [11]. ATXs have been reported to be highly potent mutagens and more acutely toxic to mice than AOH and AME and cause DNA strand breaks. Recently, high genotoxic potency of ATX II in both mammalian and human cells was demonstrated, and it was described as the most potent substance within the ATX group, capable of different mechanisms of action [34]. Nevertheless, data concerning the underlying modes of action are still limited [20].

Alternaria alternata lycopersici toxins (AALs) exhibit mostly phytotoxic effects but have been shown to disturb the sphingolipid metabolism in a similar way to fumonisins, which have been correlated with esophageal cancer and other animal diseases [35, 36].

Altenusin (ALN) is a biphenyl derivative isolated from different species of fungi, which presents antioxidant proper-ties and the ability to inhibit several enzymes, such as myosin light chain kinase, sphingomyelinase, acetylcholinesterase, HIV-1 integrase and trypanothione reductase, cFMS kinase, and pp60c-SRc kinase in the low micromolar concentration range, and

it may serve as a chemotherapeutic agent to treat trypanosomiasis and leishmaniasis [37, 38]. The biphenyl basic skeleton of ALN containing a salicylic moiety and a catechol moiety could be the important part because of its interesting azole-synergistic activity.

Altersetin (ALS) was reported to possess broad antimi-crobial activity against several multidrug-resistant bacterial demonstrating potent activity against several pathogenic Gram-positive bacteria and moderate in vivo efficacy in a murine sepsis model [39].

Anthraquinones and tetrahydroanthraquinone analytes are secondary metabolites widely distributed in natural biosources, which show significant biological activity. So far, several compounds of the alterporriol and altersolanol families have been reported, including the emerging *Alternaria* toxins macrosporin A and altersolanol A (As-A) [40], which exhibited antibacterial activity [41].

The cyclic tetrapeptide TEN is one of the major *Alternari*a toxins produced, along with dihydrotentoxin (DH-TEN) and isotentoxin (iso-TEN). Their structures differ at the unsaturated bond of the N-methyl dehydrophenylalanine moiety, which is hydrogenated into a single bond in DH-TEN and E configured in iso-TEN.All three compounds are considered to be phytotoxins, with TEN being the most potent, inhibiting photophosphorylation and inducing chlorosis. However, no toxicological data are available for mammals, and the data on the occurrence of this toxin in food and feed are limited as well [11].

The altenuic acids consist of three closely related isomeric colorless substances (altenuic acids I, II, and III) containing one carbon-methyl and one methoxyl group. The structures of the altenuic acids I and III remain

unknown, but their molecular formula (C15H14O8), which is identical to that of altenuic acid II, has been determined [42]. Williams and Thomas determined the chemical structure of altenuic acid II in 1973 by X-ray crystallographic analysis [43].

Monocerin is a polyketide metabolite isolated from sev-eral fungal species showing antifungal, phytotoxic, insec-ticidal, and plant pathogenic properties [44]. Monocerin and its analogues were proven to be non specific toxins and nonspecific seed germination inhibitors by their interference with selected stages of cell division cycles. In recent years, they have attracted greater interest; consequently, several syntheses of this molecule have been reported [45].

Other *Alternaria* metabolites were reported to be phytotoxins, that is, being toxic to plants, while the role of many others, such as infectopyrones, phomapyrones, and novaezelandins, is still unknown. However, *Alternaria* s produce a variety of other metabolites for which no reports are available due to the lack of pure substances, of which only AME, TeA, AOH, TEN, and ALT are now available on the market [12]. Figure 1 shows the chemical structure of the main *Alternaria* mycotoxins.

1.3. Stability of *Alternaria* Mycotoxins

The stability of *Alternaria* toxins has not been studied in detail. Current information reveals that *Alternaria* mycotoxins were barely degraded during wet baking, while significant degradation occurs upon dry baking, with stability decreasing in the ratio of AME > AOH > ALT [46]. Dibenzo-α-pyrones AOH and AME were stable to heating at 100∘C in sunflower flour, while heat treatment at 121∘C for 60 min significantly reduced the concentrations of

these toxins in *Alternaria* contaminated sunflower flour. However, the heat-treated material caused some toxic effects when fed to rats [2]. AOH and AME were very stable in spiked apple juice at room temperature for up to five weeks and at 80ºC after 20min. They were also stable in spiked white wine for almost 8 days at room temperature.

ATX-1 contents were stable when added to apple juice for up to 27 days at room temperature [10]. Overall, some *Alternaria* toxins remain difficult to degrade or decontaminate in fruits and their processed products.

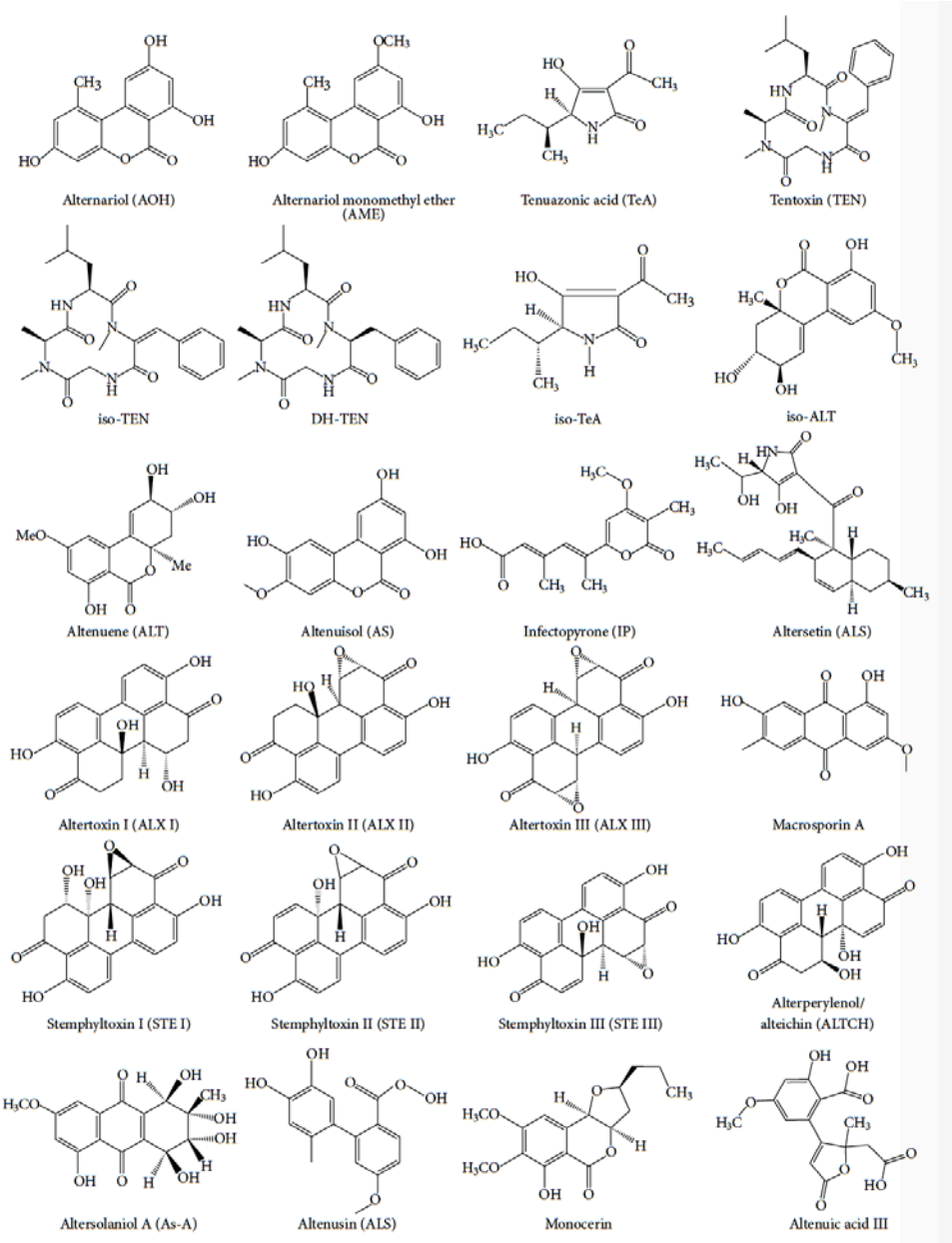

Figure 1. Chemical structure of the main *Alternaria* mycotoxins.

1.4. Metabolism of *Alternaria* Mycotoxins

Mycotoxins can be partially metabolized in living organisms, leading to the formation of conjugated toxins by the conjugation of the parent compound with glucose, sulfates, and other sugar moieties. The term "masked mycotoxins" firstly appeared defining a mycotoxin derivative that may be cleaved during digestion in living organisms to release its parent form. However, these conjugated mycotoxins are currently known as "modified mycotoxins" after a more recent comprehensive classification [47]. It is not clear if these modified mycotoxins can be hydrolyzed and absorbed in the gastrointestinal tract, thereby further contributing to the overall exposure. Since current information on the bioavailability of modified mycotoxins is very limited, EFSA has recommended to national agencies that they gather occurrence data on these modified forms using properlyvalidated, robust, and sensitive routine analytical methods [48], a prerequisite being the availability of reference standards for these compounds. Over the last two decades, formation of glycosides and sulfated mycotoxins has been reported and (bio)organic synthesis has already been applied to obtain reasonable amounts of selected conjugates as reference materials for further studies [49]. Little information about the metabolism of AOH is available, but since all products of aromatic hydroxylation of AOH are catechols or hydroquinones, which can create reactive semiquinones and quinones or undergo redox cycle, its inactivation by methylation or glucuronidation/sulfation is considered to be a possible toxicological product that may cause toxic effects on cells lines [50]. In the mammalian organism, formation of glucuronides is a major pathway of detoxification and excretion. Both AOH and AME have free hydroxyl groups available for metabolic conjugation. Consequently, modified *Alternaria*

mycotoxins such as alternariol-3-glucoside (AOH3G), alternariol-3-sulfate (AOH3S), alternariol monomethyl ether-3-glucoside (AME3G), and alternariol monomethyl ether-3-sulfate (AME3S) have received more attention during the last decade [49]. In vitro studies have shown that AOH and AME were readily converted to glucuronides upon incubation with hepatic and intestinal microsomes from humans, rats, or pigs in the presence of UDP-glucuronosyl transferases. AME was predominantly converted to the 3-O-glucuronide whereas AOH gave rise to comparable amounts of 3-O-glucuronide and 9-O-glucuronide [51]. The hydroxylation of AOH and AME was studied under in vivo-like conditions in precision-cut rat liver slices of rats. The pattern of in vivo metabolites was comparable to that of in vitro metabolites of AOH, clearly supporting the relevance of an oxidative in vivo metabolism [20]. AOH and AME were extensively conjugated in suspension cultures of tobacco BY-2 cells, demonstrating that masked mycotoxins of AOH and AME can be formed in plant cells [51]. Five AOH conjugates were isolated and identified by MS and NMR spectroscopy as β-D-glucosides (attached in AOH 3- or 9-position). For AME, conjugation resulted in β-D-glucoside (mainly attached in the AME 3-position) [52]. Knowledge of the toxicity and disposition of the oxidative AME and AOH metabolites is mandatory for a better understanding of the health risks posed by these *Alternaria* toxins, since toxicity properties may be enhanced or attenuated for their metabolites [53].

Toxicokinetic studies have so far focused on toxins with a dibenzo-α-pyrone structure, in particular, AOH and AME. Only very little information is available on the occurrence and toxicology of *Alternaria* toxins with a perylene quinone struc-ture, such as ATXs, ALTCH, and STEs. After studying the absorption of four Alternaria toxins with perylenequinone structures in

the Caco-2 cell Transwell system (a widely accepted in vitro model for human intestinal absorption and metabolism), it was shown that ATX I and ALTCH were not metabolized in Caco-2 cells, while ATX II and STE III were partly biotransformed by reductive deepoxidation to ATX I and ALTCH metabolites, respectively [54]. Very low ATX-II absorption and partial metabolism of ATX-I was observed in the intestinal Caco-2 Transwell system [20].

A comparative toxicokinetic study showed that TeA was completely bioavailable after oral administration in both pig and broiler chicken. Absorption was deemed to be slower in broiler chickens (t.max 0.32 h in pigs versus 2.60 h in chick-ens) and TeA was more slowly eliminated in broiler chickens (t 1/2el 0.55 h in pigs versus 2.45 h after oral administration). These observations were mainly due to the significantly lower total body clearance (Cl 446.1 ml/h/kg in pigs versus 59.2 ml/h/kg in chickens after oral administration) [55]. Concerning human exposure, the presence of TeA in the urine of six human volunteers in concentrations ranging from 1.3 to 17.3 µg/L was reported. It was observed that 87–93% of orally consumed TeA was excreted as parent compound into the urine of two human volunteers within 24 h after ingestion [28].

2. Material and Methods

A systematic literature search was conducted using the databases Medline, Web of Science, and Scopus with the focus on the following keywords: *Alternaria* mycotoxins analysis, determination, occurrence, toxicity, stability, metabolism, etc. The period of time framed was 2005–2017. Fifty-eight arti-cles, which met the criteria to be included into the study, were analyzed and classified. To facilitate data presentation three groups were

established based on the analyzed food/feed matrices, namely, (i) cereals and cereal by-products, (ii) vegetables, fruits, and derived products (including juices, wine, vegetable seeds, spices, herbal infusions, and dry fruits), and (iii) mixed matrices studies, which combine the analysis of different foods belonging to both groups (i) and (ii), and products non classifiable into groups (i) and (ii).The information was double-checked to select bibliographies of relevant literature, and a thorough evaluation was performed to summarize the information about extraction method, analytical methodology, and studied mycotoxins limits of detection quantitation.

3. Results and Discussion

3.1. Analytical Methods for *Alternaria* Mycotoxins Determination

Mycotoxin regulations are based on risk assessment (hazard and exposure), the parameters of which are still hard to establish, meaning that a concrete interpretation of the consequences for consumer's health remains elusive. Furthermore, it remains difficult to detect toxin metabolites at low levels in complex food matrices. Therefore, validated analytical methods ensuring robustness, sensitivity, and reli-ability are needed.

In the lasts years, *Alternaria* toxins were usually extracted from solid or liquid matrices by the classic solid-liquid/liquid-liquid extraction (SLE/LLE) with organic solvents mainly acetonitrile (ACN; 68%), ethyl acetate (EtOAc; 17%), methanol (CH3OH; 6%), and solvent mixtures such as ACN/EtOAc (2%). The chlorinated solvents dichloromethane (CH2Cl: 2%) and chloroform (CHCl3: 5%) were also used for SLE/LLE procedure. In the case of cereals and cereal by-products the choice of ACN as extraction solvent raised to 83%, followed by EtOAc (13%) and CH2Cl2 (4%), whereas in fruits and

vegetables ACN represented 50%, followed by EtOAc (24%), CHCl3 (12%), and CH3OH (10%). For other food matrices not included in the mentioned groups the solvents used for extraction procedure were limited to ACN (75%) and CH3OH (25%).

Although basic SLE/LLE represented approximately 50%of the extraction techniques for *Alternaria* mycotoxins anal-ysis in food and feed, other used methodologies such as QuEChERS (14%), dilution and/or direct injection (11%), and the combination of SLE-SPE (14%) were of relevant importance. Direct SPE, microscale extraction, dispersive liquid-liquid microextraction (DLLME), and countercurrent chromatography (CCC) technique were also used (all <5%). Variations in the predominance of each methodology were observed depending on the analyzed food group. Thus, *Alternaria* mycotoxins were mainly extracted from cereals and derived products by SLE (64%) QuEChERS procedure (18%) and SLE-SPE (14%), while fruits and vegetables were mainly represented by SLE/LLE (43%), dilution-injection (16%), and SLE-SPE and direct SPE (both 14%). Other matrices or food combinations were mainly extracted by SLE/LLE and dilution-injection (both 29%), followed by direct SPE and SLE-SPE (both 14%).

With regard to mycotoxins determination, liquid chro-matography (LC) was by far the most used technique, from other generally reported chromatographic techniques for, such as thin-layer chromatography (TLC) or gas chro-matography (GC). High- and ultra-performance LC (HPLC and UPLC) have provided new possibilities allowing high-throughput screening by shortening the analysis time, while maintaining the chromatographic principles and improving the speed, sensitivity, and resolution [56]. In the

early years, atmospheric pressure chemical ionization (APCI), electro-spray ionization (ESI), and LC-tandem mass spectrometry (MS/MS) have become the methods of choice for the identification and quantification of these toxins [57].

Consequently, UPLC and HPLC, coupled to MS/MS (including high resolution MS; HRMS and time-of-flight MS; QTOF-MS), represented the 80% of the determinations, rising to 95% when only considering cereals and cereal by-products. Other detectors such as ultraviolet (UV: 7%) and diode array detection (DAD: 7%) were also used, followed by the enzyme immunoassay technique (EIA: 4%). In the case of *Alternaria* mycotoxins detection in fruits and vegetables the main representative one also was MS/MS (64%), followed by UV and DAD (both 14%) and finally EIA (7%).

Table 2, divided into cereals and cereal by-products (Table 2(a)), vegetables and fruits (Table 2(b)), and other food products (Table 2(c)), describes the analytical method, extraction methodology, analyzed mycotoxins, and limits of detection/quantitation for the analyzed studies.

Matrix	Analytical method	Extraction method	Mycotoxins	LOD (µg/kg)	LOQ (µg/kg)	Reference
Bread	HPLC-MS/MS	SLE:EtOAc	AOH	20	60	46
			AME	5	15	
			ALT	10	30	
Barley	U-HPLC-OrbitrapMS	QuEChERS	AOH	-	-	58
			ALT	-	-	
Barley	LC-HRMS	QuEChERS	AOH	-	-	59
			AME	-	-	
Wheat	HPLC-DAD	SLE:ACN/KCl, CH2Cl2	AOH	-	10	60
			AME	-	10	
			ALT	-	50	
			TeA	-	60	
Wheat	LC-ESI-MS/MS	SLE:H2O, EtOAc/HCl	AOH	0.75	2.5	61
			AME	0.1	0.3	
			TeA	2.5	7.5	
Wheat	UHPLC-MS/MS	SLE:ACN/H2O	AOH	0.3	-	62
			AME	0.02	-	
			ATX-I	0.3	-	
			Infectopyron	n.d.	-	
			Macorsporin	0.5	-	
			TEN	0.15	-	

Wheat	HPLC-MS/MS	SLE: ACN/H2O/FA	AOH AME TEN	3 8 2.5	7 15 5	63
Wheat	HPLC-MS/MS	SLE: ACN, SPE clean-up	AOH AME TEN TeA	1 5-6 0.5-0.7 0.1	4-5 15-20 2 0.3-0.5	64
Wheat and wheat-based food	UPLC-MS/MS	SLE: ACN-SPE	TeA AOH TEN AME	2 8 0.8 0.2	- - - -	65
Maize	LC-MS/MS	SLE: ACN/H2O/AA	AME Macrosporin Monocerin	0.02 0.05 0.05	- - -	66
Maize	HPLC-MS/MS	SLE: ACN/H2O/AA	AOH AME TEN Macrosporin	- - - -	- - - -	67
Maize and wheat	HPLC-ESI-MS/MS	SLE:ACN/H2O	AOH AME Macrosporin TEN	0.75 0.1 0.05 0.4	2.25 0.3 0.15 1.2	68

Rice	UPLC-ESI-MS/MS	SLE: ACN/H2O	AOH AME TEN	- - -	0.3 0.3 0.1	69
Rice, oat flakes and barley	UPLC-MS/MS	SLE: ACN/H2O/AA, hexane	AOH AME ALT TeA TEN ATX I AOH3S AOH3G AME3S AME3G	0.50-0.85 0.67-1.32 1.19-2.21 0.61-1.18 0.46-0.91 1.01-1.81 1.24-2.41 0.74-2.11 0.79-4.16 0.77-2.50	1.00-1.70 1.35-2.52 2.38-4.43 1.21-2.35 0.93-1.82 2.02-3.62 2.48-4.82 1.49-4.23 1.58-8.32 1.53-4.99	70
Sorghum and millet	HPLC-ESI-MS/MS	SLE: ACN/H2O/AA	AOH AME TEN ATX-I TeA Macrosporin A	- - - - - -	- - - - - -	71
Sorghum and infant cereals	HPLC-MS/MS	SPE stable isotope dilution assay	TeA	0.2	0.6	72

Cereals	LC-DAD/ MALDI-TOF-MS	SLE: ACN/H2O/AA-SPE	AOH AME TEN	- - -	- - -	57
Complex feed matrices	UPLC-MS/MS	QuEChERS	AOH AME ALT TEN	- - - -	1.0-5.0 0.5-2.5 1.0-10.0 2.5-5.0	73
Maize, wheat, feed, silage, feed ingredients	U-HPLC-orbitrapMS	SLE: ACN/H2O/AA	AOH AME ALT TeA TEN	- - - -	- - - -	74
Beer	HPLC-ESI-IT-MS	LLE: EtOaC	TeA	2	8	75
Soya beans	HPLC-UV	modified QuEChERS, clean-up	AOH AME	8 16	24 48	76

Table 2a. *Alternaria* mycotoxins analysis in cereal and cereal derived products: analytical method, extraction method, analyzed mycotoxin, and limits of detection/quantitation.

Matrix	Analytical method	Extraction method	Mycotoxins	LOD (µg/kg)	LOQ (µg/kg)	Reference
Tomato	LC-UV	SLE: CHCL3	AOH AME TeA	1 0.6 2.6	5 2 11	77
Tomato	LC-MS/MS	SLE: MeOH, derivatization, SPE Strata-XL	ALT AOH TEN TeA AME	10-20 2 1 10 0.3-1	20-50 5 2.5-5 20 1-2	78
Tomato	HPLC-DAD	SLE: acidified ACN, clean-up	AOH AME ALT	3.94 0.074 0.34	- - -	79
Tomato products	HPLC-MS/MS	QuEChERS	TeA allo-TeA	0.38 0.29	1.28 0.97	31
Tomato and tomato products	UPLC-MS/MS	SLE: acidified ACN, clean-up	AOH AME ALT TEN TeA ATX-I	4 1 2 2 2 2	- - - - - -	80
Tomato and tomato products	LC-ESI-MS/MS	DLLME: ACN/CHCl3	AOH AME TEN	1.4 1.4 0.7	3.5 3.5 1.75	81

Tomato and pepper products	HPLC-MS/MS stable isotope dilution assay	QuEChERS	TeA	0.86	2.89	82
Sweet pepper	UPLC-MS/MS	SLE: EtOAc, clean-up	AOH AME ALT	3.3 12 0.6	6.6 25 1.2	83
Sweet pepper	LC-UV	SLE: CHCL3	AME AOH TeA	1.1 2.9 2.8	- - -	84
Sunflower seeds	LC–MS	SLE: MeOH	AOH and AME	1.25	-	85
Tea and herbal infusions	UPLC-MS/MS	LLE: EtOAc/FA, NH2-SPE, C18-SPE	AOH AME ALT	5.8-13 20-30 1.3-2.1	12-26 41-60 2.6-4.1	86
Maca, soy isoflavones, garlic, black radish, St John's wort, ginkgo biloba	UPLC-MS/MS	SLE: EtOAc, clean-up	AOH AME ALT	8 30 2	25 100 6	87
Apple	HPLC-DAD	micro-scale extraction	AOH, AME, TEN	20	50	88
Apple juice and wine	HPLC-UV	CCC: EtOAc/H2O	AME TeA	0.04 0.03 0.14	- - -	89
Apple juice concentrate	UPLC-MS/ MS	dilution-analysis	AOH, AME, ALT and TEN	-	1.0-5.0	90

Strawberries	HPLC-MS/MS	SLE:EtOAc	AOH AME TEN	0.75 2 0.25	1.75 3.5 0.75	3, 91
Blueberries	HPLC-UV	SLE:EtOAc/FA	AOH AME	6 2	10 4	92
Berries and field samples	HPLC-MS/MS	SLE:ACN	ATX-I ALT TeA	- - -	- - -	93
Pomegranate fruits and juices	HPLC–DAD	QuEChERS	AOH AME TEN	15 15 20	50 50 66	94
Wine, fruit juices	HPLC-DAD	direct injection or SPE, clean-up	AOH AME	2.0-6.0 0.1-2.0	3.3-10 2-3.1	95
Wine (red, white and rosé), cider (white and rosé) and their cork stoppers	LC-ESI-MS/MS	dilution (ACN)-filtration	AOH AME	- -	0.6-5.0 0.2-2.2	96
Tomato products and apple	enzyme immunoassay	dilution-centrifugation-analysis	AOH	1-2	-	97
Tomato products and apple	enzyme immunoassay	dilution-centrifugation-analysis	TeA	25-150	-	98

Tomato- and citrus-based foods	UPLC-ESI-MS/MS	SLE: ACN, SPE clean-up	TeA AOH TEN AME	1 5-6 0.5-0.7 0.1	4-5 15-20 2 0.3-0.5	99
Tomato Products, Fruit and Vegetable Juices	UPLC-MS/MS	QuEChERS	AOH AME ALT TeA TEN ATX I AOH3S AOH3G AME3S AME3G	3.0-18.3	1.1-5.7 9.8-61.5	47
Tomato, apple, sweet cherry, and orange fruits	UPLC-MS/MS	SLE:ACN (NaCl)-SPE (MCX and NH2)	AOH AME TeA ALT TEN	- - - - -	1 1 1 1 1	100
Tomatoes, tomato products, bell peppers, onions, soft red fruits	LC-TOF-MS	SLE: ACN/EtOAc	AOH AME	7.4-17.4 4.7-90	14.8-34.8 9.4-180	101

Nuts	UPLC-MS/MS	SLE: ACN/H2O/AA (79:20:1, v/v/v)	AOH AME ATX-I TEN	- - - -	3 2.9 14 1.2	102
Vegetable juices, fruit juices, wine	HPLC–MS/MS	SPE	AOH AME ALT/iso-ALT TeA TEN ATX-I ATX-II AA-III AALs AS	- - - - - - - - - -	0.5-0.6 0.4-0.5 2.2-3.1 0.9-1 0.9-1 0.8-1 1.2-1.3 1-1.1 1.4-1.9 0.8	103

Table 2b. Alternaria mycotoxins analysis in vegetables and fruits: analytical method, extraction method, analyzed mycotoxin, and limits of detection/quantitation.

Matrix	Analytical method	Extraction method	Mycotoxins	LOD (µg/kg)	LOQ (µg/kg)	Reference
Fruit juices and spices and cereals	HPLC-MS/MS by stable isotope dilution assay	derivatization, clean-up	TeA	0.15-17	0.5-50	27
Tomato, blueberry, walnut and wheat	Semi-quantitative UPLC-HRMS	micro-scale extraction	AOH, AME, TEN, ATX, ALT, TeA, ALN, AIA, PyA	-	-	12
Tomato, wheat, juice and sunflower seeds	LC-MS/MS	SLE: MeOH, derivatization for TeA. SPE clean-up	AOH AME ALT TEN TeA	- - - - -	10 1-10 10 1-5 5	104
Tomato products, bakery products, sunflower seeds, fruit juices, and vegetable oils	HPLC-MS/MS	ACN/H2O/FA. Dilute-and-shoot	AOH AME TeA ALT isoALT TEN ATX-I AAL TA1 AAL TA2	0.2–2.8 0.04–0.4 3.6–34 0.8–24 1.3–19 0.1–2.0 2.1–14 2.8–5.4 1.2–17	0.6–9.3 0.1–1.2 12–110 2.5–81 4.4–62 0.5–6.6 6.9–48 9.3–18 3.8–55	105

Beers, tomato products, apple juices, olive and dried basil	LC/APCI-MS/MS	SPE	AOH	620-8080	2080-29920	106
			AME	160-1380	540-4600	
			ALT	2220-12310	7400-41000	
			TEN	200-4140	680-13700	
			TeA	1220-7120	4060-23730	
Wine, apples, apple juices, tomatoes, tomato sauces, citrus, dried figs, olives, sunflower seeds and cereals	UPLC Xevo-TQ-S MS	SLE: ACN-($MgSO_4$) SLE: ACN/H_2O/FA	ALT	-	1.5-2	107
			AME	-	1-2	
			AOH	-	2	
			TEN	-	2-2.5	
			TeA	-	5	
Bread, cereals, chips, juice, nuts, oil, sauce, seeds, and spice	LC-MS/MS	SLE: ACN/H_2O-(-hexane)-SPE LLE: EtOAc-SPE	TEN	0.18-0.99	0.54-2.94	108
			DH-TEN	0.19-0.35	0.62-1.05	
			isoTEN	0.10-0.45	0.33-1.32	

Table 2c. Alternaria mycotoxins analysis in other food products: analytical method, extraction method, analyzed mycotoxin, and limits of detection/quantitation.

3.2. Prevalence of *Alternaria* Mycotoxins.

In the lasts years different food matrices were analyzed for the presence of *Alternaria* mycotoxins, including cereals (wheat, barley, rice, oat, maize, sorghum, bakery products, and bread); fruits and vegetables (tomato, tomato products, pepper, garlic, onions, black radish, apple and apple juice, sweet cherry, strawberries, berries and blueberries, red fruits, orange fruits, citrus, and pomegranate fruits);beverages (beer; wine: red, white, and rosé; tea and herbal infusions; cider; fruit; and vegetable juices); other products (spices, nuts, walnuts, dried figs, olives, oilseeds, sugar beet pulp, maca, soy isoflavones, soya meal, and ginkgo biloba); silage, feed, and feed ingredients.

Among all of the analyzed matrices, tomato, followed by cereals and fruits, especially apples and berries, were the most studied foodstuff in recent years for *Alternaria* mycotoxins. *Alternaria* toxins are, together with aflatoxins, ochratoxin A, and patulin, the most commonly found mycotoxins in fruits and their processed products. Due to the limitations of current industrial processes to completely eliminate the rotten tissues and the reported stability of some *Alternaria* mycotoxins (i.e., AOH, AME, and ATXs) in fruit juices and during tomato processing, it is obvious that these mycotoxins are likely to be present in commercial end products [47].

Consequently, *Alternaria* mycotoxins were detected in cereals and derived products [46, 57–76], feed [74, 109–113], dairy products [114], grapes and by-products [27, 77, 115], tomatoes and derived products [31, 47, 77, 81, 106, 113], nuts and by-products [102, 116], spices [117], soybeans [76, 116, 118], sunflower seeds [85, 104, 107], vegetables [27, 101], vegetable oils [105], strawberries [3, 91, 93], red fruits [92, 101], several fruit juices [27, 72, 90, 99], peppers [119], apples [88, 90, 109], peanuts [109], tea and herbal

infusions [86], wine [89, 103], beer [75], fermented beverages [96], and spices and food supplements [84, 87, 120, 121].

The maximum concentrations of *Alternaria* toxins reported in commercial food products ranged from 1 up to 8700 μg/kg. The highest levels were found in samples visibly infected with *Alternaria* rot, that is, in products obviously not suitable for human consumption. According to the European Food Safety Authority (EFSA), major contributors to Alternaria mycotoxins dietary exposure are grains and grain-based products, especially wheat. The highest levels of AOH and AME were found in lentils, oilseeds, and tomato paste, followed by fruit and vegetable juices, wines, cereals, and vegetables (tomatoes and carrots). The highest concentrations of TeA were reported for cereals, followed by commercial beers, while AOH, AME, TeA, and TEN were found in legumes, nuts, and oilseeds, particularly in sunflower seeds [6].

Moreover, it was observed that the cooccurrence of *Alternaria* toxins with other mycotoxins is plausible. Owing to the different physical and chemical properties of all the prevalent mycotoxins in fruits, the determination of trace amounts of all them in food represents an extremely challenging task [100].

3.3. Most Reported Mycotoxins.

Among the 58 studies included in Table 2, morethan80% analyzed AOH and AME, and around 50% included TEN and TeA, followed by ALT (36%). These were followed by *Alternaria* toxins ATXs included in 16% of the analyzed studies, whereas only a few publications reported other compounds such as macrosporin (9%), monocerin, ALN, AALs, and infectopyrone (all <3%). Notably, limited information was found about pyrenochaetic acid A

(PyA), alternarienonic acid (AIA), and alloTeA and isoALT, recently reported in food samples for the first time [31, 105] and metabolites such as AOH3S, AOH3G, AME3S, and AME3G [47, 69]. In this way, AOH, AME, TeA, and TEN have been shown to occur in food samples frequently [82, 97, 108], while the occurrence of ALT, isoALT, ATX-I, and AAL toxins is of much lower incidence, mainly due to shortcomings in current analytical methodologies.

Slight differences were observed based on the analyzed food matrices. Thus, AOH and AME (both 86%) were the most analyzed ones in cereals and cereal by-products, followed by TEN and TeA (59 and 41%, resp.). Other mycotoxins studied in these matrices were ALN (27%), macrosporin (23%), ALT (14%), and less extent monocerin and infectopyrone (both5%). The same trend was observed in fruits, vegetables, and derived products, with predominance of AOH and AME (both 79%), followed by TEN (41%), TeA (38%), ALT (34%), and ATXs (14%).

4. Concluding Remarks

In the last years, several food matrices were analyzed for the presence of *Alternaria* mycotoxins, with tomatoes and derived products, cereals and cereals by-products, and fruits such as apples and berries as the most representatives. The most common extraction technique was SLE/LLE with several organic solvents mainly ACN, EtOAc, and CH3OH. Other methodologies of relevant importance were QuEChERS, dilution-direct injection and SLE-SPE. LC-MS/MS systems were by far the most used analytical techniques for *Alternaria* mycotoxins determination. AOH and AME, followed by TEN, TeA, and ALT were the most analyzed mycotoxins in food and feed.

With regard to the widespread occurrence of *Alternaria* mycotoxins in various food and feedstuffs intended for human or animal consumption and their high *toxicity*, more toxicological studies are needed on the field, transport, stor-age, and processing stages. The monitoring of their incidence at low concentrations is warranted to find out the extent of human exposure to these contaminants, as well as the influence of cooccurring microorganisms and their produced metabolites on the subsequent enhancement or inhibition of *Alternaria* species growth. Considerable attention should be paid to the mycotoxin production process in order to take adequate measures for their suppression and decontamination within national and international programs. Moreover, it remains important to examine the potential correlation among these toxic compounds, as different toxicity profiles may occur. Furthermore, the action of purified molecules in various in vitro systems needs to be studied in depth. Fungal population genetics also deserve attention in order to elucidate their role as mycotoxin producers and to understand the *Alternaria* secondary metabolism (producing parent or conjugated analytes) when subjected to different ecophysiological factors (mainly temperature and water activity), which are able to modulate their toxicological profiles. The use of predictive models to determine the factors that influence *Alternaria* mycotoxin contamination in the field and across the different processing stages may result in a notable decrease of this contamination throughout the production chain.

Although there are no specific international regulations for any of the *Alternaria* mycotoxins, EFSA has provided a scientific opinion on the risks for animal and public health related to the presence of *Alternaria* toxins in food and feed [6]. Risk assessment related to food safety is frequently hampered by

the lack of quantitative data. Up to now, results on *Alternaria* mycotoxins risk assessment proved to be inconclusive due to limited representative occurrence and toxicity data. However, application of the threshold of toxicological concern (TTC) approach indicated that there might be a possible risk for human health related to the presence of *Alternaria* toxins in foodstuffs. Maximum levels admitted for these toxins should be released by EFSA and regulated by the European Union in the near future. As these contaminants could be found in a wide range of food products, their current and future determination is essential for regulatory bodies with the purpose of improving the quality of products and preserving consumers' health.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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3.3 Mycotoxins Analysis of Human Urine by LC-MS/MS: a Comparative Extraction Study

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Mycotoxins Analysis of Human Urine by LC-MS/MS: a Comparative Extraction Study

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Abstract

The lower mycotoxin levels detected in urine make the development of sensitive and accurate analytical methods essential. Three extraction methods, namely salting-out liquid–liquid extraction (SALLE), miniQuEChERS (quick, easy, cheap, effective, rugged, and safe), and dispersive liquid–liquid microextraction (DLLME), were evaluated and compared based on analytical parameters for the quantitative LC-MS/MS measurement of 11 mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ZEN, BEA, EN A, EN B, EN A1 and EN B1) in human urine. DLLME was selected as the most appropriate methodology, as it produced better validation results for recovery (79–113%), reproducibility (RSDs < 12%), and repeatability (RSDs <15%) than miniQuEChERS (71–109%, RSDs < 14% and < 24%, respectively) and SALLE (70–108%, RSDs < 14% and < 24%, respectively). Moreover, the lowest detection (LODS) and quantitation limits (LOQS) were achieved with DLLME (LODs: 0.005–2 μg L^{-1}, LOQs: 0.1–4 μg L^{-1}). DLLME methodology was used for the analysis of 10 real urine samples from healthy volunteers showing the presence of ENs B, B1 and A1 at low concentrations.

1. Introduction

Toxic fungal secondary metabolites, known as mycotoxins, frequently contaminating food and feed are of concern due to their association with a wide array of adverse health effects [1,2]. The diversity of mycotoxins leads to a broad range of acute and chronic toxic effects in animals and humans, such as vomiting, hematotoxicity, immunosuppression, hepatotoxicity, nephrotoxicity, teratotoxicity, immunotoxicity, and hormonal or reproductive effects, although potencies vary depending on species and sex [3]. Humans are

often simultaneously exposed to mycotoxins mixtures along with other contaminants such as pesticides or heavy metals, making multi-mycotoxin exposure study relevant from a public health perspective. Actual exposure to mycotoxins is difficult to measure using an indirect approach based on mycotoxin occurrence in food combined with data on food consumption. Individual exposure is influenced by the heterogeneous distribution of mycotoxins, under- and overestimation of food consumption data, the presence of masked mycotoxins, and individual differences in absorption, distribution, metabolism and excretion (ADME) [4]. To overcome these disadvantages, detecting the presence of mycotoxins in biological fluids such as blood and urine could be useful and reliable in short- and long-term exposure assessment, and may make it possible to predict future adverse health consequences [2]. Urine is the body fluid most often used to measure mycotoxin exposure due to large amounts being easily and non-invasively collected, although blood (serum, plasma) has also been used [4]. Hence, detection of mycotoxins in human or animal urine allows more accurate and objective exposure assessment at an individual level since it covers exposure from all sources, thus reducing uncertainties related to occurrence and consumption rates [5]. On the other hand, urine analysis may provide widely valuable information, from mycotoxins toxicokinetics, ADME and bioavaliability studies, to human biomonitoring and exposure assessment. Urine analysis of mycotoxins can be used to establish population reference ranges and identify vulnerable consumer groups and individuals with higher exposures [6]. Moreover, the relationship between urinary mycotoxin levels and some diseases such as nephropathy has been also investigated [7].

Since the advent of the latest generation of high-performance LC-MS/MS and GC-MS/MS instruments, a clear trend towards the development and application of multi-analyte methods in mycotoxin research has been observed [2]. Recent reviews show that the great majority of mycotoxin determination in urine performed in the last year was based on LC-MS/MS [8]. However, a major challenge in urine mycotoxin analysis is the extremely low analyte concentrations present following dietary exposure, in the range of few μg L^{-1}. Thus, effective, sensitive, and accurate methods for mycotoxin detection in urine are required. Appropriate sample preparation protocols are crucial to accomplish the desired sensitivity while obtaining acceptable limits of detection (LODs) and quantitation (LOQs). Most of the methods available in the literature are based on traditional extraction techniques such as liquid–liquid extraction (LLE) or solid–liquid extraction (SLE), which have several disadvantages, mainly the high solvent volumes, high amounts of sample, and the long times required for the analysis [9]. In recent years, method simplification and miniaturization was one of the most important trends in sample preparation allowing the use of low sample and solvent volume, fast analysis, and greater efficiency [10,11]. In classical LLE, the addition of an inorganic salt into a miscible mixture forces the formation of a two-phase system leading to selective analytes movement into the organic phase, in a technique known as salting-out liquid–liquid extraction (SALLE) [5]. The well-established QuEChERS (quick, easy, cheap, effective, rugged, and safe) method, based on ACN extraction followed by a salting-out and quick dispersive solid-phase extraction (d-SPE), expands the polarity range of the amenable compounds and allows extract purification by using small amounts of non-chlorinated organic solvents [8]. Dispersive liquid–liquid micro

extraction (DLLME), which is based on a ternary component solvent system, enhances the surface area between the organic and the aqueous phase, facilitating the achievement of an equilibrium state, markedly reducing extraction time and enhancing enrichment factors [12,13].

In this study, three extraction methods, namely SALLE, miniQuEChERS, and DLLME, have been evaluated and compared based on analytical parameter data for the quantitative measurement by LC-MS/MS of 11 mycotoxins (aflatoxin B1; AFB1, aflatoxin B2; AFB2, aflatoxin G1; AFG1, aflatoxin G2; AFG2, ochratoxin A; OTA, zearalenone; ZEN, beauvericine; BEA, enniatin A; EN A, enniatin B; EN B, enniatin A1; EN A1 and enniatin B1; EN B1) in human urine.

2. Results and Discussion

2.1. Method Optimization

Several parameters were evaluated in terms of extraction efficiency for each tested methodology: type and volume of extraction solvent, salt amount, and C18 clean-up. Method optimization was performed by recovery experiments in three replicates using blank urine samples (1 mL) spiked at 20 μg L^{-1} (ENs-BEA), 40 μg L^{-1} (AFs) and 80 μg L^{-1} (OTA and ZEN) for each single compound.

2.1.1. Optimization of SALLE

The following parameters affecting extraction efficiency were evaluated: type and volume of extraction solvent, salt amount, and C18 clean-up step. Single modifications were added to the initial extraction conditions (1 mL of ACN-0.5 g NaCl) to study each single parameter, keeping the non-studied parameters fixed.

Type of extraction solvent. Three different solvents, namely ACN, EtOAc and $CHCl_3$, were tested and compared with regard to extraction efficiency for the studied mycotoxins. As shown in Figure 1, the best recovery ranges were obtained with ACN (83–107%), compared to EtOAc (46–108%) and $CHCl_3$ (44–87%). For some mycotoxins, such as ENs and ZEN, similar recoveries were obtained with both ACN and EtOAc. However, recoveries of AFs decreased from 83–107% (ACN) to 46–96% when using EtOAc. Moreover, EtOAc and $CHCl_3$ did not successfully extract OTA from urine, leading to non-defined chromatographic peaks in terms of symmetry and resolution, therefore reporting inconsistent results. In addition, ACN showed better reproducibility results (RSD $<$ 7%), than EtOAc (RSD $<$ 23%), and $CHCl_3$ (RSD $<$ 35%). $CHCl_3$ was the least appropriate solvent for mycotoxin extraction in urine using the SALLE method, showing the lowest recovery ranges, even lower than 50% for some mycotoxins (EN B1 and BEA). In keeping with these results, it has been reported that ACN (polarity index: 5.8) is often preferred as the SALLE solvent over other candidates such as acetone, MeOH and ethanol (polarity index: 5.1, 5.1 and 5.2, respectively) [5,14]. Therefore, ACN was selected as the extraction solvent for subsequent optimization testing.

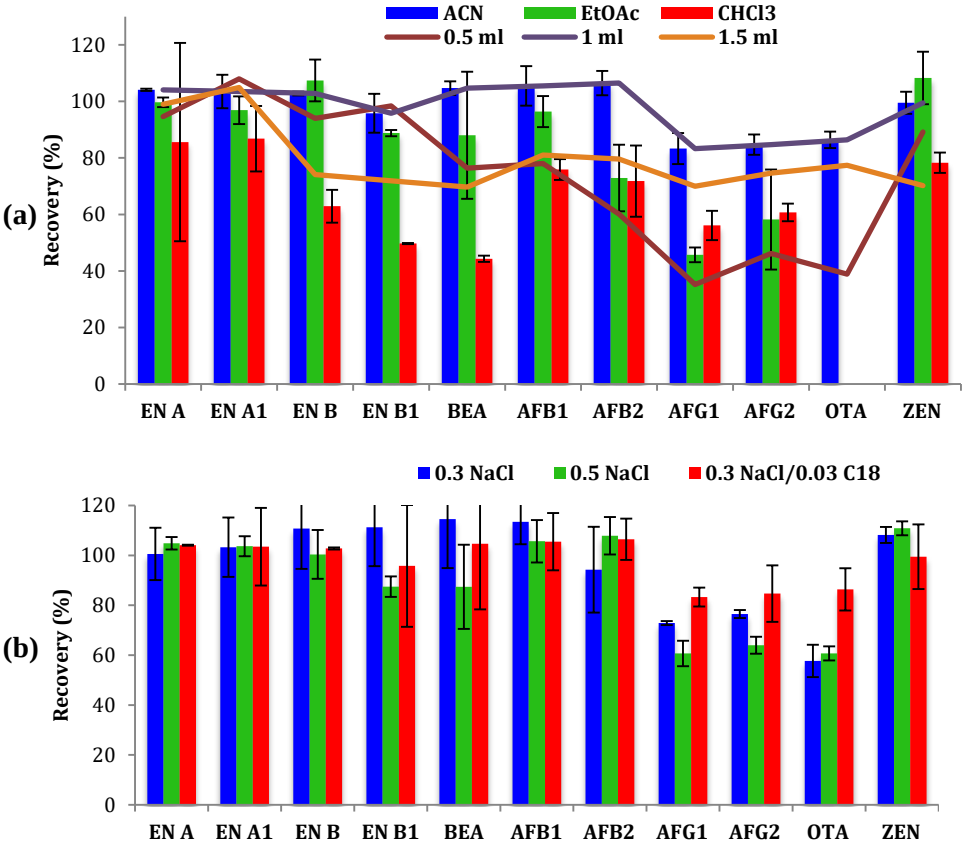

Figure 1. (**a**) Recovery assays performed by salting-out liquid–liquid extraction (SALLE) in urine comparing acetonitrile, ethyl acetate, and chloroform as extraction solvents (vertical bars); acetonitrile volumes of 0.5, 1, 1.5 mL (horizontal lines); and (**b**) different NaCl amounts in the presence/absence of C18 (0.5 g NaCl, 0.3 g NaCl, and 0.3 g NaCl/0.03 g C18, respectively) performed with 1 mL of acetonitrile.

Volume of extraction solvent. The effect of the volume of solvent on mycotoxin extraction from 1 mL of urine was evaluated. Volumes of 0.5, 1 and 1.5 mL of ACN were tested (Figure 1). The use of 0.5 mL resulted in an unclear limit between ACN and the saline phase, impeding suitable sampling of the

organic phase after centrifugation, thus hindering removal of the upper organic layer. Even so, acceptable recoveries were obtained for some mycotoxins (60–108%, RSD < 11%), but AFGs and OTA did not attain admissible recoveries (<50%). Optimal recovery values were achieved using both 1 mL (83–107%; RSDs < 7%) and 1.5 mL (70–105%; RSDs < 13%) of ACN. Thus, 1 mL of ACN was selected for mycotoxin extraction.

Sodium chloride amount and C18 clean-up. SALLE is an alternative sample preparation technique, based on the salting-out effect, to separate water-miscible organic solvents such as ACN. The NaCl amount was first optimized, based on previous studies [5,15,16]. In this way, extraction efficiency was evaluated after adding 0.3 g or 0.5 g of NaCl to urine samples. Since non-significant differences were observed ($p > 0.05$), 0.3 g of NaCl was preferred for miniaturization purposes. Secondly, the influence of the clean-up step was tested by evaluating improvement/worsening after 0.03 g addition of C18 sorbent. The results showed constant recovery results for the great majority of mycotoxins, but an improvement in extraction efficiency (from 58–114% to 83–107%) was observed for AFGs and OTA (Figure 1). Moreover, the addition of C18 led to partial removal of interferences leading to more clean extracts and repeatability improvement (RSD < 7%). Hence, the combination of 0.3 g NaCl followed by 0.03 g C18 clean-up was finally selected for the SALLE procedure performed with ACN.

2.1.2. Optimization of miniQuEChERS

Type and volume of extraction solvent. Since previous studies reported that ACN permitted good recoveries of target mycotoxins and the partial removal of unwanted material from urine, it was proposed as the extraction solvent [17,18]. The extraction efficiency of different ACN volumes

(0.5, 1 and 1.5 mL) was evaluated. Similarly to the results obtained in the SALLE procedure, lower volumes (0.5 mL) hampered the organic phase collection, and resulted in lower recovery ranges for some compounds, such as AFs, especially those of group G (25–37%). Moreover, RSDs < 40% revealed that repeatability should be improved for some compounds, such as BEA. Optimal recoveries were achieved using 1 mL (73–107%, RSDs < 18%) and 1.5 mL (73–102%, RSDs < 20%) of ACN (Figure 2). As non-significant differences were found between them ($p > 0.05$), 1 mL of ACN was selected for mycotoxin extraction for the miniQuEChERS procedure.

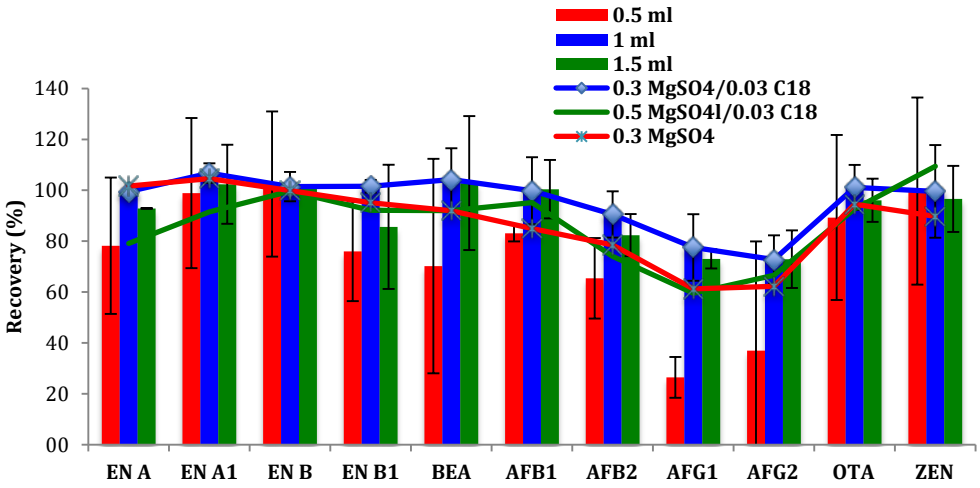

Figure 2. Recovery assays performed by the miniQuEChERS (quick, easy, cheap, effective, rugged, and safe) method in urine comparing acetonitrile volumes of 0.5, 1, 1.5 mL (vertical bars); and using different amounts of $MgSO_4$ and C18 (0.5 g NaCl/0.03 g C18; 0.3 g NaCl/0.03 g C18; 0.3 g $MgSO_4$, respectively) (horizontal lines).

Magnesium sulfate amount and C18 clean-up. QuEChERS is a high effective extraction method, which also allows purification of the extracts [19]. This technique offers different alternatives by re-adjusting protocol

according to the analyzed matrix [18]. In the proposed miniQuEChERS methodology, a small volume of 1 mL instead of 10–15 mL as reported for typical QuEChERS method was used. To optimize conditions for the desired purpose, the influence of the $MgSO_4$ amount was evaluated by comparing extraction efficiency after addition of 0.3 g and 0.5 g. In this case $MgSO_4$ addition was directly combined with the C18 sorbent clean-up step. Thus, the recoveries obtained after ACN extraction with 0.5 g $MgSO_4$/0.03 g C18 or 0.3 g $MgSO_4$/0.03 g C18 were calculated. As Figure 2 shows, better results were obtained with the lower $MgSO_4$ concentration (0.3 g), probably due to its great efficiency in phase separation. $MgSO_4$ has more ionic strength (4 mol L^{-1}) per unit concentration in aqueous phase than other salts, demonstrating that it is very efficient for phase separation of urine and water-miscible ACN [5]. Finally, in order to check the positive effect of C18 addition, ergo, the clean-up process, the recoveries obtained with 0.3 g NaCl in the absence of C18 were evaluated. As expected, the recovery values decreased for all compounds, except the emerging mycotoxins ENs and BEA, which remained constant (Figure 2). Accordingly, the combination of 0.3 g $MgSO_4$ followed by 0.03 g C18 clean-up was selected for the miniQuEChERS procedure.

2.1.3. Optimization of DLLME

The DLLME technique is based on a ternary component solvent system where a disperser solvent and an extraction solvent are combined. The surface area between extraction solvent and aqueous sample is infinitely large; therefore, the equilibrium state is quickly achieved and the extraction time is very short.

Type of disperser solvent. First, the type of extraction and disperser solvents was investigated. The miscibility of the disperser solvent with the

extraction solvent and aqueous solution is a critical factor in DLLME. With regard to the disperser solvent, it has been reported that when using ACN a cloudy state was correctly formed. Moreover, although other solvents such as acetone, MeOH and ethanol showed suitable properties as disperser solvents, ACN extracts are highly compatible with LC/MS applications resulting in the fewest interferences as large amounts of lipophilic material are not extracted. Hence, based on previous works and other studies reported in the scientific literature [12,14,20,21] 1 mL of ACN was directly selected as the optimum disperser solvent for a 1 mL urine sample size.

In conventional DLLME the density of the extraction solvent was higher than water, therefore its application was limited to water samples and the volume of the sedimented phase was in some cases dependent on the surrounding temperature. Some modification techniques were performed with lower density organic solvents, resulting in improvements in DLLME methods. Thus, DLLME may be classified into two broad categories, depending on the extraction solvent used; lower-density or higher-density solvents [13].

Type of extraction solvent. The type of extractant solvent is one of the most important parameters that affect the efficiency of DLLME. Therefore, the ability of two extraction solvents to extract the studied mycotoxins from urine was compared: one representative high-density solvent, $CHCl_3$ (density 1.49 g/cm^3); and the other a low-density solvent, EtOAc (density 0.90 g/cm^3). As shown in Figure 3, better results in terms of extraction efficiency were achieved by EtOAc, showing an improvement from 34–74% ($CHCl_3$) to 86–102% (EtOAc) for the great majority of mycotoxins. The highest increase was observed for BEA, which showed low extraction efficiency with $CHCl_3$ (34%) but achieved higher recoveries using EtOAc (102%). Only for OTA did the type

of extraction solvent show no effect on extraction efficiency and the obtained recoveries (69% for both solvents) remained constant. The better performance of EtOAc compared to $CHCl_3$ could be explained by the fact that EtOAc (and other ethers) are hydrogen bond acceptor molecules and therefore extract electron donor solutes more readily than $CHCl_3$ [10]. However, some already reported methods achieved good recoveries using $CHCl_3$ as the extractant solvent, possibly due to an extraction step being performed before DLLME [21], or the different target compounds [14].

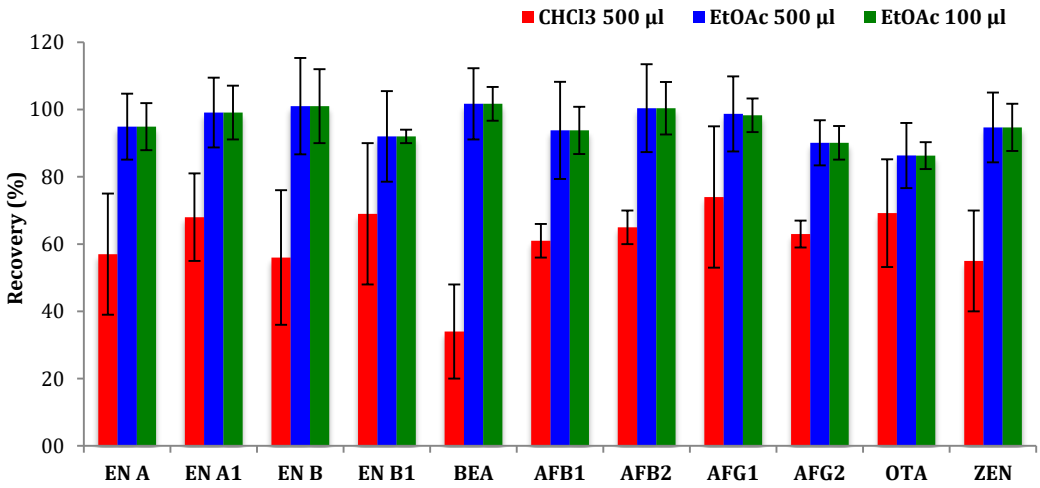

Figure 3. Recovery assays performed by dispersive liquid–liquid microextraction (DLLME) in urine comparing chloroform and ethyl acetate (500 µL), and ethyl acetate (100 µL) as extractant solvents.

Volume of extraction solvent. The effect of the volume of extraction solvent on extraction efficiency was evaluated by different disperser-extraction solvent ratios (1/2 and 1/10), selected on the basis of previous studies [10,21]. Volumes of 500 µL or 100 µL of each extraction solvent (EtOAc, $CHCl_3$) were combined with a fixed volume (1 mL) of the disperser

solvent (ACN). Results confirmed that better recoveries were obtained with the mixture of ACN containing EtOAc. Moreover, reduction of the extraction volume from 500 µL to 100 µL did not show significant variations in mycotoxin extraction efficiency (Figure 3), with comparable recovery values (86–102% and 70–98%, respectively). On the other hand, a lower volume enhances the enrichment factor of the DLLME process, and reduces the supernatant volume, allowing faster extract evaporation and therefore shortening the extraction time. Thus, 100 µL of EtOAc (extraction solvent) and 1 mL of ACN (disperser solvent) were selected to carry out the DLLME methodology.

Salt amount influence. The salting-out effect may significantly improve the analyte extraction in DLLME. It usually increases both the analytes solubility in the organic phase and the drop volume by reducing the solubility of the extractant [16,21]. In this way, the effect of different amounts of added salt (0.1, 0.3 and 0.5 g) on extraction efficiency was tested. In accordance with other authors, it was observed that a small amount of salt (0.1 g) led to an unclear separation phase whereas with larger amounts (0.3 and 0.5 g) the separation between the phases, as well as the peak shape, was better defined [10,22]. Non-significant differences were observed between 0.3 and 0.5 g of NaCl ($p > 0.05$); therefore 0.3 g was selected in order to miniaturize the methodology as much as possible.

2.2. Evaluation of the Methods

2.2.1. Salting-Out Liquid–Liquid Extraction (SALLE)

In the SALLE methodology, ACN-NaCl/C18 clean-up was used for mycotoxin extraction. Recoveries ranged between 70–108%, with intra-day

and inter-day precision lower than 14 and 24%, respectively. Signal enhancement was observed (116–144%) except in the cases of EN B and AFB1, which showed signal suppression (83–85%). Good linearity was observed for all compounds ($r^2 > 0.99$). LODs ranged from 0.1 to 10 μg L^{-1}, and LOQs were between 0.5–40 μg L^{-1} for all mycotoxins (Table 1).

Song et al. [5] compared the SALLE procedure with two fast techniques: (i) dilute-and-shoot; (ii) dilute-evaporate-and-shoot. The authors reported that dilute-evaporate-and-shoot had better sensitivity and response than dilute-and-shoot because of the concentration step (evaporation); however, both techniques showed more serious signal suppression and required a high analyte concentration for a significant signal to be seen. In contrast, the SALLE approach based on two LLE steps with EtOAc and ACN, respectively, gave the highest slope values for all the compounds, which indicated that the matrix effect was minimal. Recoveries ranged from 70–108%, with the intra- and inter-day RSD < 25% for most of the compounds and LOQs between 0.07–3.3 μg L^{-1}.

Rodriguez-Carrasco et al. [14] selected the SALLE methodology after comparing it with DLLME, for trichothecenes extraction from urine due to its slightly higher extraction efficiency, obtaining recoveries ranging from 84 to 96%, intra-day precision <14%, and LODs/LOQs between 0.12–4 and 0.25–8 μg L^{-1}, respectively.

Table 1. Analytical parameters for *SALLE, miniQuEChERS*, and DLLME method validation: limits of detection (LOD), limits of quantitation (LOQ), recoveries at three spiked concentration levels, intra-day and inter-day precision, matrix effect and linearity for the studied mycotoxins. [a] Spiked levels: low (2 LOQ), medium (5 LOQ), high (10 LOQ)

Mycotoxin	LOD	LOQ	*Recovery (%)*			Intra-Day (RSD%)			Inter-Day (RSD%)			Matrix effect (%)	Linearity (r^2)
	(µg L⁻¹)	*(µg L⁻¹)*	*[a] Spiked Level (µg L⁻¹)*			*[a] Spiked Level (µg L⁻¹)*			*[a] Spiked Level (µg L⁻¹)*				
SALLE													
			low	*medium*	*high*	*low*	*medium*	*high*	*low*	*medium*	*high*		
EN A	1	5	97.8	93.2	86.4	14.1	8.0	6.2	12.0	20.0	19.7	115.6	0.99719
EN A1	1	5	104.4	100.5	89.5	11.3	8.4	4.9	17.2	17.8	19.0	122.8	0.99391
EN B	0.1	0.5	92.2	95.6	85.6	8.0	8.3	4.5	11.6	16.7	18.8	85.1	0.99944
EN B1	0.3	1	88.7	100.7	86.5	6.1	5.0	6.7	7.1	8.7	14.2	124.3	0.99946
BEA	3	9	108.4	108.2	89.2	8.6	12.1	8.7	13.5	19.6	19.2	76.3	0.99102
AFB1	0.4	1.2	90.0	93.8	82.2	7.6	8.4	6.7	11.5	20.3	19.5	83.3	0.99051
AFB2	0.4	1.2	91.3	95.3	85.0	5.2	5.5	6.3	16.2	14.3	19.1	101.5	0.99692
AFG1	1.3	4	90.3	91.7	82.3	8.5	7.4	1.5	16.7	15.8	20.0	138.9	0.99519
AFG2	1	3	96.7	103.9	84.5	7.7	6.7	5.1	17.4	10.1	15.2	137.7	0.99208
OTA	8	20	77.1	72.7	69.7	10.5	8.6	8.1	13.7	18.8	24.4	125.3	0.99360
ZEN	10	40	100.4	98.7	87.9	4.9	6.2	3.8	6.3	11.3	14.5	143.8	0.99224
miniQuEChERS													

EN A	1	5	87.9	89.1	91.3	11.2	4.7	12.4	20.4	11.1	17.0	94.6	0.99615
EN A1	0.8	2.5	71.2	106.2	86.9	9.8	8.2	12.9	9.0	15.2	14.5	89.6	0.99473
EN B	0.1	0.5	74.9	103.2	91.9	5.4	8.3	4.9	15.2	7.8	9.3	71.2	0.99893
EN B1	0.1	0.5	78.2	108.9	79.0	8.7	5.8	9.1	15.2	13.3	24.1	105.1	0.99946
BEA	2	6	79.4	100.5	91.8	11.5	6.5	8.6	11.1	8.3	11.3	60.8	0.99715
AFB1	0.5	1.5	86.4	94.7	87.4	9.1	5.3	6.0	17.7	20.8	20.0	68.9	0.99804
AFB2	0.5	1.5	83.8	98.2	84.5	7.3	6.3	4.3	13.4	19.1	20.4	70.1	0.99854
AFG1	1	3	92.3	73.3	76.9	8.7	2.8	8.8	19.3	16.2	22.9	79.0	0.99583
AFG2	1	3	85.9	95.8	86.6	8.4	4.3	13.2	15.7	21.1	20.5	87.3	0.99583
OTA	15	35	83.8	83.9	74.4	14.4	10.1	4.5	14.7	17.6	15.9	110.3	0.99470
ZEN	12	20	99.4	100.7	103.8	5.5	4.5	4.5	11.2	14.1	8.5	110.7	0.97626
DLLME													
EN A	0.2	0.5	93.7	96.8	94.9	9.9	11.7	7.1	10.2	14.5	10.2	98.3	0.99921
EN A1	0.1	0.3	92.6	101.4	99.1	3.9	4.5	6.1	7.0	11.8	10.8	72.0	0.99886
EN B	0.05	0.1	99.4	100.7	101.0	11.6	4.3	6.2	8.3	14.6	14.9	99.8	0.99326
EN B1	0.05	0.1	93.8	94.9	92.0	9.6	8.7	3.7	9.3	8.1	14.2	79.2	0.99262
BEA	0.3	1	93.1	101.2	101.7	5.7	11.6	4.9	14.3	14.6	11.1	70.6	0.99549
AFB1	0.1	0.2	94.8	103.5	93.8	7.7	6.1	4.6	12.0	7.5	15.1	116.2	0.99066
AFB2	0.2	0.4	94.6	100.7	100.4	3.6	3.0	7.8	6.9	6.5	13.7	109.9	0.99049
AFG1	0.2	1	92.3	98.1	98.7	6.0	4.3	5.4	15.4	12.4	11.7	97.2	0.99008
AFG2	0.2	0.4	88.0	113.1	90.1	4.7	8.5	5.0	5.7	8.9	7.1	102.6	0.99629
OTA	2	4	78.5	82.3	87.2	9.3	9.7	7.6	8.7	10.2	14.5	109.9	0.99792
ZEN	1.8	4	93.4	96.9	94.7	4.3	6.5	5.8	7.8	9.7	10.9	109.1	0.99947

2.2.2. MiniQuEChERS

In the miniQuEChERS methodology, extraction using ACN/$MgSO_4$/C18 clean-up was optimized to achieve process simplification in one single step, allowing faster analysis. Table 1 shows the recoveries obtained (71–109%) with intra-day and inter-day precision lower than 14 and 24%, respectively. Signal suppression was observed for the majority of mycotoxins (69–95%) but EN B1, OTA and ZEN showed slight signal enhancement (105–111%). Good linearity was observed for all compounds ($r^2 > 0.99$). LODs ranged from 0.1 to 2 μg L^{-1} for all compounds except for ZEN and OTA, which showed the highest values (12 and 15 μg L^{-1}, respectively). LOQs ranged between 0.5–6 μg L^{-1} for all mycotoxins except ZEN and OTA (20 and 35 μg L^{-1}, respectively). Rodríguez-Carrasco et al. [15] developed a methodology involving
solvent extraction at high ionic strength (ACN/$MgSO_4$/NaCl) followed by dispersive solid phase extraction (d-SPE: $MgSO_4$/C18) and GC-MS/MS analysis to determine 15 mycotoxins and metabolites in human urine. The methodology was applied by the same authors to evaluate human exposure assessment through mycotoxin/creatinine ratio [23] and to estimate DON excretion through a 24 h pilot study [19]. Similar recoveries to those shown in the present study were achieved (72–109%), with intra and inter-day RSDs < 15%, and LOQs between 0.25 and 8 μg L^{-1}. QuEChERS extraction followed by UHPLC-HRMS detection was applied to evaluate mycotoxins and metabolites in human breast milk, showing recoveries ranging from 64% to 93%, RSD < 20%, and lowest calibration levels (LCLs) between 1.25–50 μg L^{-1} [17].

2.2.3. Dispersive Liquid–Liquid Microextraction (DLLME)

DLLME consists of a simple microextraction technique based on the use of an extraction solvent mixed with a disperser solvent. Since DLLME is an

efficient, economical, and environmentally responsible methodology, it has gained importance in recent years. It also has other advantages such as simplicity of operation, rapidity, and high recovery and enrichment factors. Nevertheless, there are a limited number of studies that determine mycotoxins using DLLME methodology. Single compounds have been analyzed in different liquid matrices, such as ZEN in beer [12], OTA in wine [24,25], or patulin in apple juice [26]. However, multi-mycotoxin studies applying DLLME methodology are scarce.

In the present study, a DLLME method, based on a low-density extraction solvent (EtOAc) combined with ACN (disperser solvent) in the presence of NaCl, was optimized and successfully validated for the extraction of 11 mycotoxins in urine. As Table 1 shows, satisfactory recoveries were obtained (79–113%) with intra- and inter-day precision lower than 12 and 15%, respectively. Values for the matrix effect ranging from 72–117% indicated that both signal enhancement and signal suppression were observed. LODs were between 0.05–2 μg L^{-1}, and LOQs ranged from 0.5 to 4 μg L^{-1} for all studied mycotoxins. Good linearity was observed for all compounds ($r^2 > 0.99$).

Tolosa et al. [21] performed a multi-mycotoxin analysis in water and fish plasma based on a low density-DLLME method using the same solvent mixture ACN/EtOAc (disperser/extraction) with NaCl addition. In line with the results presented here, the authors reported differences in recovery assays when different extraction solvents were employed, achieving better recovery results for the majority of the analyzed mycotoxins when using EtOAc, except for some AFs, which showed better recoveries with $CHCl_3$.

Serrano et al. [10] developed a high density DLLME for determining emerging *Fusarium* mycotoxins (ENs and BEA) in water. The extraction was performed in carbon tetrachloride (CCl4, density: 1.59 g/cm^3) using ACN as the disperser solvent, in the presence of NaCl. Similar recoveries to those obtained in the present study were reported (79 and 100%), with RSD values lower than 14%, and LOQs ranging from 0.06–0.17 μg L^{-1}.

2.3. Selection of the Most Appropriate Methodology

Three extraction methods, namely SALLE, miniQuEChERS and DLLME, were optimized and validated for the extraction of 11 mycotoxins in urine. All the methodologies started from a small sample size (1 mL) in order to follow the current method requirement of simplification and miniaturization. Thus, low sample and solvent volumes were used, with fast analysis and high efficiency. It must be borne in mind that the establishment of feasible multi-biomarker methods is hindered by the absence of the latest generations of QTRAP. Due to the extremely low analyte concentrations present in urine following dietary exposure, the challenge of obtaining acceptable LODs and LOQs must be overcome to achieve the desired sensitivity. The present study therefore focused on parent toxins, to evaluate the efficiency of their extraction through different methodologies. It is expected that the study will be expanded to phase II metabolites in the near future, since it is well known that mycotoxins are widely metabolized in humans and animals. The main analytical parameters for all tested methodologies are compared in Table 1.

Recovery and matrix effect. As Table 1 shows, all the studied methodologies showed recoveries within the appropriate range according to the limits set in Commission Decision 2002/657/EC (European Commission

2002) (recoveries: 70–120% and RSDs < 20%). Recoveries for SALLE and miniQuEChERS ranged from 70 to 109%, while DLLME showed recoveries in the range of 73–103%. The matrix effect was evaluated for all the methodologies, showing values from 49% (signal suppression) to 144% (signal enhancement). Since matrix effects represent a major drawback of mycotoxins analysis in complex samples, matrix-matched calibrators were performed to compensate matrix effects and to obtain effective sample quantitation.

Precision. Intra-day (repeatability) and inter-day (reproducibility) precision was calculated as the RSD% of triplicate sample measurements ($n = 3$) analyzed on three different non-consecutive days ($n = 3$). Values for intra-day precision were <14% (miniQuEChERS), <14% (SALLE), and <12% (DLLME), indicating better repeatability for the DLLME method. On the other hand, values for inter-day precision were <24% while DLLME showed higher reproducibility (<15%).

Similar recovery and precision results were obtained for trichothecene extraction in urine by SALLE and miniQuEChERS [15,19,21] but higher values of repeatability and reproducibility (<31%) were reported by DLLME [14]. However, DLLME performed for emerging mycotoxins extraction in water and serum samples showed similar precision, as well as recovery values than the obtained in the present study [10,21].

Sensitivity. The LODs and LOQs were calculated using the criteria of S/N = 3 and S/N = 10, respectively. Comparable sensitivity results were obtained by the SALLE and miniQuEChERS methods showing LODs between 0.1–10 μg L^{-1} and 0.1–15 μg L^{-1}, and LOQs ranging between 0.5–40 μg L^{-1} and 0.5–35 μg L^{-1}, respectively. Although satisfactory values were achieved for

some mycotoxins, higher LOQs were obtained for OTA and/or ZEN using both methodologies. Sensitivity in multi-mycotoxin methods is hampered by the chemical diversity of analytes, which lead to a necessary compromise between sensitivity, resolution, and analyzed mycotoxins. The lowest LODs and LOQs were achieved by DLLME showing values of a few μg L^{-1} for all the mycotoxins, ranging between 0.05–2 μg L^{-1} and 0.1–4 μg L^{-1}, respectively. These values are in concordance with previous studies based on DLLME techniques [10,14,21].

Consequently, DLLME was selected as the most appropriate methodology to extract the studied mycotoxins from urine, achieving high recoveries (73–113%) with RSDs lower than 15% in all cases, and the greatest sensitivity with the lowest reported LOD and LOQ values.

Figure 4 shows the LC-MS/MS chromatograms of a spiked urine sample at 2.5 μg L^{-1} (ENs-BEA), 5 μg L^{-1} (AFs), 20 μg L^{-1} (OTA, ZEN).

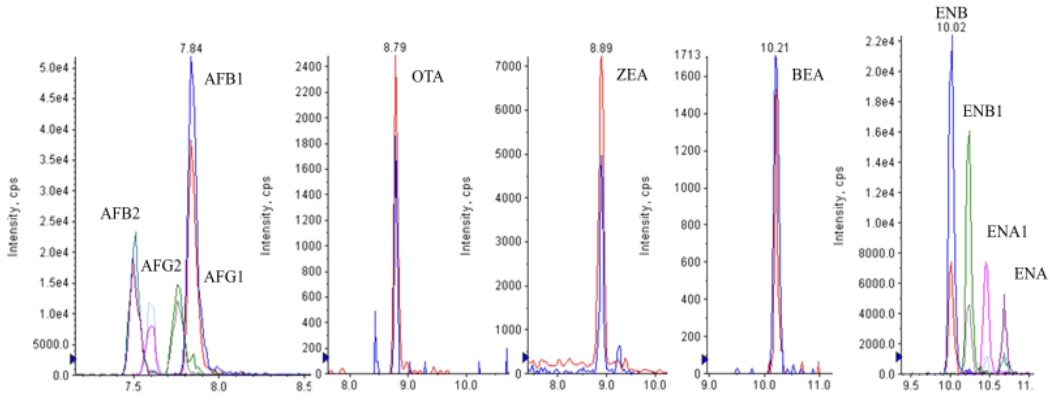

Figure 4. LC-MS/MS chromatograms of a spiked urine sample at 2.5 μg L^{-1} (ENs, BEA), 5 μg L^{-1} (AFs), 20 μg L^{-1} (OTA, ZEN).

2.4. Human Urine Samples Analysis

The developed method was successfully applied to real urine samples from 10 volunteers of both genders (5 female, 5 male) in an age range of 18–58 years. Urine samples were analyzed in triplicate (n = 3) using the developed DLLME methodology. Results showed the presence of ENs B, B1 and A1 in low concentrations. Four samples were positive for EN B (0.1–0.54 μg L^{-1}), four detectable but not quantifiable (<LOQ) and two non-detectable (<LOD). With regard to EN B1, two samples reached concentrations of between 0.1 and 0.34 μg L^{-1}, while the other four were <LOQs. Finally, EN A1 was only detected in one sample in concentrations below LOQs. EN A, BEA, AFs, OTA and ZEN were not detected in any urine sample. Two samples showed the simultaneous presence of two mycotoxins in a concentration sum of 0.61 and 0.88 μg L^{-1}.

3. Conclusions

A new dispersive liquid–liquid microextraction method with a lower-density extraction solvent has been developed for the pre-concentration and quantitative determination of 11 mycotoxins in urine, including AFs, OTA, ZEN, ENs and BEA. Optimized DLLME was selected ahead of miniQuEcHERS and SALLE techniques, as it reported better validation results in terms of recovery, precision and sensitivity. Moreover, the DLLME method has been demonstrated to offer further advantages, such as low operational cost, short extraction time, the use of minimal laboratory material, and environmental friendliness, mainly due to the low solvent volume required. To verify the applicability of the selected methodology, human urine samples from an ongoing pilot survey were analyzed for their mycotoxin levels. Thus, the

method was applied to the analysis of real urine samples from healthy volunteers from Valencia, demonstrating its usefulness in human mycotoxin exposure assessment studies, alongside other applications such as toxicokinetics or ADME (absorption, distribution, metabolism, elimination) studies. Further investigations are needed in order to broaden the range of mycotoxins studied and to include their metabolites, conjugates and masked mycotoxins.

4. Material and Methods

4.1. Chemicals and Reagents

Acetonitrile (ACN), ethyl acetate (EtOAc), methanol (MeOH), chloroform ($CHCl_3$) and hexane (HPLC gradient grade, 99.9%) were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., Danbury, CT, USA).

4.2. Analytical Standards

The standards of AFB1, AFB2, AFG1, AFG2, OTA, ZEN, BEA, EN A, EN B, EN A1 and EN B1 (purity: 99%) were obtained from Sigma-Aldrich. Solid standards were dissolved and combined into a multi-standard working solution for preparation of calibrants and spiking experiments. The standards were stored in darkness and kept at −20 °C until the HPLC-MS/MS analysis.

4.3. Sample Collection

Blank urine samples for recovery and validation studies were obtained from five volunteers (age: 25–40) who avoided the consumption of presumably mycotoxin-contaminated food such as cereal-based products for four days. Since no difference ($p > 0.05$) was observed between the blank samples, the validation process was performed by spiking a pooled sample deriving from those five individuals. Additionally, samples from volunteers in Valencia ($n = 10$), including both genders (5 female, 5 male; age 18–58), who were taking part in a larger ongoing human pilot study, were obtained. The Ethics Committee of the University of Valencia approved the project. Informed written consent was obtained from all participants prior to inclusion in the study. The volunteers did not consume any special diet on the days prior to sample donation, but a food questionnaire based on their diet record over four consecutive days was requested in conjunction with urine collection. Participants were screened for their medical history, smoking behavior and pathologies in order to check sampling representativeness. After collection, the samples were stored at −20 °C until analysis.

4.4. Samples Extraction Procedures

Several methodologies based on currently employed extraction techniques were tested, optimized, and evaluated in terms of validation data with respect to the main analytical parameters' linearity, extraction recovery, repeatability, reproducibility, LODs, LOQs and matrix effect, as determined by the European Union (European Commission 2002).

4.4.1. Salting-Out Liquid–Liquid Extraction (SALLE)

One mL of urine was centrifuged at 10,621× *g* for 3 min at 4 °C and the upper layer was placed into a 15 mL test tube. Then, 0.3 g sodium chloride (NaCl), 1 mL of ACN, and 30 mg of C18 sorbent were added in different steps after appropriate mixing. The samples were finally vortexed for 1 min and centrifuged at 4500× *g* for 3 min at 4 °C. The extract was collected as above, filtered and 10 µL were injected into the LC-MS/MS instrument.

4.4.2. MiniQuEChERS

One mL of urine was centrifuged at 10,621× *g* for 3 min at 4 °C and the upper layer was placed into a 15 mL test tube. In this case, 0.3 g magnesium sulfate ($MgSO_4$), 1 mL of ACN, and 30 mg of C18 sorbent were added after vigorously mixing between steps. Then, the solution was vortexed again for 1 min and centrifuged at 4500× *g* for 3 min at 4 °C. Finally, the upper layer was collected, filtered and 10 µL were injected into the LC-MS/MS instrument.

4.4.3. Dispersive Liquid–Liquid Microextraction (DLLME)

One mL of centrifuged urine was placed into a 15 mL test tube and 0.3 g sodium chloride was added. After mixing, the mixture of 1 mL of ACN and 100 µL of EtOAc was quickly added. The mixture was vortexed for 1 min and centrifuged at 10,621× *g* for 3 min at 4 °C to achieve the two phases of separation. The supernatant phase was collected, transferred to a vial and evaporated to dryness under a gentle stream of nitrogen. Afterwards, the dry extract was reconstituted with 100 µL of MeOH/water (70:30, *v*/*v*), filtered and 10 µL were injected into the instrument.

4.5. HPLC-MS/MS Analysis

HPLC-MS/MS analysis was performed using an Agilent 1200 liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200

QTrap® mass spectrometry system (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo electrospray ionization (ESI) interface. A reversed-phase analytical column (Gemini® C18 column, 3-µm particle size, 150 × 2 mm, I.D.) equipped with a C18 (4 × 2 mm, I.D.; 5 µm security guard cartridge; Phenomenex, Madrid, Spain) was used for analyte chromatographic separation. A binary gradient mode was selected for elution with a constant 0.250 mL/min flow rate. Mobile phases consisted of (A) water/formic acid 99:1 (*v*/*v*) and (B) MeOH/formic acid 99:1 (*v*/*v*), both containing 5 mM ammonium formate. The gradient was programmed as follows: started with 90% A and 10% B (3 min), followed by several linear gradients progressively reaching 70% B (3 min), 80% B (6 min) and 90% B (14 min). The gradient was finally switched back to 90% A (5 min). The injection volume was 10 µL. The QTRAP was used to function as a triple quadrupole mass spectrometry detector (MS/MS) in the multiple selected reaction monitoring (SRM) and using the Turbo V ion spray in positive ionization mode (ESI+). The instrument was operated using the following settings: source temperature 350 °C; ion source gas 1 (sheath gas) 50 psi; ion source gas 2 (drying gas) 55 psi, ion spray voltage 5500 V. The precursor ion of each mycotoxin was confirmed in the product ion scan mode. For each compound, the precursor ion and two characteristic product ions were monitored; using the most abundant for quantitation, and the second one for confirmation. By the acquisition of two SRM transitions per analyte the identification of positive results was confirmed. The criteria applied to confirm mycotoxin identity were: (1) a signal for each of the two SRM transitions of the analyte had to be identical in the sample and in the standard or matrix matched, obtaining four identification points for each analyte; (2) the relative ion intensity of the

mycotoxins studied in the standard solution and the spiked samples at the concentration levels used for the calibration curve were compared at tolerance of 0.5% (3) the relative retention time of the analyte in both, sample and standard solution, should be as maximum difference of 0.1 min. Analyst version 1.5.2 software (Applied Biosystem/AB sciex) was used for data acquisition and processing. The final selection of SRM precursor and product transitions, the retention time for each mycotoxin, as well as the optimal declustering potential (DP), collision energies (CE) and collision cell exit potential (CXP) are shown in Table 2.

Table 2. Optimized MS/MS parameters for the studied mycotoxins: retention time, quantitation transition, confirmation transition, declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP).

Mycotoxin	Retention Time (min)	Quantitation Transition	Confirmation Transition	DP (V)	CE (V)	CXP (V)
EN A	13.1	699.4 > 210.1	699.4 > 228.2	76	35	14
EN A1	12.2	685.4 > 210.2	685.4 > 214.2	66	37	8
EN B	11.1	657.3 > 196.1	657.3 > 214.0	51	39	8
EN B1	11.6	671.2 > 214.1	671.2 > 228.1	66	61	10
BEA	11.8	801.2 > 784.1	801.2 > 244.1	116	27	10
AFB1	8.0	313.1 > 241.0	313.1 > 284.9	46	41	4
AFB2	7.9	315.1 > 286.9	315.1 >259.0	81	33	6
AFG1	7.8	329.0 > 243.1	329.0 > 311.1	76	39	6
AFG2	7.7	331.1 > 313.1	331.1 > 245.1	61	27	6
OTA	9.3	404.3 > 102.1	404.3 > 239.0	55	97	6
ZEN	9.4	319.0 > 301.0	319.0 > 282.9	26	15	10

4.6. Method Validation

Method validation followed the guidelines established by the European Union [27]. Validation included the determination of linearity, matrix effect (ME), limits of detection (LODs), limits of quantitation (LOQs), recoveries, repeatability (intra-day precision), and reproducibility (inter-day precision). Calibration curves constructed in standard solutions (external calibrators) and in the matrix (matrix-matched calibrations) were used to evaluate linearity and matrix effects. Matrix-matched calibration curves were prepared by blank samples spiked with selected mycotoxins after extraction. Both external and matrix-matched calibration curves were built by plotting peak areas against concentration and applying linear functions. Eight concentration levels between LOQ and 100 times LOQ were employed to construct the calibration curves, and they were analyzed in triplicate. LODs and LOQs were determined by analysis of decreasing concentrations of the spiked urine, defining them as the concentration with a signal-to-noise ratio (S/N) of 3 and 10, respectively. Matrix effect was assessed by the ratio of (A) the slope of matrix-matched and (B) the slope of external calibration (B), defining matrix effect (%) as follows: A/B × 100. A value of 100% indicated that there was no matrix effect, while a value >100% or <100% represented signal enhancement or signal suppression, respectively. The method's accuracy was investigated by recovery assays by the repeated analysis of blank urine samples spiked at three concentration levels. For precision evaluation, the relative standard deviation (RSD%) of measurements of three replicates ($n >$ 9) was calculated and carried out on the same day (intra-day precision; repeatability), and on three different non-consecutive days (inter-day precision; reproducibility). The spiked levels, selected depending on the

method sensitivity, corresponded to 2 LOQs, 5 LOQs and 10 LOQs for low, medium and high levels, respectively. For statistical analysis, a student's repeated measures *t*-test ($n = 3$) was applied to analyze the results considering as significant *p*-values < 0.05.

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Author Contributions

Houda Berrada and Guillermina Font conceived and designed work, Laura Escrivá and Lara Manyes analyzed the data and wrote the paper.

Conflicts of Interest

The authors declare no conflict of interest.

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3.4 Analysis of Trichothecenes in Laboratory Rat Feed by Gas Cromatography-Tandem Mass Spectrometry

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Analysis of trichothecenes in laboratory rat feed by gas chromatography-tandem mass spectrometry

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Abstract

A method for the determination of seven trichothecenes, neosolaniol (NEO), diacetoxyscirpenol (DAS), deoxynivalenol (DON), nivalenol (NIV), fusarenon-X (FUS-X), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), in laboratory rat feed by GC-MS/MS was developed. Sample extraction and purification was performed by an acidified mixture of acetonitrile/water (80–20% v/v). Limits of quantitation (LOQs) were between 1 and 10 μg kg^{-1} for all studied trichothecenes. Eight concentration levels between the LOQ and 100 × LOQ were used for the calibration curves. Matrix-matched calibration was used for quantitation purposes to compensate the detector signal enhancement obtained for all the analytes. The method accuracy was evaluated by recovery assays at three concentration levels, 25, 50 and 100 μg kg^{-1} ($n = 9$). Recoveries ranged from 62% to 97% and precision, expressed as intra- and inter-day relative standard deviations, was evaluated for all compounds. The validated method was successfully applied to the analysis of 35 laboratory rat feed samples showing mycotoxin contamination in 66% of the samples. DON was the most prevalent trichothecene followed by 15-ADON, NIV and 3-ADON. The maximum DON concentration reached in real samples was 2156 ± 4.3 μg kg^{-1}, while NEO, DAS and FUS-X were not detected in any sample. Multi-contamination by at least two mycotoxins was observed in 17% of the analysed feed samples.

1. Introduction

The presence of mycotoxins in European feed and feed raw materials has been reported worldwide for decades (Streit et al. 2012; Kim et al. 2014). Fungal secondary metabolites, known as mycotoxins, are ubiquitous

contaminants of crop plants and forage representing the main components of compound feeds (Zachariasova et al. 2014). Mixed feeds represent an excellent substrate for fungal growth, under favourable conditions such as appropriate moisture percentage and temperature (Monge et al. 2013). Any failure to comply with good manufacturing practice (good sorting, appropriate storage conditions avoiding air access when silage-making, etc.) usually lead to an increase in mycotoxin production (Zachariasova et al. 2014) Trichothecenes are a large family of chemically related mycotoxins produced by fungi in taxonomically unrelated genera, such as *Myrothecium*, *Stachybotrys* and in particular *Fusarium* (Borutovaa et al. 2012). The broad family of trichothecenes – over 220 – is extremely prevalent and presents a potential threat to human and animal health throughout the world since their exposure can cause feed refusal, growth retardation, vomiting, skin dermatitis, neuroendocrine changes, immunosuppression and haemorrhagic lesions (McCormick et al. 2011; Escrivá et al. 2015). Chronic intake of small amounts of trichothecenes leads to an increased susceptibility to infections (Montes et al. 2012). At a molecular level, trichothecenes would display multiple inhibitory effects on the primary metabolism of eukaryotic cells including the inhibition of proteins, DNA and RNA synthesis (Alassane-Kpembi et al. 2013). Trichothecenes contain a tricyclic 12,13 epoxytrichothec-9-ene (EPT) core structure with various side-chain substitutions, allowing their classification into four types: A, B, C and D (McCormick et al. 2011). Types A and B constitute non-macrocyclic trichothecenes and are commonly found in grain, animal feed and human food produced from contaminated grain (Döll & Dänicke 2011; Tang et al. 2015). Several surveys demonstrate that type-B

trichothecenes occur more frequently and at higher concentrations compared with other types of trichothecenes (Ok et al. 2011; Rodríguez-Carrasco et al. 2014).

Among the trichothecenes, deoxynivalenol (DON) is the most frequently occurring toxin, and it is found worldwide, particularly in cereal crops such as wheat, maize, barley, oats, rye and less often in rice, sorghum and triticale (EFSA 2004; Streit et al. 2012; Shar et al. 2014b). As animal diets consist mainly of cereals, it can be assumed that animals are frequently exposed to DON-contaminated feeds (Awad et al. 2010). Cereals contaminated with DON are often also contaminated with other *Fusarium* mycotoxins (Ogiso et al. 2012) such as 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), nivalenol (NIV) and other trichothecenes (EFSA 2004). JECFA considers the major *Fusarium* toxin contaminants are DON, and its acetylated derivates 3-ADON, and 15-ADON (JECFA 2011). The ingestion of DON has been associated with alterations of the intestinal, immune, endocrine and nervous systems in laboratory animals (Escrivá et al. 2015). DON would modify the innate immune response increasing the susceptibility of the host to infection (Antonissen et al. 2014). Sub-chronic dietary exposure to DON affects the gut wall morphology of duodenum and jejunum, and increases the intestinal barrier permeability leading to intestinal inflammation (Osselaere et al. 2013). DON ingestion resulted in lipid peroxidation and oxidative stress of jejunal cells, damage to the DNA of blood lymphocytes, and alteration of non-specific parameters such as growth rate, plasma biochemistry, and villus height and apparent surface area in the duodenum (Devreese et al. 2014). Changes in the intestinal microflora were observed in DON-exposed animals, suggesting an impact of this toxin on the

dynamics of intestinal bacteria communities (Waché et al. 2009). NIV showed higher acute toxicity than DON in animal studies, therefore it is of concern for food safety although is not as prevalent as DON (Cheat et al. 2015).

The European Union established guidance values for DON ranging from 0.9 to 5 mg kg^{-1} in complete and complementary animal feeds (European Commission 2006). However, EU recommendations for maximum levels and guidance values of mycotoxins in feed are generally applied to farm animals feed (European Commission 2002, 2006, 2013). Currently there is no EU legislation establishing the maximum levels of mycotoxins in laboratory animal feed.

Over the last years, a significant number of publications about trichothecenes have focused increasingly on LC-MS and LC-MS/MS methods (Berthiller et al. 2014; Juan et al. 2014). Some papers using GC-MS have been published showing that despite its narrower analytical scope, GC-MS/MS reached to be a very useful and relatively inexpensive analytical performance with some clear advantages such as lower LODs and greater selectivity (Rodríguez-Carrasco et al. 2012; Shar et al. 2014a).

Some authors have already reported trichothecene analysis in feeds by GC. Labuda et al. (2005) analysed DON, 3-ADON, 15-ADON and NIV by GC-electrochemical detection (ECD) in poultry feed mixtures of Slovakia. Amelin et al. (2013) reported a method for the analysis of the trichothecenes T-2, HT-2, DON and NIV after QuEChERS extraction in grain and mixed feed by GC-ECD; while Waldemarson et al. (2005) determined them in rodent feeds by GC-MS. Cegielska-Radziejewska et al. (2013) reached the simultaneous determination of seven trichothecens (T-2, HT-2, DAS, DON, 3-ADON, 15-ADON and NIV) by GC-MS in feed mixtures for broiler chickens. A comparative

study of three different GC techniques – GC-flame ionisation (FID), GC-ECD and GC-MS – for the quantification of DON in poultry feed samples was also performed by Shar et al. (2014a). Better sensitivity of DON was achieved by GC-MS, which exhibited slightly higher precision, lowest coefficient of variation, and lowest LOD.

Considering the limited GC-MS/MS analytical methods developed for the determination of mycotoxins feeds and the scarcity of performed studies in laboratory animal feeds, the aim of this study was to develop a method for the determination of seven trichothecenes (type A: neosolaniol (NEO) diacetoxyscirpenol (DAS); and type B: DON, NIV, fusarenon-X (FUS-X), 3-ADON and 15-ADON) in laboratory rat commercial feed by GC-MS/MS.

2. Materials and methods

2.1 Chemical and reagents

Acetonitrile, hexane and methanol (HPLC gradient grade, 99.9%) were purchased from Fisher Scientific (Madrid, Spain). Deionised water (< 18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). The derivatisation reagent BSA (*N,O-bis*(trimethylsilyl)acetamide) +TMCS (trimethylchlorosilane) + TMSI (*N*-trimethylsilyimidazole) (3:2:3) was purchased from Supelco (Bellefonte, PA, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Química S.L.U. (Barcelona, Spain).

2.2 Analytical standards

The standards of the type A and B trichothecenes: DON, 3-ADON, 15-ADON, NIV, NEO, DAS and FUS-X, were obtained from Sigma-Aldrich. Individual stock solutions of all analytes were prepared at the same concentration by dissolving 1 mg of the mycotoxin in 1 ml of pure methanol (1000 mg l^{-1}). The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (10 mg l^{-1}). All procedures were performed following the safety recommendations reported in a USFDA document (FDA 2013). Standards were stored in darkness and kept at −20°C until the GC-MS/MS analysis.

2.3 GC-QqQ-MS/MS conditions

Chromatographic determination was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA). Quantitation data were acquired at selection reaction monitoring (SRM) mode and the mass spectrometer was operated in electron ionisation (EI, 70 eV). The transfer line and source temperatures were 280 and 230°C, respectively. The collision gas for MS/MS experiments was nitrogen (1.5 ml min^{-1}), and helium was used as the quenching gas (2.25 ml min^{-1}), both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data were acquired and processed using the Agilent Masshunter version B.04.00 software.

Analytes were separated on a HP-5MS 30 m × 0.25 mm × 0.25 µm capillary column. A total of 1 µl of extract of mycotoxins was injected in splitless mode in a programmable temperature vaporisation (PTV) inlet

(150°C for 0.1 min then 600°C min^{-1} to 250°C for 5 min) employing helium as the carrier gas at a fixed pressure of 20.3 psi. The oven temperature programme was initially 80°C, and the temperature was increased to 245°C at 60°C min^{-1}. After a 3-min hold time, the temperature was increased to 260°C at 3°C min^{-1} and finally to 270°C at 10°C min^{-1} and then held for 10 min. All analytes eluted within 12 min, reaching the requirement for a high throughout determination (SANCO 2013).

2.4 Samples

A total of 35 laboratory rat feed samples were obtained from Spanish animal research laboratories. All samples were grounded using a laboratory mill and stored in a dark and a dry place into specific plastic food containers before their analysis.

Analysed feed samples included in their composition cereals such as wheat, corn, wheat bran, barley, maize, maize gluten, corn gluten feed, wheat germ, wheat brain and corn starch; vegetal proteins and vegetal oils such as soybean oil, corn oil, sunflower seed, potato protein and extruded soybeans; derived animal products such as hydrolysed fish and egg white solids; and other ingredients such as yeasts, vitamins (A, D3, E) and minerals (Fe, Mn, Zn, Cu, I, Co).

2.5 Sample preparation

2.5.1 Extraction and clean-up

Feed (5 g) accurately weighed (precision 0.1 mg) were transferred to centrifuge tubes (50 ml) and homogenised in a vortex with 10 ml of acetonitrile/water (80:20) 0.1% HCOOH. Samples were then shaken (IKA Ks

260 basic, Stanfen, Germany) for 1 h, and the tubes were placed in an ultrasonic bath for 15 min followed by centrifugation at 4500 rpm for 10 min (Centrifuge 5810R, Eppendorff, Germany). A total of 2 ml of supernatant extract were transferred to an Eppendorff vial and stored in a freezer (for a minimum of 2 h). After this time, the extract was centrifuged again at 14 000 rpm for 10 min, filtered with a 0.22-µm filter (Phenomenex, Madrid, Spain) and dried under nitrogen flow.

2.5.2 Derivatisation

The dry extract was added with 50 µl of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at RT. The derivatised sample was diluted to 200 µl with hexane and mixed thoroughly on a vortex for 30 s. Then the hexane was washed with 1 ml of phosphate buffer (60 mM, pH 7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to an autosampler vial for chromatographic analysis.

2.6 Method validation

The analytical method was in-house-validated with respect to the main analytical parameters, namely linearity, extraction recovery, repeatability, reproducibility, LODs, LOQs and matrix effect according to the SANCO/12571/2013 document (SANCO 2013).

Linearity and matrix effects were calculated using calibration curves of the studied analytes in pure solvent and in matrix. Both external calibration curves and matrix-matched calibration curves were constructed by plotting peak areas against the concentration of the corresponding standards at eight concentration levels ranging between LOQ and 100× LOQ, and linear functions were applied to the calibration curves. All calibration curves were

performed in triplicate and linearity was expressed by the square correlation coefficient (r^2). To assess the matrix effect, the matrix-matched calibration slope (A) and external calibration slope (B) were calculated. Thus, the ratio (A/B × 100) is defined as the matrix effect (%). A value of 100% indicated that there was no matrix effect. There was signal enhancement when the value obtained was higher than 100% and signal suppression when the value was lower than 100%. Accuracy of the method was evaluated by measuring the recoveries from feed sample, previously analysed to ensure the absence of studied mycotoxins, spiked at 25, 50 and 100 μg kg^{-1} ($n = 9$). Precision evaluation (expressed as the relative standard deviation; RSD %) was determined by repeatability (intra-day) and reproducibility (inter-day). Intra-day precision was evaluated in three determinations per concentration in a single day, while inter-day precision was tested on three different working days within 20 days. The sensitivity of the method was assessed by the LODs and LOQs. The LODs and LOQs were the lowest matrix-matched calibration providing signal-to-noise ratios greater than 3 and 10, respectively, at both quantitation and confirmation transitions, and matching the intensity ratio observed for the particular compound in the standard solution.

3. Results and discussion

3.1 Optimisation of mass spectrometry conditions

The specificity and selectivity of the method relies on the chromatographic retention time of each analyte and on the SRM transition used. Two MS/MS transitions were acquired for each mycotoxin reaching four identification points with a defined SRM transitions ratio for the developed method, as is indicated in the requirements for mass spectrometry

(SANCO 2013). For each compound, the most abundant SRM transition was used for quantitation, while the other transition was used for confirmation. Table 1 shows the quantitation and confirmation transitions, the average ion ratios and the retention time obtained for each trichothecene.

Table 1. Optimised GC-MS/MS parameters of the selected mycotoxins.

Mycotoxin	Rt (min)	Quantitative transition (Q)	CE (V)	Dt (ms)	Qualitative transition (q)	CE (V)	Dt (ms)	Ratio (Q/q ± RSD %)
DON	8.45	392 > 259	10	25	407 > 197	10	25	64 ± 4
3-ADON	9.45	392 > 287	5	35	467 > 147	10	25	34 ± 4
15-ADON	9.65	392 > 217	20	35	392 > 184	20	35	37 ± 5
NIV	9.95	289 > 73	15	35	379 > 73	15	35	24 ± 4
NEO	11.30	252 > 195	10	25	252 > 167	15	35	58 ± 2
DAS	9.56	350 > 229	15	35	378 > 124	10	25	42 ± 7
FUS-X	9.55	450 > 260	10	35	450 > 245	20	35	12 ± 11

3.2 Method performance

To assess the possible matrix effect on the chromatographic response, the slopes of the calibration in standard solutions with those obtained in matrix-matched standards were compared. The results obtained showed a signal enhancement for all analytes except for FUS-X, which showed irrelevant matrix effect. Matrix-matched calibration was used for quantitation in order to compensate for the matrix effect (Table 2).

The results obtained demonstrated a good linearity between LOQ and 100× LOQ for all derivatised compounds. Satisfactory results in terms of recoveries were found for all the compounds at three fortification levels (25, 50 and 100 μg kg^{-1}). The studied trichothecenes showed recovery results within the range 62–97% (Table 2). Intra- and inter-day precision studies showed that the method was repeatable and reproducible for all analytes. Similar recoveries were obtained by other authors in feeds using GC-MS (Cegielska-Radziejewska et al. 2013; Shar et al. 2014b). LODs and LOQs obtained ranged from 0.5 to 5 μg kg^{-1} and from 1 to 10 μg kg^{-1}, respectively, and showed the suitability of the developed method for the determination of trace amounts of the selected mycotoxins in feed samples (Table 2).

Table 2. Limits of detection and quantitation (LODs, LOQs), matrix effects (ME %), average recoveries and RSD at different feed spiked concentrations.

Mycotoxin	LOD	LOQ	Recovery (%) Spiked level (µg kg−1)			Intra-day (RSD%) Spiked level (µg kg−1)			Intra-day (RSD%) Spiked level (µg kg−1)			Matrix effect	Linearity
	(µg kg−1)	(µg kg−1)	**25**	**50**	**100**	**25**	**50**	**100**	**25**	**50**	**100**	**(%)**	**(*r*2)**
DON	0.5	1	85	89	86	16	7	3	16	11	8	178	0.9993
3-ADON	1	3	95	72	62	1	9	8	11	11	12	146	0.9998
15-ADON	1	3	69	69	62	15	13	10	12	15	21	154	0.9966
NIV	1	2.5	97	86	65	4	2	7	4	14	7	114	0.9972
NEO	2	5	80	69	62	10	5	3	7	24	14	100	0.9962
DAS	2.5	5	69	68	81	24	19	9	10	2	22	144	0.9885
FUS-X	5	10	84	73	67	9	21	10	29	12	21	144	0.9429

LOQs of all studied mycotoxins were lower than those obtained by Labuda et al. (2005) using GC-ECD (LOQs: 28, 23, 23 and 41 μg kg^{-1} for DON, 3-ADON, 15-ADON and NIV, respectively) and by Shar et al. (2014a) using the three GC-tested methods, which reported LOQs for DON of 36 μg kg^{-1} (GC-FID), 30 μg kg^{-1} (GC-ECD) and 18 μg kg^{-1} (GC-MS). LOQs achieved in this study were also lower than those reported by previous studies on different feed samples categories using several techniques such as HPLC-ultraviolet detection (UV) (Rodrigues et al. 2011), LC-MS/MS (Åberg et al. 2013; Błajet-Kosicka et al. 2014; Kim et al. 2014) and TubroFlow-LC-MS/MS (TLX-LC-MS/MS) (Ates et al. 2013) which reported LOQs for DON of 150, 20, 35, 115 and 375 μg kg^{-1}, respectively.

Thus, according to the obtained results, the developed method is robust and suitable for its purpose, and is presented as an alternative method for the determination of trichothecenes in laboratory rat feed.

Figure 1 shows the SRM chromatograms of DON, NIV, 3-ADON, 15-ADON, NEO, DAS and FUS-X obtained from a spiked feed sample (100 μg kg^{-1}) indicating the quantitation (Q) and confirmation (q) transitions for each compound.

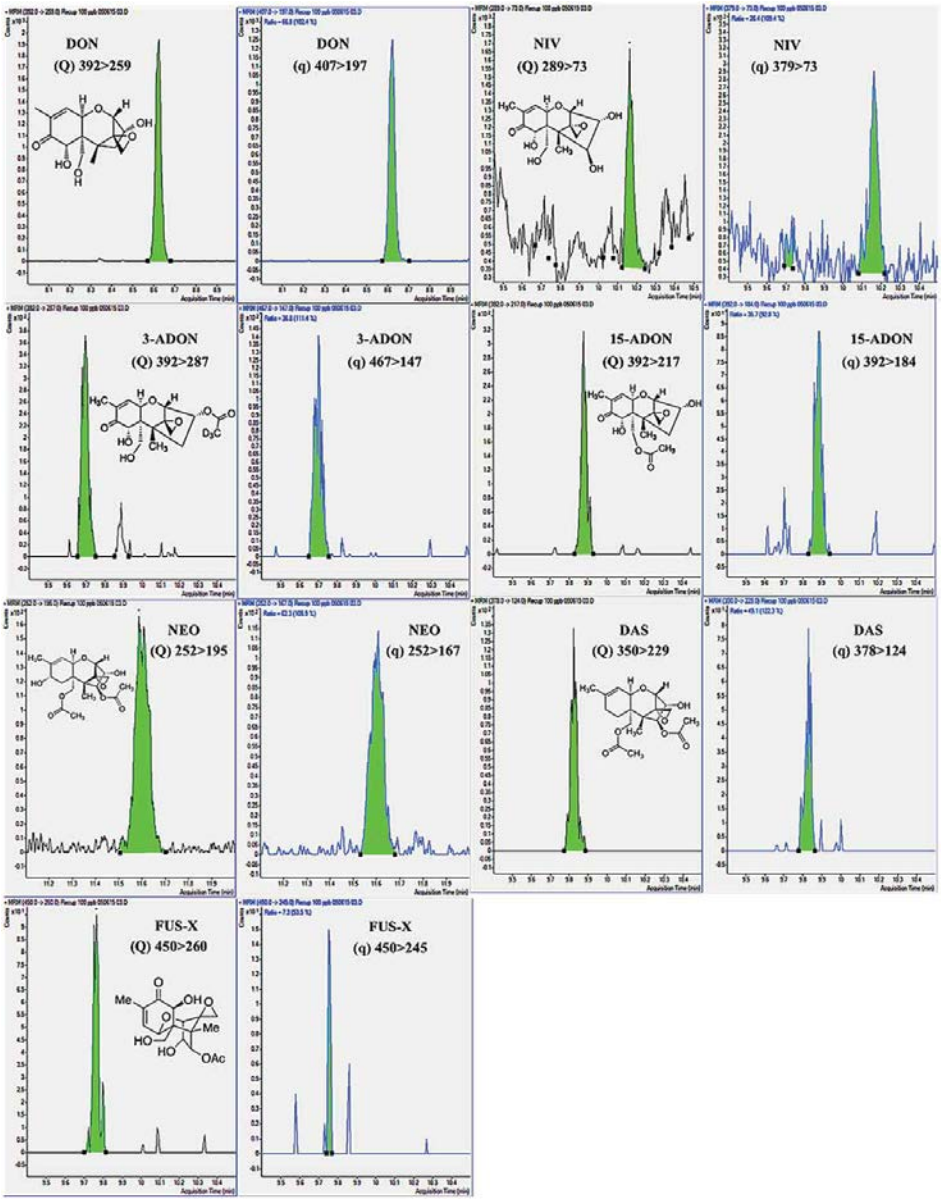

Figure 1. SRM chromatograms of DON, NIV, 3-ADON, 15-ADON, NEO, DAS and FUS-X obtained from a spiked feed sample (100 µg kg^{-1}), showing the molecular structure and the quantitation (Q) and confirmation (q) transitions for each compound.

3.3 Analysis of feed samples

Thirty-five samples of commercial feed normally given to laboratory rats were obtained from different animal facilities and research centres in Spain. Feed samples were grounded, homogenised and analysed in triplicate by the method described above (see the Sample preparation section). The results obtained for the seven studied trichothecenes in the 35 analysed laboratory rat feed samples are shown in Table 3.

A total of 66% of the 35 analysed samples showed mycotoxin contamination. A total of 17 samples (49%) showed contamination with a single mycotoxin. DON was the most prevalent trichothecene with an incidence of 62.9%. The high incidence of DON is in agreement with some published data. Labuda et al. (2005) detected 56% of positive samples for DON among the analysed poultry feed samples in concentrations between 64 and 1230 μg kg^{-1} (average of 303 μg kg^{-1}). Kim et al. (2014) reported DON contamination in 53% and 91% of the total feed ingredients and compound feeds analysed, respectively.

Among compound feeds, 100% of the cattle feed, 88% of the swine feed and 86% of the poultry feed samples analysed were contaminated with DON, showing levels of contamination between 131 and 1000 μg kg^{-1}, between 37 and 982 μg kg^{-1}, and between 35 and 1492 μg kg^{-1}, respectively. Li et al. (2014) described DON (among ZEN) as the most prevalent mycotoxin found in complete feeds with an overall incidence of 97% (mean of 650 μg kg^{-1}). The detection rate of DON ranged from 86% up to 100% and the averages concentrations were between 280 and 850 μg kg^{-1} in several pig feeds categories.

Table 3. Detected concentrations of DON, 3-ADON, 15-ADON, NIV, NEO, DAS and FUS-X in the 35 analysed laboratory rat feed samples.

	Concentration (µg kg–1) ± RSD (%)						
Sample	**DON**	**3-ADON**	**15-ADON**	**NIV**	**NEO**	**DAS**	**FUS-X**
1	2155.9 ± 4.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	993.0 ± 1.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	397.8 ± 0.8	6.2 ± 18.2	33.2 ± 1.1	n.d.	n.d.	n.d.	n.d.
4	214.3 ± 15.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
5	56.4 ± 58.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	715.8 ± 6.7	n.d.	57.0 ± 9.7	n.d.	n.d.	n.d.	n.d.
7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	490.3 ± 14.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
10	315.2 ± 3.3	n.d.	28.6 ± 3.1	25.6 ± 0.2	n.d.	n.d.	n.d.
11	352.2 ± 38.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12	474.0 ± 29.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
13	44.8 ± 20.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	342.2 ± 30.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
15	145.0 ± 20.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	110.8 ± 19.9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17	150.2 ± 26.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18	300.4 ± 50.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19	30.1 ± 35.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20	268.9 ± 1.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21	341.8 ± 10.5	n.d.	22.1 ± 31.7	n.d.	n.d.	n.d.	n.d.
22	340.6 ± 10.3	n.d.	16.4 ± 21.8	n.d.	n.d.	n.d.	n.d.
23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
24	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
26	n.d.	n.d.	n.d.	39.6 ± 4.8	n.d.	n.d.	n.d.
27	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
28	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
30	4.8 ± 13.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
31	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
32	422.1 ± 5.5	n.d.	24.5 ± 5.7	n.d.	n.d.	n.d.	n.d.
33	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
34	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
35	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

As shown in Table 4, the average content of DON in the positive samples was 393.9 μg kg^{-1}, reaching the maximum concentration of 2156 ± 4.3 μg kg^{-1}. Similar maximum concentrations of DON were reported by Li et al. (2014) with the highest concentration of 2310 μg kg^{-1} detected in starter pig feeds. Ezekiel et al. (2012) reported that DON concentrations exceeded 1000 μg kg^{-1} in 10% of 58 analysed poultry feed samples.

Table 4. Incidence, positive samples and minimum, maximum and average concentrations of the studied mycotoxins in the analysed laboratory rat feed samples.

Mycotoxin	Incidence	Positive samples (%)	Concentration (µg kg–1) Minimum ± RSD (%)	Maximum ± RSD (%)	Average ± SD
DON	22/35	62.9	4.8 ± 13.3	2155.9 ± 4.3	393.9 ± 456.6
3-ADON	1/35	2.9	6.2 ± 18.2	6.2 ± 18.2	6.2 ± *
15-ADON	6/35	17.1	16.4 ± 21.8	57.0 ± 9.7	30.3 ± 14.3
NIV	2/35	5.7	25.6 ± 0.2	39.6 ± 4.8	32.6 ± 9.9
NEO	0/35	0	n.d.	n.d.	n.d.
DAS	0/35	0	n.d.	n.d.	n.d.
FUS-X	0/35	0	n.d.	n.d.	n.d.

After DON, the most prevalent mycotoxin in the present study was 15-ADON (17%) followed by NIV (5.7%) and finally 3-ADON (2.9%). Neither NEO, nor DAS, nor FUS-X were found in any of the analysed samples. Other authors reported the absence of NEO and FUS-X in sow feeds (Monbaliu et al. 2010), and DAS in poultry feeds (Monge et al. 2013). The maximum detected concentrations were 57.0 ± 9.7 μg kg^{-1} (15-ADON), 39.6 ± 4.8 μg kg^{-1} (NIV) and 6.2 ± 18.2 μg kg^{-1} (3-ADON). Consistent with these results Vanheule et al. (2014) reported that the acetylated forms of DON are usually co-occurring with DON as a certain low percentage of the total DON load, with dominance of 15-ADON over 3-ADON. Ogiso et al. (2012) reported that the general order of concentration in contaminated feed and feed ingredients was DON > 15-ADON > 3-ADON; and most analysed mixed feed samples contained DON (150−1200 μg kg^{-1}) and 15-ADON (30−290 μg kg^{-1}), but only one mixed feed sample contained 3-ADON (10 μg kg^{-1}). In the study performed by Labuda et al. (2005) the acetyl-derivatives of DON were detected in 8% of the samples, and NIV was not detected in any of the investigated feed samples.

Co-occurrence of *Fusarium* mycotoxins is of great of interest as mycotoxigenic fungi are capable of producing more than one mycotoxin and feed raw materials might be infected with various fungal species. Simultaneous occurrence of mycotoxins appears to exert greater negative effects on health and productivity than single mycotoxins (Kim et al. 2014). In this study multi-contamination was observed in six of the 35 analysed samples (17%). As shown in Table 5, co-occurrence with two and three different compounds was observed in four and two laboratory rat feed samples, respectively (11% and 6%). The binary combination detected was

DON, 15-ADON; while the ternary combinations were DON, 3-ADON, 15-ADON and DON, 15-ADON, and NIV.

Table 5. Co-occurrence mycotoxins data based on the sum of the concentrations found in the same sample.

Co-occurrence	Number of samples	Sum. C.min (µg kg–1)	Sum. C.max (µg kg–1)
Two mycotoxins			
DON, 15-ADON	4	357.0	772.8
Three mycotoxins			
DON, 3-ADON, 15-ADON	1	437.2	*
DON, 15-ADON, NIV	1	369.4	*

Other authors reported similar trichothecenes co-occurrence. Waldemarson et al. (2005) detected the binary combination of DON–NIV in rodent feeds. Labuda et al. (2005) described the binary and ternary combinations of DON and its acetylated derivates 3-ADON and 15-ADON detected in poultry feeds. Monbaliu et al. (2010) reported the same combinations cited above and the quaternary combination of DON, NIV, 3-ADON and 15-ADON.

As far as the sum concentration of trichothecenes found in the same positive sample was concerned, 20% of the samples contained values higher than 400 µg kg^{-1}, and almost half of the analysed samples (49%) reached concentrations higher than 100 µg kg^{-1}.

Figure 2 shows an SRM chromatogram of naturally contaminated feed sample (no. 22) with DON (340.6 ± 10.3 µg kg^{-1}) and 15-ADON (16.4 ± 21.8 µg kg^{-1}).

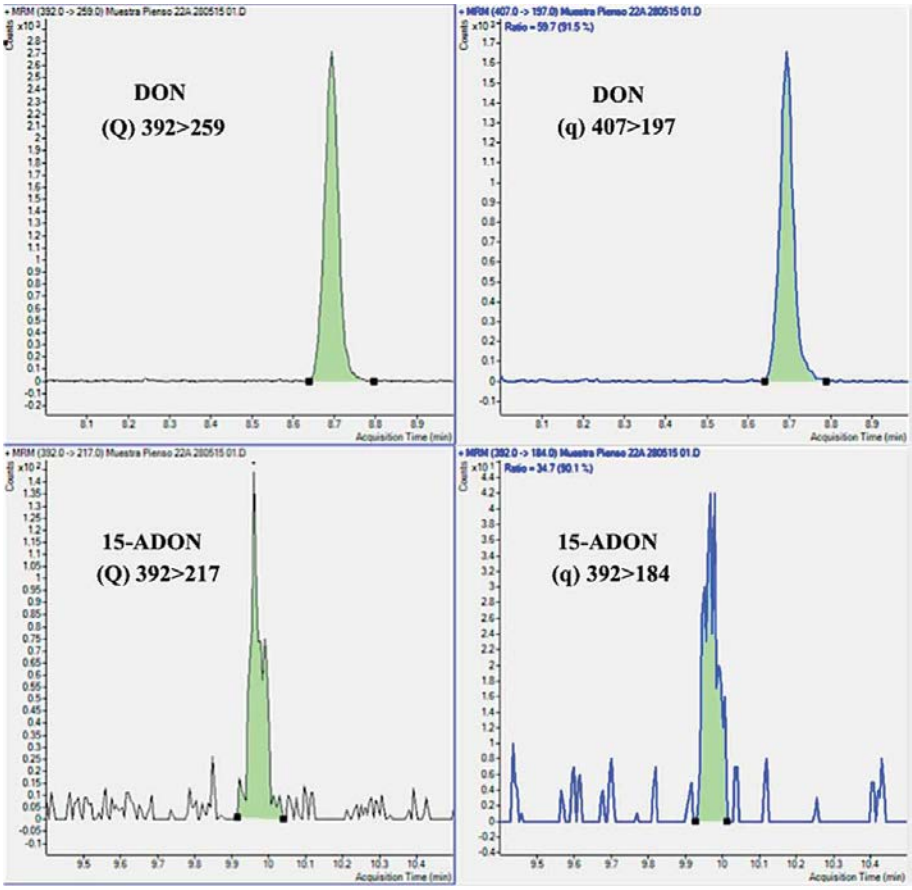

Figure 2. SRM chromatograms obtained from a feed sample (no. 22) contaminated by DON (340.6 ± 10.3 μg kg^{-1}) and 15-ADON (16.4 ± 21.8 μg kg^{-1}).

4. Conclusions

An accurate, precise and sensitive GC-MS/MS method was developed for the determination of seven trichothecenes: DON, 3-ADON, 15-ADON, NIV, DAS, NEO and FUS-X in feed. LOQs lower than 10 μg kg^{-1} and acceptable recoveries (between 62% and 97%) at three different spiked levels were obtained for all mycotoxins. The validated method was successfully applied to

35 laboratory rat feed samples showing mycotoxin contamination in 66%. DON was the most prevalent trichothecene followed by 15-ADON, NIV and 3-ADON, and reached the maximum concentration of 2156 ± 4.3 μg kg^{-1}. NEO, DAS and FUS-X were not detected in any sample. Multi-contamination by two and three different compounds was observed in 17% of the analysed feed samples.

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3.5 Mycotoxin Contamination in Laboratory Rat Feeds and their Implications in Animal Research

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Mycotoxin contamination in laboratory rat feeds and their implications in animal research.

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Abstract

Compound feed is particularly vulnerable to multi-mycotoxin contamination. A method for the determination of 12 mycotoxins; enniatins A, A1, B, B1; aflatoxins B1, B2, G1, G2; OTA; ZEN; T-2 and HT-2 by liquid chromatography-tandem mass spectrometry has been developed and applied for the analysis of laboratory rat commercial feeds. The method trueness was checked by recovery assays at three different spiked levels ($n = 9$). Recoveries ranged from 73% to 112%, and the intra-day and inter-day precision were lower than 9% and 13%, respectively. Limits of quantitation were lower than 15 μg/kg. Twenty-seven laboratory rats feed samples showed multi-contamination by at least three up to six different mycotoxins. ENNs B and B1, followed by ZEN were the most prevalent mycotoxins. T-2, HT-2, and OTA were not detected. ZEN showed the highest concentration levels reaching 492 μg/kg. The results underline the importance of implementing mycotoxin regular surveillance programs for laboratory animal feeds.

1. Introduction

Mycotoxins are fungi secondary metabolites mainly produced by *Aspergillus*, *Penicillium*, or *Fusarium* genus that can occur in all agricultural commodities in field or storage under appropriate fungi growth conditions. Mycotoxin contamination depends on climatic and environmental conditions before and after harvest (Anfossi et al., 2014). Other factors such as improper processing, packaging, drying techniques, and transport activities influence fungal growth and increase the risk of mycotoxin production (Afsah-Hejri et al., 2013).

Mycotoxins are unavoidable contaminants in crops (Zhang et al., 2009), therefore they commonly contaminate a wide range of cereals

and cereal-derived products (Abysique et al., 2015). The Food and Agriculture Organization (FAO) reported that approximately 25% of crops worldwide are affected by mycotoxins (FAO/WHO, 2001). Furthermore, most mycotoxins are thermostable, resistant to milling, processing, and heating, which make difficult their elimination; therefore they readily enter the food and feed chains (Grenier & Oswald, 2011). Since fungal toxins have been detected in various food commodities from many parts of the world they have been recognized as one of the most prevalent toxic contaminants of food and feed (Bhat et al., 2010; Cheli et al., 2014).

Compound feed is particularly vulnerable to mycotoxin contamination as it typically contains a mixture of several raw materials mainly cereals and seed proteins. The high prices and scarcity of protein sources for animal feeds have led to the use of alternative protein sources such as distillers dried grains with solubles (DDGS) (Zachariasova et al., 2014). DDGS suppose at even greater risk of containing higher levels of mycotoxins, as they are about three times more concentrated than the original grains (Oplatowska-Stachowiak et al., 2015). Those procedures inevitably lead to the contamination of the final mixed feeds, manufactured by mixing different raw materials from different origins to make a totally new matrix with a new risk profile (Binder et al., 2007; Tolosa et al., 2014). In addition, as most feeds are made from annual crops, mycotoxin content may vary from year to year, and distribution of mycotoxin contamination is very heterogeneous existing highly contaminated sites among good quality material (Alonso et al., 2013). As a consequence, the available feed is frequently contaminated with mycotoxins (Bryden, 2012; Streit et al., 2013a).

On the other hand, animal feed is frequently contaminated simultaneously by several fungi, which are able to produce several mycotoxins leading to co-occurrence of mycotoxins (Bhat et al., 2010;Grenier & Oswald, 2011). Multi-mycotoxin contamination is of particular concern due to potential additive or synergistic effects (Cheli et al., 2013). Special attention should be paid to both, feed composition and contamination risk while storing (Arroyo-Manzanares et al., 2015).

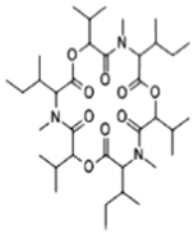
In 2014, out of the 3097 original notifications counted in RASFF (Rapid Alert System for Food and Feed), 309 concerned feed, about 10% of the total, rising in number for the first time in several years. Notifications concerning feed have been increasing for a few specific categories with mycotoxin as the second most important (RASFF, 2014). RASFF. (2014). Regarding the feed manufacturing process, some mycotoxins are subject to legal regulations; aflatoxin B1 (AFB1), or recommendations; deoxynivalenol (DON), zearalenone (ZEN), sum of fumonisins B1 and B2 (FB1 + FB2), ochratoxin A (OTA) and sum of T-2 and HT-2 toxins (T-2 + HT-2), in the European Union (UE) (European Commission, 2002, 2006, 2013a,b).

Mycotoxin maximum levels or guidance values are established in different feedstuffs, depending on the animal feed (e.g. ZEN has different guidance value for piglets and gilts (0.1 mg/kg), sows and fattening pigs (0.25 mg/kg), calves, dairy cattle, sheep, lamb, and goats (0.5 mg/kg)). Also individual values have been established for animal feeds with specific destination (e.g. FBs in pet animal feeds: 5 mg/kg). However, laboratory animal feeds are still not mentioned in EU legislation.

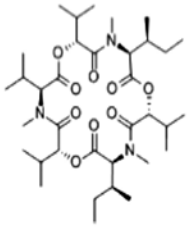
There is an increased information demand for mycotoxins presents in feed, even at levels well below the legal limits (Monbaliu et al., 2010).

Nevertheless, very few studies address laboratory animal feed mycotoxin contamination (Streit et al., 2013a). Mycotoxins were found in mixed feed samples for laboratory rodents, namely DON, nivalenol (NIV), OTA, and ZEN, showing co-occurrence of two (DON or NIV and OTA or ZEN) or three (DON, OTA, and ZEN) different mycotoxins, and indicating that feed ingredients were contaminated by these compounds (Waldemarson et al., 2005). A preliminary screening to evaluate the presence of AFB1 in laboratory rat feed samples was also performed (Guerra et al., 2007) and the fungal contamination and natural occurrence of OTA were previously evaluated in mice feed samples showing contamination with filamentous fungi as *Cladosporium sp.*, *Aspergillus niger*, and *Penicillium* (Almeida et al., 2010). Recently, seven trichothecenes were analyzed in laboratory rat feeds showing multi-mycotoxin contamination with DON, its acetylated metabolites 3-ADON and 15-ADON, and NIV (Escrivá et al., 2016). These mycotoxicological researches put in evidence the importance of the use of contaminant-free experimental animal feed in order to prevent any interference on the experimental animal health, and emphasizes the need for systematic control of the feed as a key issue in animal experimentation.

The aim of this study was to develop a method for the determination of twelve mycotoxins (enniatins A, A1, B, B1; aflatoxins B1, B2, G1, G2; OTA; ZEN; T-2 and HT-2) by liquid chromatography-tandem mass spectrometry (LC-MS/MS) for laboratory rat commercial feeds analysis. The chemical structure, empirical formula, CAS (Chemical Abstracts Service) number and molecular weight for the studied mycotoxins are shown in Figure 1



ENN A
681.92 g/mol
$C_{36}H_{63}N_3O_9$
Cas No. 2503-13-1



ENN A1
667.87 g/mol
$C_{35}H_{61}N_3O_9$
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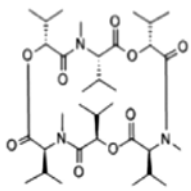

ENN B
639.82 g/mol
$C_{33}H_{57}N_3O_9$
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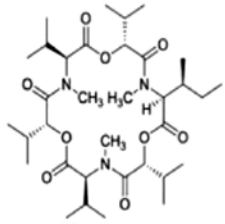
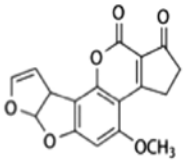
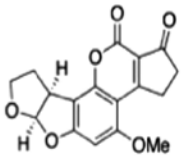


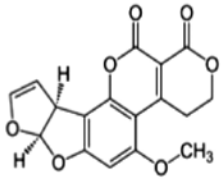
ENN B
653.85 g/mol
$C_{34}H_{59}N_3O_9$
Cas No. 19914-20-6



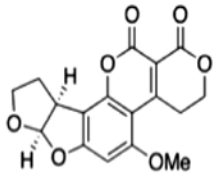
AFB1
312.27 g/mol
$C_{17}H_{12}O_6$
Cas No. 1162-65-8



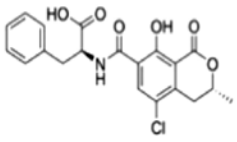
AFB2
314.29 g/mol
$C_{17}H_{14}O_6$
Cas No. 7220-81-7



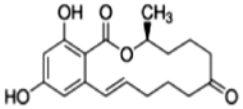
AFG1
328.27 g/mol
$C_{17}H_{12}O_7$
Cas No. 1165-39-5



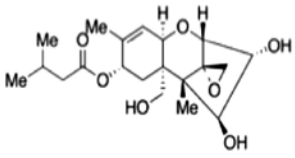
AFG1
330.29 g/mol
$C_{17}H_{14}O_7$
Cas No. 7241-98-7



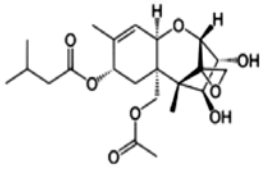
OTA
403.81 g/mol
$C_{20}H_{18}ClNO_6$
Cas No. 303-47-9



ZEA
318.36 g/mol
$C_{18}H_{22}O_5$
Cas No. 17924-92-4



T-2
466.52 g/mol
$C_{24}H_{34}O_9$
Cas No. 21259-20-1



HT-2
424.48 g/mol
$C_{22}H_{32}O_8$
Cas No. 26934-87-2

Figure 1. Chemical structure, molecular weight, empirical formula, and CAS (Chemical Abstracts Service) number and for ENN A, ENN A1, ENN B, ENN B1, AFB1, AFB2, AFG1, AFG2, OTA, ZEN, T-2, and HT-2.

2. Materials and methods

2.1 Chemical and reagents

Acetonitrile and methanol were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Ammonium formiate (HCO_2NH_4, 97%) and formic acid (HCOOH, 98%) were supplied by Sigma-Aldrich (Madrid, Spain). All solvents were passed through a 0.22 µm cellulose filter from Membrane Solutions (Dallas, TX). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., Danbury, CT).

The stock standard (purity: 99%) of ENN A, ENN B, ENN A1, ENN B1, AFB1, AFB2, AFG1, AFG2, OTA, ZEN, T-2, and HT-2 toxins were purchased from Sigma Aldrich (Madrid, Spain) and individual stock solutions with concentrations of 1000 mg/L were prepared by dissolving 1 mg of mycotoxin in 1 ml of pure methanol. These stock solutions were diluted with methanol in order to obtain the appropriate multi-compounds working standard solutions. All procedures were performed following the safety recommendations reported in a USFDA document (FDA, 2013). All standards were stored in darkness and kept at 4 °C.

2.2 Analyzed samples

Twenty-seven laboratory rat feed samples were purchased from Spanish animal facilities and research centers. Feed samples analyzed included as nutrient composition: crude protein (15–21%), crude fat (3–6%), ashes (5–6%), crude fiber (4–5% as cellulose), calcium (0.8–0.9%), phosphorus (0.4–0.6%), and sodium (0.2–0.3%). Their composition included

cereals such as wheat, corn, wheat bran, barley, maize, maize gluten, corn gluten feed, wheat germ, wheat brain, corn starch; vegetal proteins, and vegetal oils such as soybean oil, corn oil, sunflower seed, potato protein, extruded soybeans; derived animal products such as hydrolyzed fish, and egg white solids; and other ingredients such as yeasts, vitamins (Vit A, Vit D3, Vit E) and minerals (Fe, Mn, Zn, Cu, I, Co).

2.3. Sample preparation and extraction

All samples were grounded and homogenized before their analysis. Briefly, 5 g of feed was accurately weighed (precision 0.1 mg), transferred to centrifuge tubes (50 ml), and homogenized in a vortex with 10 ml of acetonitrile/water (80:20, v/v) 0.1% HCOOH. After, the samples were shacked (IKA Ks 260 basic, Stanfen, Germany) for 1 h, and the tubes were placed in an ultrasonic bath for 15 min followed by centrifugation at 4500 rpm for 10 min (Centrifuge 5810R, Eppendorf, Germany). Two milliliters of supernatant extract were transferred to an eppendorff vial and stored in a freezer (minimum 2 h). Afterward, the extract was centrifuged again at 14,000 rpm for 10 min. Finally, the supernatant extract was filtered with a 0.22 μm filter (Phenomenex, Madrid, Spain) prior to their LC-MS/MS analysis.

2.4. Instrumental and chromatographic conditions

Detection and quantitation were performed with an Agilent 1200 liquid chromatograph (Agilent Technologies, Palo Alto, CA) coupled to a 3200 QTrap®mass spectrometry system (Applied Biosystems, Foster City, CA) equipped with a Turbo electrospray ionization (ESI) interface. The QTRAP analyzer combines a fully functional triple quadrupole and a linear ion trap

mass spectrometer. Chromatographic separation of analytes was performed with a reversed-phase analytical column (Gemini® C18 column, 3-µm particle size, 150 × 2 mm, I.D.), equipped with a C18 (4 × 2 mm, I.D.; 5 µm security guard cartridge) all from Phenomenex, Madrid, Spain. Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium formiate and were composed of water/formic acid 99:1 (v/v; eluent A) and methanol/formic acid 99:1 (v/v; eluent B). The flow rate was 0.250 ml/min. The gradient program started with 90% A and 10% B and was kept until 3 min, afterwards a linear gradient was applied, reaching 70% B after 1.5 min (holding time, 3 min). Then, the linear gradients 80% B (6 min) and 90% B (14 min) were included. Finally, gradient switched back (5 min) to 90% A. The injection volume was 10 µl. MS/MS was performed in the selected reaction monitoring (SRM) and using the Turbo V ion spray in positive ionization mode (ESI+). The instrument was operated using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature, 350 °C; desolvation temperature 270 °C, curtain gas 20 psi; ion source gas 1 (sheath gas) 50 psi; ion source gas 2 (drying gas) 55 psi, ion spray voltage 5500 V and collision gas energy 5 eV. Nitrogen served as nebulizer and collision gas. Analyst version 1.5.2 software (Applied Biosystem/AB sciex) was used for data acquisition and processing.

2.5. Method validation

Linearity and matrix effects were studied using standard solutions and matrix-matched calibrations. Matrix-matched calibration curves were built by spiking blank samples with selected mycotoxins after extraction. Both external calibration curves and matrix-matched calibration curves were

constructed by plotting peak areas against concentration and linear functions were applied to the calibration curves. Limit of detection (LOD) and quantitation (LOQ) were defined as the concentration with a signal-to-noise ratio (S/N) of 3 and 10, respectively. These parameters were determined by analysis decreasing concentrations of the spiked feed. Eight concentration levels between LOQ and 100 times LOQ were employed for constructing the calibration curves, analyzing them in triplicate. To assess matrix effect the slope of feed matrix-matched (A) and the slope of external calibration (B) were calculated. Thus, the ratio ($A/B \times 100$) is defined as the matrix effect. A value of 100% indicated that there was no matrix effect. There was signal enhancement if the value was higher than 100% and signal suppression if the value was lower than 100%. The method's recovery and precision were calculated by the repeated analysis of feed samples spiked at three concentrations levels; and by the relative standard deviation (RSD %) of measurements of three replicates ($n = 9$) carried out in the same day (intra-day precision) and in three different days (inter-day precision). The spiked levels were 20, 50, 100 μg/kg (ENNs) and 50, 100, 200 μg/kg (AFs, OTA, ZEN, T-2, and HT-2).

3. Results

3.1 Optimization of mass spectrometry conditions

The optimization of MS/MS parameters was performed by flow injection analysis of each compound and entrance potential (EP) was set at 8 V. For each compound, the precursor ion and two characteristic product ions were monitored; the first and most abundant one was used for quantitation (Q), while the second one was used for confirmation (q). The acquisition of

two SRM transitions per analyte allowed confirming the identity of the positive results. Table 1 shows the precursor and product ions, the retention time, and the main MS/MS programed parameters for each mycotoxin.

Table 1. Optimized MS\MS parameters for the studied mycotoxins.

Mycotoxin	Retention time (s)	Precursor ion (*m/z*)	Products ions (*m*/z)	DP (V)	CE (V)	CXP (V)
ENN A	17.9	699.4 [M + NH4]+	210.1Q	76	35	14
			228.2q		59	16
ENN A1	17.5	685.4 [M + NH4]+	210.2Q	66	37	8
			214.2q		59	10
ENN B	16.7	657.3 [M + NH4]+	196.1Q	51	39	8
			214.0q		59	10
ENN B1	17.0	671.2 [M + NH4]+	214.1Q	66	61	10
			228.1q		57	12
AFB1	15.0	313.1 [M + NH4]+	241.0Q	46	41	4
			284.9q		39	4
AFB2	14.1	315.1 [M + NH4]+	286.9Q	81	33	6
			259.0q		39	6
AFG1	14.7	329.0 [M + NH4]+	243.1Q	76	39	6
			311.1q		29	6
AFG2	14.2	331.1 [M + NH4]+	313.1Q	61	27	6
			245.1q		39	4
OTA	15.3	404.3 [M + NH4]+	102.1Q	55	97	6
			239.0q		27	6
ZEN	16.0	319.0 [M + NH4]+	301.0Q	26	15	10
			282.9q		19	4
T-2	15.9	484.3 [M + NH4]+	185.1Q	76	22	4
			215.1q		29	4
HT-2	15.6	442.2 [M + NH4]+	215.4Q	61	19	8
			262.8q		19	4

Product ions: "Q" indicates quantification transition, and "q" indicates qualification transition. DP: declustering potential; CE: collision energy; CXP: collision cell exit potential.

3.2 Method validation

The validation process was carried out using blank feed samples with no detectable mycotoxin. Linearity, evaluated by preparing matrix-matched calibration curves (LOQ-100LOQ), showed a linear trend with a correlation coefficient (r^2) > 0.9941 for all compounds.

Matrix effect, calculated as the percentage of the slopes ratio for standard and matrix calibration curves, varied depending on the studied mycotoxin, showing in all cases signal suppression (<100%). Strong signal suppression was observed for AFs (22.5–30.1%), followed by T-2, HT-2, and ENN A1 (65.5–68.7%); ENN B, ENN B1, ZEN (79.4–79.4%); and finally ENN A and OTA (82.0–90.8%) (Table 2). The results obtained justified the use of matrix-matched calibration for quantitation to compensate matrix effect in order to have reliable and accurate results.

LODs were between 1 and 5 μg/kg, and LOQs between 5 and 15 μg/kg. The intra-day and inter-day precision was evaluated on spiked samples at three concentration levels (ENNs: 20, 50, 100 μg/kg; AFs, OTA, ZEN, T-2, and HT-2: 50, 100, 200 μg/kg). The values <10% for the intra-day test and <13% for the inter-day test demonstrated good repeatability and reproducibility for LC-MS/MS. Recoveries, evaluated by analyzing spiked feed samples at three concentration levels (ENNs: 20, 50, 100 μg/kg; AFs, OTA, ZEN, T-2, and HT-2: 50, 100, 200 μg/kg) were satisfactory for all mycotoxins, with values between 73.3 and 111.6%, and RSD <15.6% in all cases. Table 2 shows the LODs, LOQs, recoveries, intra-day and inter-day precision, matrix effect, linearity, and linear range for all mycotoxins (Table 2).

Table 2. Analytical parameters obtained in the validation of the method: limits of detection (LOD), limits of quantitation (LOQ), recoveries at three concentration spiked levels, intra-day and inter-day precision, matrix effect, and linearity for the studied mycotoxins.

Mycotoxin	LOD (µg/kg)	LOQ (µg/kg)	Recovery (R%)			Precision (%RSD)			Linearity (r^2)	
			20 µg/kg	50 µg/kg	100 µg/kg	Intra-day	Inter-day	Matrix effect (%)	Standard	Matrix
ENN A	1	5	105.1 ± 8.0	110.8 ± 7.6	102.8 ± 12.3	5.2	7.7	82.0	0.9960	0.9979
ENN A1	1	5	97.8 ± 14.6	111.6 ± 11.8	103.9 ± 11.0	5.2	11.9	67.5	0.9943	0.9960
ENN B	1	5	99.6 ± 14.6	106.4 ± 10.3	106.1 ± 9.3	5.7	10.7	75.4	0.9930	0.9955
ENN B1	1	5	98.1 ± 11.5	104.9 ± 9.0	99.6 ± 6.5	7.9	5.4	70.4	0.9933	0.9984
			50 µg/kg	100 µg/kg	200 µg/kg					
AFB1	3	10	96.7 ± 12.7	99.5 ± 10.3	94.4 ± 8.0	8.4	7.9	30.1	0.9951	0.9983
AFB2	3	10	88.4 ± 10.0	90.9 ± 9.4	94.1 ± 11.7	6.5	9.5	24.8	0.9924	0.9991
AFG1	3	10	84.0 ± 15.6	106.7 ± 5.6	104.1 ± 6.5	6.3	7.7	22.5	0.9908	0.9953
AFG2	3	10	85.7 ± 10.8	98.2 ± 11.5	102.3 ± 9.8	9.4	7.4	27.3	0.9909	0.9972
OTA	3	10	85.7 ± 14.8	83.0 ± 13.0	74.4 ± 6.0	7.3	10.4	90.8	0.9945	0.9964
ZEN	3	10	97.8 ± 14.7	90.8 ± 17.7	73.3 ± 9.2	7.6	12.7	79.4	0.9943	0.9957
T-2	5	15	107.0 ± 14.9	102.3 ± 9.2	98.9 ± 8.4	9.1	7.4	65.5	0.9936	0.9968
HT-2	5	15	88.0 ± 10.3	100.3 ± 13.9	96.2 ± 11.4	8.8	9.2	68.7	0.9948	0.9941

3.4 Analysis of feed samples

Twenty-seven laboratory rat feed samples were grounded, homogenized, and analyzed by triplicate by the method described above. Statistical analysis (Student's *t*-test) of repeated measures ($n = 3$) was applied to analyze the results. No significant statistical differences for a confidence interval of 95% were found in terms of recovery results for the same analyzed sample.

100% of the analyzed feed samples were mycotoxin multi-contaminated. Although co-contamination was such common, co-occurrence of 2 mycotoxins was not observed in any sample. The detected combinations were from three up to six different mycotoxins, being the most frequent combinations of six (33%) followed by three and five different mycotoxins at the same prevalence (26%). The *Fusarium*mycotoxins ENNs B and B1 were the most prevalent mycotoxins, with 100% of incidence followed by ZEN, detected in 81% of the analyzed samples. Minor prevalence was observed for ENN A1 (59%) and ENN A (19%). Among the AFs, the most detected were AFB2 (56%) and AFG2 (30%), followed by AFG1 (19%) and AFB1 (4%). OTA, T-2, and HT-2 were not detected in any sample. The total mycotoxin prevalence observed in the present study followed the order ENN B and ENN B1 > ZEN > ENN A1 > AFB2 > AFG2 > ENN A and AFG1 > AFB1 (Table 3).

ZEN was detected in considerable concentrations, reaching the maximum concentration of 492 μg/kg, followed by AFB2, AFG2, and ENN B with the maximum concentrations of 324, 226, and 104 μg/kg, respectively (Table 3). The minimum detected concentrations were in all cases less than

50 μg/kg except for ZEN (191 μg/kg), which reached by far the highest average value of the detected concentrations (304 μg/kg) (Table 3).

Considering the total amount of detected mycotoxins (sum of mycotoxins) concentrations higher than 300 μg/kg were observed in over half of the samples analyzed in this study (67%) reaching in the worst-case scenario the large amount of 1049 μg/kg (Figure 2).

Table 3. Positive samples, incidence, and minimum, maximum, and average concentration of the studied mycotoxins in the analyzed feed samples.

			Concentration (µg/kg)		
Mycotoxin	**Positives**	**Prevalence (%)**	**Minimum ± RSD (%)**	**Maximum ± RSD (%)**	**Average ± SD**
ENN B	27/27	100	14.8 ± 6.8	103.5 ± 1.3	59.3 ± 21.7
ENN B1	27/27	100	10.3 ± 3.6	37.5 ± 7.4	21.9 ± 7.3
ENN A	5/27	19	13.9 ± 4.4	24.1 ± 9.7	17.7 ± 4.0
ENN A1	16/27	59	5.1 ± 8.9	9.2 ± 3.3	6.4 ± 1.2
AFB1	1/27	4	40.4 ± 4.0	–	–
AFB2	15/27	56	4.2 ± 7.4	324.2 ± 6.7	92.1 ± 106.6
AFG1	5/27	19	12.9 ± 1.0	31.9 ± 1.2	21.4 ± 7.1
AFG2	8/27	30	26.8 ± 9.7	226.3 ± 0.5	118.6 ± 79.0
ZEN	22/27	81	191.1 ± 12.3	492.1 ± 7.2	303.6 ± 103.8
OTA	0/27	nd	nd	nd	nd
T-2	0/27	nd	nd	nd	nd
HT-2	0/27	nd	nd	nd	nd

nd: non detected.

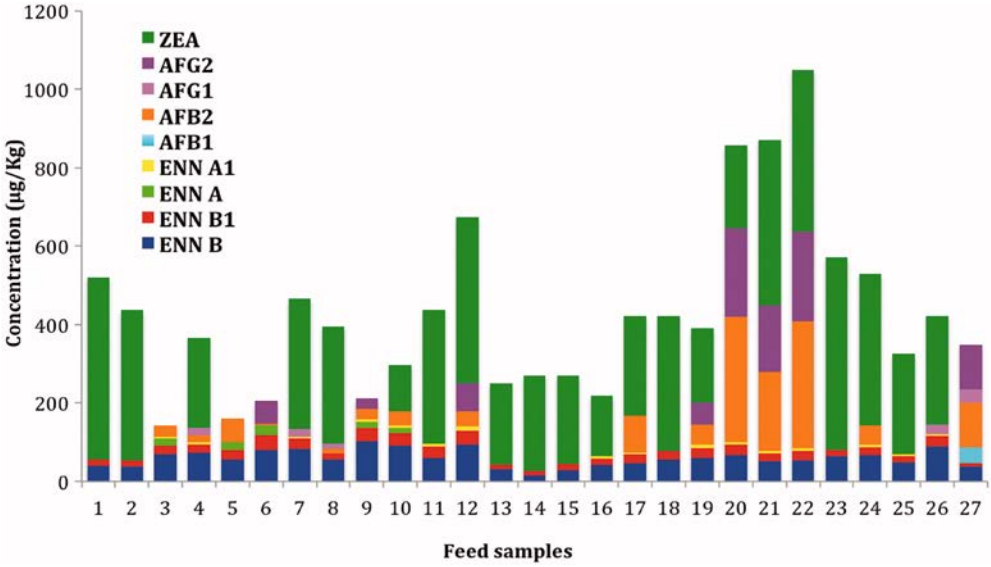


Figure 2. Sum of the detected concentrations of ZEN, ENN B, ENN B1, ENN A, ENN A1, AFB1, AFB2, AFG1, AFG2 in the analyzed feed samples.

4. Discussion

4.1 Mycotoxin occurrence in feeds

Similar high mycotoxin incidence (79–100%) was reported in feedingstuffs and complex compound mixtures for various groups of animals (Zachariasova et al., 2014). It has been previously reported that 75%–100% of the analyzed feeds contained more than one mycotoxin (Streit et al., 2012). Several authors reported that *Fusarium* mycotoxins were the most common feed contaminants (Binder et al., 2007; Streit et al., 2013b).

Fusarium genus is globally one of the most important genera of fungi and its species are probably the most prevalent toxin-producing fungi. Spontaneous outbreaks of *Fusarium* mycotoxicosis have been reported in Europe, Asia, Africa, New Zealand, and South America (Escrivá et al., 2015).

Regarding to the mycotoxin prevalence, the reported incidences described in this work were in concordance with the results reported by other authors. ENNs were very habitual contaminants in feed and feed ingredients showing incidences between 87% and 95% (Streit et al., 2013b; Zachariasova et al., 2014), up to 100% in feed for farmed fish (Tolosa et al., 2014). Previously reported ENNs incidences in feeds followed the order ENN B and B1 > ENN A1 > ENN A (Abia et al., 2013; Mendes de Souza et al., 2013).

Moreover, these mycotoxins – reported as present in all of the investigated feed sample categories – reached concentrations for ENN B, B1, A, and A1 of 799 μg/kg, 405 μg/kg, 2816 μg/kg, and 223 μg/kg, respectively in compound feeds for pigs; and 142 μg/kg, 55 μg/kg, 1455 μg/kg, and 106 μg/kg for ENN B, B1, A, and A1, respectively in compound feeds for chickens and laying hens, as well as, concentrations of 36 μg/kg and 11 μg/kg for ENNs B and B1, were respectively detected in compound feeds for birds and rodents (Zachariasova et al., 2014).

Other authors also reported ZEN as a high prevalent mycotoxin in feeds and feedstuffs, reaching considerable concentrations in some cases. For instance, ZEN was found in 88% of the analyzed poultry feed samples (Labuda et al., 2005). In complete feeds for growing pigs and sows 67% positive samples for ZEN were detected with a maximum concentration of 571 μg/kg (Thieu et al., 2008). 50% of the piglet samples and 67% of the analyzed suckling pig samples showed ZEN levels over the recommended limits (Pereyra et al., 2011), and 48% of feed samples surveyed in Central Europe were positive for ZEN reaching the maximum concentration of 1045 μg/kg (Rodrigues & Naehrer, 2012).

On the other hand, AFB2, that reached in the present work 56% of prevalence in concentrations up to 324 μg/kg, was previously reported with 40% of prevalence in poultry feed samples in concentrations higher than the maximum tolerance level established for poultry feeds (20 μg/kg) (Anjum et al., 2011). AFs were detected in 74% of the analyzed animal feeds and feed ingredients reaching concentrations up to 156 μg/kg (Mngadi et al., 2008).

Concerning the non-detected mycotoxins in the present study, the absence of detected levels of OTA, T-2, and HT-2 were also reported in laboratory rodent feed (Waldemarson et al., 2005), fattening pig feed (Pereyra et al., 2011), and broiler and poultry feed (Mngadi et al., 2008).

With regard to the EU legislation, the established mycotoxin maximum levels for AFB1 range from 5 μg/kg for finish feed for dairy animals to 20 μg/kg for raw materials and other finish feed. Thus, although AFB1 was only found in one sample the detected concentration (40.4 ± 4.0 μg/kg) exceeds these levels. The recommended levels set for ZEN were from 100 μg/kg for complementary and complete feedingstuff for piglets and gilts to 3000 μg/kg in maize by-product feed material. The ZEN levels found in the analyzed laboratory rat feeds were between 191 and 492 μg/kg, therefore in some cases it could be above the established recommendations. Unfortunately, it is not possible to accurately compare in the absence of specific values for mycotoxins in experimental animal feeds. The recommended limits established for OTA range 50–250 μg/kg, and were between 250 and 2000 μg/kg for the sum of T-2 and HT-2. Nevertheless, as it was discussed above, these mycotoxins were not found in the analyzed feeds. Regulatory limits have not yet been established for emerging mycotoxins like ENNs, widely prevalent in the laboratory rat feeds.

4.2 Implications in animal experimental research

Common experimental animals like rats, mice, and guinea pigs are very important for *in vivo* assays. Rodent feeding trials are the most widely used experiments in biomedical research and are particularly used to evaluate the potential side effects of commercial products in mammals. The rat may also be considered as a toxicological model for small mammals, either wild or kept as farm animals or pets. The quality of the rodent diet is thus crucial (Mesnage et al., 2015). Standardizing the physical, health, and welfare conditions of experimental animals is an imperative for the accuracy of *in vivo* research, which can be unintentionally affected by feed contamination (Guerra et al., 2007). Moreover, the use of quality-controlled diets, free of undesirable substances such as mycotoxins, may improve reproducibility and standardization, reduce both the inter-individual variability of the studied parameters and the number of animals needed in experimental research, as well as, prevent any negative effect on the animal health (Almeida et al., 2010).

Regarding the mycotoxin concentrations reported here, some calculations were made in order to estimate the mycotoxin daily exposure of laboratory rats in the hypothetical situation they consume the analyzed feeds. Considering the general conditions for the adult rat of 20 g/day feed intake and an average body weight of 300 g, the calculated mycotoxin daily intake range from 0.28 to 12.74 μg/kg bw/day (minimun mycotoxin detected levels) to 0.61–32.81 μg/kg bw/day in the worst case (maximum mycotoxin detected levels). Taking into account the average mycotoxin detected values the calculated mycotoxin daily intake range between 0.43 and 20.24 μg/kg bw/day. Hence, considerable concentrations of ZEN (12.74–32.81 μg/kg

bw/day), AFB2 (21.61 µg/kg bw/day), and AFG2 (15.09 µg/kg bw/day) could be ingested throughout the whole animal life as a result of consumption of the analyzed feeds. No NOAELs/LOAELs for ZEN in animals were derived by EFSA due to the inadequate available data; however, NOAEL of 10 µg/kg bw/day for estrogenic effects in pigs, considered the most susceptible specie, was used as a reference to calculate the human tolerable daily intake (TDI) (European Commission, 2014). Regarding AFs, since carcinogenicity is the basis for concern as it, it is assumed that it is not possible to identify a dose threshold of effect for genotoxic compounds; any small dose will have a proportionally small probability of inducing the effect (European Commission, 2012).

Moreover, recent data obtained in our laboratory reported that several trichothecenes are commonly present in this kind of laboratory rat feeds. Levels of DON from 4.8 µg/kg up to 2.2 mg/kg, accompanied in some cases by 15-ADON (16.4–57.0 µg/kg), NIV (25.6–39.6 µg/kg) or 3-ADON (6.2 µg/kg) were found in the laboratory rat feeds analyzed by GC-MS/MS (Escrivá et al., 2016). Based on the same scenario as above, the consumption of 20 g/day of contaminated feed by a standard laboratory rat (300 g body weight) could mean in the worst case (maximum detected concentrations) a DON daily intake of 143.73 µg/kg bw. A provisional TDI for DON was set in 2002 by the Scientific Committee for Food (SCF) at 1 µg/kg body weight (b.w.) per day on the basis of a NOAEL of 100 µg/kg b.w. per day for decreased body weight gain reported in a 2-year feeding study in mice (SCF, 2002Scientific Committee on Food (SCF). (2002). Opinion of the Scientific Committee on Food on Fusarium toxins. Part 6: Group evaluation of T-2 toxin, HT-2 toxin, nivalenol and deoxynivalenol, adopted on 26 February 2002.). In 2010, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) extended it to

the group of DON and its acetyl derivatives 3-ADON and 15-ADON and also derived an Acute Reference Dose (ARfD) at 8 µg/kg bw (European Commission, 2013a,b).

A TDI of 1.2 µg/kg bw per day for NIV was established based on a lower 95% confidence limit for a benchmark response of 5% extra risk (BMDL50) of 350 µg/kg bw per day derived from reduced white blood cell counts observed in a 90-day rat study and by applying an UF of 300 to account for inter- and intra-species differences (European Commission, 2014).

Although the estimated mycotoxin intake levels are not alarming concentrations, it has to be noted that in some studies low doses of mycotoxins were administered. Kouadio et al. (2013) investigated the lipid metabolism disorders, lymphocytes cells death, and renal toxicity after DON administration (45 µg/kg bw/day) for 7 days in mice. Firmin et al. (2011) administered a single dose of AFB1 (18 µg/kg bw) to evaluate the effect of a yeast cell wall-based preparation on mycotoxin toxicokinetics in rats. González et al. (2014) performed a 60-day subchronic oral toxicity study in rats feeded by AFB1 (7–17 µg/kg bw) and AFG1 (3.5–5.8 µg/kg bw) to evaluate genotoxicity and cytotoxicity of AFs and the ability of dietary yeast to reduce the toxic effect. Mitchell et al. (2014) administered a single dose of 125 µg/kg bw AFB1 to rats to evaluate the mycotoxin bioavailability reduction by a montmorillonite clay. It has been reported 37.5% incidence of lung adenocarcinoma in NIH mice when DON (1.5 µg/kg bw/day) was administered orally for 24 weeks (Mishra et al., 2014).

It is well known that the major concern associated with mycotoxin contaminated animal feed is chronic disease, and low level mycotoxin multiple exposition will may cause an array of metabolic disturbances. A combination

of mycotoxins at low concentration may produce negative effects, even though the concentrations of individual mycotoxins are below the concentrations reported to cause negative effects (Bryden, 2012; Grenier & Oswald, 2011).

On the other hand, the amount of feed intake can be influenced by several factors, such as energy diet content, temperature, light cycle, physiological status, etc. Rats exposed to temperatures below their neutral thermal zone increase feed intake to satisfy their increased energy requirement to maintain a constant body temperature (Krinke, 2000). The average daily consumption of a pregnant female rat increases from a manteinance rate of 30 g/day up to 65 g/day, and can reach 72 g/day during lactation period (Baker et al., 2013). Therefore, higher exposures than the reported here may occur in certain situations and cause an increased mycotoxin exposure of several weeks or even months.

Anywise, efforts toward safer agricultural practices and better control of environmental contaminants have to be made in order to feed laboratory rodents with healthy diets, to not only improve the reliability of toxicity tests, but also the value of animal feeding trials in biomedical research (Mesnage et al., 2015).

The high incidence of mycotoxins found in the analyzed laboratory rat feeds highlights the need of periodic monitoring of mycotoxin contamination in feedstuffs and complete feeds (Thieu et al., 2008; Zachariasova et al., 2014). The results of this study reiterate the importance of mycotoxin testing prior to the feeding of experimental animals and underline the suggestion to establish some regulations in laboratory animal feeds regarding the mycotoxin contamination.

Figure 3 shows a LC-MS/MS SRM chromatogram of a feed sample (No. 15) indicating the presence of ENN B, ENN B1, and ZEN in concentrations of 29.2 ± 2.9 µg/kg (ENN B), 15.3 ± 8.1 µg/kg (ENN B1), and 225.6 ± 4.6 µg/kg (ZEN).

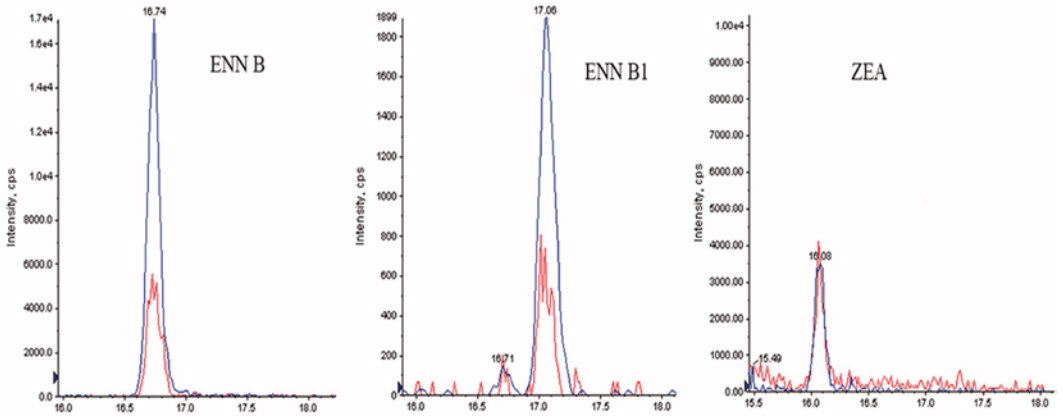


Figure 3. LC-MS/MS MRM chromatogram of a feed sample (No. 15) indicating the presence of ENN B, ENN B1, and ZEN in concentrations of 29.2 ± 2.9 µg/kg (ENN B), 15.3 ± 8.1 µg/kg (ENN B1), and 225.6 ± 4.6 µg/kg (ZEN).

5. Conclusions

The developed method was accurate, precise, and sensitive for the detection and quantitation of twelve mycotoxins; ENNs A, A1, B, B1; AFs B1, B2, G1, G2, OTA, ZEN, T-2, and HT-2 in feed. The validated method was applied to real samples of laboratory rat feeds, showing high average of multi-mycotoxin contamination. The frequently detected combinations were from three up to six different mycotoxins. ENNs B and B1 along with ZEN were the most prevalent mycotoxins. ZEN contents range from 191 to 492 µg/kg showing the highest concentration levels. T-2, HT-2, and OTA were not detected in any sample. The high percentage of positive feed samples

indicates that the laboratory rat commercial feeds are commonly contaminated by mycotoxins. It is recommendable to control the quality of laboratory animal feeds, highly susceptible to contamination by mycotoxins, to obtain reliable *in vivo* test results.

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3.6 Effects of Quercetin against Mycotoxin Induced Cytotoxicity: a Mini-Review

Current Food Science and Nutrition (2017)

Effects of quercetin against mycotoxin induced cytotoxicity: a mini-review

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Abstract

Quercetin (QUER) is a bioactive phytochemical belonging to a large group of polyphenolic flavonoid substances and one of the most abundant flavonoids in the human diet. The antioxidant and pro-oxidant activity of QUER has been extensively investigated because of contradictory findings about its ability to protect mammalian cells from cytotoxicity. QUER is concomitant with mycotoxins in numerous food and foodstuff. In the 80s, experimental approaches started to evaluate *in vitro* its protective effects against cytotoxicity related to mycotoxin ingest. The aim of this mini-review was to analyze how QUER treatment modifies mycotoxin effects in *in vitro* experimental models. In literature they are described the following: ochratoxin A, zearalenone and its metabolites α- and β-zearalenol, alternariol, alternariol monomethyl ether, T-2 toxin, patulin, aflatoxin B-1, deoxynivalenol, enniatins and beauvericin. Regarding the experimental models, they were used human hepatocellular (HepG2) and colorectal carcinoma (HCT116 and Caco-2), human peripheral blood mononuclear cells (hPMBC), embryonic kidney (HEK293), chinese hamster ovary (CHO-K1) and African green monkey kidney (Vero) cell lines. In summary, QUER showed promising results that made researchers move up to *in vivo* experiments. As future implementation, a combination of dietary non-enzymatic antioxidant products in food and feed could reduce the effects of mycotoxins in humans and animals.

1. Introduction

Flavonoids are polyphenolic compounds integrated in the human diet produced exclusively by plants, widely distributed in fresh fruits, berries,

black tea, red wine, purple grape juice or medicinal herbals [1]. The protective effect of these compounds includes antiproliferative and anti-inflammatory activity and stimulation of the immune system, mainly due to a wide variety of mechanisms, including lightening oxidative stress by scavenging free radicals, promoting cellular survival by modulating intracellular signals, regulating gene expression or enzymes activity, etc. [2].

The flavonol 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one (QUER; Figure 1) is a secondary metabolite in higher plants, commonly found glycosylated in vegetables and fruits, showing exceptional high concentration in onions, apples, tea, broccoli, peppers, berries, cherries, grapes, and red wine. QUER is a major polyphenol ingested with a dietary intake of 20-100 mg, which may increase to 200-500 mg in case of high intake of fruits and vegetables [3-4]. Principally, QUER exhibits a broad spectrum of properties, such as anti-inflammatory, immunomodulatory, antitumoral, antiplatelet, and vasoprotector effect, and modulator of enzyme activities and gene expression through both direct interaction or indirect modification of signal transduction [5]. Moreover, the regular intake of QUER is suggested to exert beneficial health effects including protection against osteoporosis, certain forms of cancer, pulmonary and cardiovascular diseases, possibly due to its ability to scavenge reactive oxygen oxygen species (ROS) and free radicals [6].

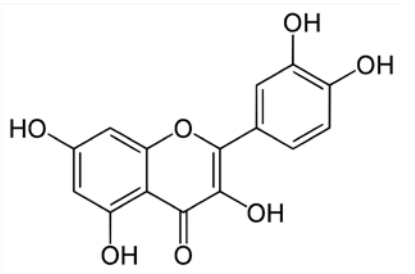

Figure 1. Chemical structure of Quercetin

QUER is considered the most potent scavenger of free radicals within the flavonoid family and the most plentiful one, showing both antioxidant and pro-oxidant effects, largely related to its dosage and the particular biological system [7]. The antioxidants are essential for protecting the cells from undergoing chemical damage due to oxidation, while the pro-oxidant properties can either be favourable or detrimental to biological systems and can affect the progress of chronic diseases in human beings [8]. In this sense, cell viability depends on a balance between pro-oxidant and antioxidant compounds [9]. QUER showed excellent free radical scavenging activity for both ROS such as superoxide anion and hydroxyl radicals, H_2O_2, singlet oxygen and lipid peroxyl radicals; and reactive nitrogen species (RNS), like nitric oxide and peroxinitrite anion. Moreover, significant protective effects of QUER against oxidative stress in both cell and animal-based models have been reported, being able to activate cellular defense systems such as superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and reduced glutathione (GSH) [4].

Mycotoxins are low-molecular-weight molecules produced as secondary metabolites by filamentous fungi, mainly including *Aspergillus, Penicillium, Fusarium, Alternaria,* and *Claviceps* genera [10]. Mycotoxins represent a major issue in food safety among natural food contaminants, and currently they suppose critical challenges in food toxicology. They are related to human chronic effects like cancer induction, liver and kidney toxicity, and immunosuppression [11].

Based on the effects on human and animal health, aflatoxins (AFs), fumonisins (FBs), zearalenone (ZEN), ochratoxin (OTA), patulin (PAT) and thichothecenes such as deoxynivalenol (DON), nivalenol (NIV), T-2 toxin (T-

2), and HT-2 toxin (HT-2) are recognized as the most dangerous mycotoxins in food and feed comodities [12]. Out of the several hundreds of identified mycotoxins, only a small proportion has been investigated in animals and cell lines about their toxicity. Many mycotoxins entry in the cell with unknown mechanisms triggering oxidative stress, which leads to high levels of ROS and causes different cellular disorders. Thus, mycotoxins and their metabolites may elicit adverse effects such as immunosuppression, lipid peroxidation (LPO), inhibition of translation, DNA damage, genome instability, changes of cell organization and morphology, and cell death -necrosis and/or apoptosis- causing several kinds of cytotoxicities either by similar pathways or specific activities; eg. ribotoxic stress response (T-2 and DON) and protein synthesis inhibition (OTA) [13].

The cytoprotective effects of QUER against mycotoxin toxicity have been widely investigated since the 80s. The aim of this mini-review was to analyze the studies of QUER as potential protective compound to mycotoxin-induced cytotoxicity, focused on *in vitro* experimental models.

1.1 QUER among other bio-antioxidants

Based on the structure-activity relationships for antioxidant effects, QUER appears to be one of the most active flavonoids [14]. Some *in vitro* studies were exclusively focused on QUER as single compound of interest for protecting against mycotoxins induced-cytotoxicity, whereas other authors also investigated the effect of several bio-antioxidants including QUER metabolites such as QUER-3-β-d-glucoside (QUER-3βd-G) and isorhamnetin (ISH); other flavonols such as rutin (RUT), myricetin (MYR), chrysin (CHR), catechin (CAT), epicatechin (EC), and epigallocatechin gallate (EGCG); and

isoflavones such as genistein (GEN), biochanin A (bioA), daidzein (DAI), and equol. Other studied compounds in combination with mycotoxins were resveratrol (RES), t-pterostilbene (PTE), α-tocopherol (α-TOC), α-tocopherolphosphat (α-TOCP), gallic acid (GA), crocin (CRO), and rosmarinic acid (ROSAC). All classified studies reported below included QUER either alone or in combination with other bio-antioxidant compounds. QUER has been the most extensively studied dietary polyphenol in the last decades probably because it is widely available and easy to extract, isolate and detect [15]. Moreover, among several phenolic phytochemicals QUER was reported to hold one of highest antioxidant activities in the Trolox equivalent antioxidative activity test, only superated by EGCG, and epicatechin gallate (ECG) [4]. Table 1 shows the studied bio-antioxidants for their protective effect against mycotoxin-induced cytotoxicity, their chemical classification, as well as, the studied mycotoxins.

Regarding the addition of QUER -or other bio-antioxidants- to the cell cultures, pre-treatment (24h) or simultaneous treatment of antioxidant agent at non-cytotoxic concentrations and mycotoxins were usually reported. QUER tested doses ranged from 1 up to 100 μM, reporting in most cases effectiveness at contentrations of 10 μM [5,16-18]. Different doses of mycotoxins -normally based on their calculated IC50- were used to evaluate the protective effect of the studied antioxidant compounds, either after or simultaneously to the antioxidant treatment. The mycotoxins doses generally reported ranged between 3 and 50 μM, eventually rising up to 400 μM (α-ZOL and β-ZOL) [7]. Mycotoxin exposure time is generally 24h, extending in some cases the time of exposure to 48h, or even 72h [18].

Table 1. Studied bio-antioxidants toghether with QUER regarding their protective effect against mycotoxins cytotoxicity: antioxidant name, tested mycotoxin(s) and reference.

Bio-antioxidant (+QUER)	Tested MTs	Reference
Resveratrol	OTA	[19]
t-pterostilbene	ENs	[2, 12]
α-tocopherol	OTA	[20, 21]
α-tocopherolphosphat	OTA	[21]
Gallic Acid	OTA	[19]
Crocin	ZEN, PAR	[21, 22]
Rosmarinic Acid	OTA	[21]
Flavonoids		
Quercetin-3-β-d-glucoside	ENs	[2]
Isorhamnetin	AFB1	[9]
rutin/rutoside	ENs	[2]
Myricetin	ENs	[2]
Chrysin	OTA	[19]
Catechin	OTA	[19, 20,21]
Epigallocatechin Gallate	OTA	[19, 20,21]
Epicatechin	OTA	[20]
Genistein	OTA	[19,20]
Biochanin A	OTA	[19]
Daidzein	OTA	[20]
Equol	OTA	[20]

2. QUER effects on *in vitro* studies

The contradictory results about the ability of QUER to protect mammalian cells were crucial to hold the interest in its research. In this way, several *in vitro* cellular models have been selected to investigate the protective role of QUER for counteract the cytotoxic effects exerted by mycotoxins. The most representative cell lines were human hepatocellular carcinoma (HepG2), human colorectal carcinoma (HCT116), human colorectal

adenocarcinoma (Caco-2), embryonic kidney (HEK293), human peripheral blood mononuclear (hPMBC), chinese hamster ovary (CHO-K1) and African green monkey kidney (Vero) cells.

Among the studied mycotoxins for these purpose OTA was the most reported one followed by ZEN and its metabolites α-ZOL and β -ZOL. Other studied mycotoxins in combination with QUER were alternariol (AOH) and alternariol monomethyl ether (AME), PAT, AFB-1, T-2, DON, enniatins (ENs) and beauvericin (BEA).

2.1 Mycotoxins from *Penicillum sp*.

2.1.1 Ochratoxin A

The effect of polyphenols on OTA absorption was investigated across the Caco-2 cells as an *in vitro* model of the human intestinal barrier, reporting very significant increase in both OTA absorption and cellular accumulation upon co-incubation with QUER and other antioxidants (CHR, GEN, BioA, RES), at concentrations that should be encountered in the gastrointestinal tract. Moreover, an increase in the OTA transport was observed maybe due to OTA efflux impairment by competitive inhibition of MRP-2 efflux pump. The authors suggested a greater bioavailability of OTA in the bloodstream with their possible associated adverse effects [19].

The same year, the effect of α-TOC and different polyphenols including QUER, CAT, DAI, EC, EGCG, GEN at concentrations ranging 50-100 μM, was assessed on OTA-induced cytotoxicity in HepG2 liver cells. Their results did not show counteract effect for OTA-induced cytotoxicity indicating that the tested polyphenols did not alter the cytotoxic effect produced by OTA in HepG2 cells [20].

Accordingly, the effects of different dietary antioxidants on the viability of OTA-exposed liver HepG2 cells were investigated, showing that pre-treatment for 24h with QUER, among other polyphenols, such as CAT and ROSAC, did not prevent OTA-induced toxicity in HepG2. Furthermore, QUER and a-TOC at concentration of 25-100 μM even amplified the cytototoxic effects of OTA. QUER acts as antioxidant and as pro-oxidant partly due to the formation of hydrogen peroxide, while α-TOC inhibits cell proliferation and modulates membrane fluidity, which might be the reason for a facilitated uptake of OTA into HepG2 cells, and thus, for the observed synergistic toxic effects [21].

In 2013, the cytoprotective effect of QUER (5-15 μM) on OTA-induced toxicity in Vero cells was evaluated, with specific reference to oxidative stress, intracellular calcium flux, and levels of protective antioxidant enzymes. The authors observed that QUER pre-treatment suppressed OTA-induced cytotoxicity, oxidative stress, and apoptosis by inhibiting the activation of caspases cascade that leads to DNA fragmentation. Moreover, QUER attenuated OTA-induced DNA damage and micronucleus formation exhibiting antigenotoxic potential [16]. In second place, the protective effect of QUER against OTA-induced cytotoxicity, genotoxicity, and inflammatory response in human peripheral blood mononuclear (hPBMC) cells was studied, demonstrating its protector effect. QUER (5-10 μM) offered cytoprotection by maintaining cell viability, combated OTA-induced oxidative stress by restoring antioxidant enzyme activity LPO and PCC levels, and reduced OTA-induced chromosomal aberration and DNA damage. Further, antiinflammatory effect of QUER was evident from significant reduction of proinflammatory cytokines release [17]. Finally, the sequence of some molecular mechanisms of OTA

toxicity in HepG2 cells and the cytoprotective effect of QUER on OTA-induced toxicity was investigated. Pre-treatment with QUER (5-15 μM) ameliorated the ROS generation, calcium release and NF-κB nuclear translocation and expression triggered by OTA. Moreover, QUER antiinflammatory property was exhibited as it downregulated COX-2. Antigenotoxic effect of QUER was evident in prevention of DNA damage and micronucleus formation, concluding that QUER modulated OTA-induced oxidative stress and redox-signaling, thus preventing OTA cytotoxicity in HepG2 cells [18].

2.1.2 Patulin

The influence of QUER on PAT-induced toxicity was evaluated in HEK293 and HCT116 cells combining non cytotoxic concentrations of QUER and PAT at the concentrations of 15 and 20 μM, respectively. Cell pretreatment with QUER lead to an efficient preventive effect against PAT induced mortality by a marked decrease in ROS generation and apoptosis, prevention of ER stress activation and LPO, and cells protection from DNA fragmentation by its ability to re-establish the loss of the mitochondrial membrane potential [5].

2.2 Mycotoxins from *Aspergillus sp*.

2.2.1 Aflatoxins

The effects of QUER and ISH on AFB1 -treated HepG2 cells were also investigated, showing that both antioxidants inhibited ROS production and cytotoxicity, and blocked the decrease of GSH levels, with stronger inhibitory ability on LPO of ISH. QUER suppressed the cell decreased viability induced by AFB1 in a dose dependent manner and reduced ROS to basal levels [9].

2.3 Mycotoxins from *Fusariums sp*.

2.3.1. Zearalenone and metabolites

Recently, ZEN and its metaboiltes α/β-ZOL were studied. The authors investigated the involvement of endoplasmic reticulum (ER) stress in ZEN mediated toxicity in human intestine (HCT116) and kidney (HEK293) cells and evaluated the effects of QUER and CRO. ZEN exposure induced ER stress associated with activation of the mitochondrial pathway of apoptosis; increase in ROS generation and LPO, loss of mitochondrial transmembrane potential, caspases activation, and DNA damages. However, the antioxidant properties of QUER and CRO (5 μM) helped to prevent the ER stress and reduce ZEN-induced apoptosis in both HCT116 and HEK293 cells [22]. The same authors investigated the underlying mechanism of toxicity triggered by the ZEN metabolites α-ZOL and β-ZOL, as well as, the QUER cytotoxic protecction in HCT116 cells. α-ZOL and β-ZOL generated ER stress and activated the unfolded protein response as evidenced by XBP1 mRNA splicing and up-regulation of GADD34, GRP78, ATF4 and CHOP. Apoptosis was triggered by ZEN metabolites-induced ER stress, and executed though a mitochondria-dependent pathway, characterized by a loss of mitochondrial transmembrane potential, a downstream generation of superoxide anion, and caspase-3 activation. Cellular deficiency of the pro-apoptotic proteins Bax and Bak protected cells against α/β-ZOL-induced toxicity. Besides, treatment with α-ZOL and β-ZOL combined with QUER significantly reduced damage induced by both mycotoxins in all tested markers, concluding that QUER exerted protective effect against ZEN metabolites cellular toxicity in HCT116 cells [7].

2.3.2 Trichothecenes; Deoxynivalenol and T-2 toxin

QUER and PTE showed protective effects against DON and T-2 induced cytotoxicity in CHO-K1 cells with a general positive correlation between antioxidant concentration and protective cell effect [23].

2.3.3 Emerging mycotoxins: Enniatins and Beauvericin

The cytoprotective effect of QUER among other four polyphenols (QUER-3βd-G, RUT, MYR and T-PTE) was assessed in CHO-K1 cells simultaneously exposed at low levels of ENs (A, A1, B and B1) showing a wide variety of cytoprotective effects, depending on type of polyphenol, concentration in food commodity, simultaneous presence, and interaction between polyphenols and other contaminants. Lower doses of QUER (<100 μM) did not cause cytotoxicity and showed cytoprotective effect for EN A1 and EN B1 in CHO-K1 cells with viability increasement from 24 to 84 % [2]. Protective effects against BEA induced cytotoxicity in CHO-K1 cells was also reported by QUER and PTE treatment [23].

2.4 Alternaria mycotoxins; Alternariol and Alternariol monomethyl ether

In 2016, the cytoprotective effect of QUER exposed simultaneously with AOH, AME in Caco-2 cells was evaluated. The authors demonstrated that AOH and AME showed very low cytotoxicity in Caco-2 cells; and QUER preserved the effect, favoring that event in mycotoxins combination, which does not increase the cytotoxic effect [1]. Table 2 shows the studies of the protective effect of QUER against mycotoxins *in vitro.*

Table 2. Cytoprotective effect of QUER in cell lines exposed to mycotoxins: mycotoxin, cell line, mycotxin doses, tested antioxidants, QUER doses (effective concentration*), pre-treatment and exposure time, protective QUER effect, and reference.

Mycotoxin (MT)	Cell line	MT Doses (μM)	Antioxidants	QUER Conc.(μM)	(Pre-treatment) Exposure time	Cytoprotective effect of QUER	Reference
OTA	Vero	10-50	QUER	5, 10*, 15	(24 h) 24 h	Respect to cytotoxicity, oxidative stress, apoptosis and genotoxicity	[16]
OTA	hPMBC	10-50	QUER	5, 10*	(24 h) 24, 48 h	Respect to cell viability, oxidative stress, chromosomal aberration, DNA damage and antiinflammatory effect	[17]
OTA	HepG2	10-50	QUER	5, 10*, 15	(24 h) 24, 48, 72 h	Respect to ROS, calcium release, NF-κB induction/expression, genotoxicity, and antiinflammatory effect	[18]
OTA	HepG2	20	QUER, α-TOC, EGCG, CAT, EC, DAI, EQU, GEN	50	48 h	No	[20]
OTA	HepG2	0.25-50	QUER, α-TOC, α-TOCP, EGCG, CAT, ROSAC	25, 50, 100	24 h	No, increased cytotoxicity	[21]

OTA	Caco-2	0.75, 7.5, 10 nM	QUER, RES, GA, CHR, CAT, EGCG, GEN, BioA	50	24 h	No, increased bioavailability	[19]
α-ZOL, β-ZOL	HCT116	100-400	QUER	5*	24 h	Positive for ER stress and unfolded protein response activaiton	[7]
ZEN	HCT116 HEK293	25-200	QUER, CRO	5*	24 h	Positive for ER stress and apoptosis	[22]
AOH, AME							
PAT	HEK293 HCT116	15 20	QUER, CRO	1-10*	(2 h) 24 h	Respect to ROS, apoptosis, ER stress, LPO, and DNA fragmentation	[5]
ENs	CHO-K1	0.5-15	QUER, QUER-3βd-G, RUT, MYR, PTE	5, 25*, 50	24 h	Cell viability	[2]
BEA, DON, T-2	CHO-K1	0.03-2.6	QUER, PTE	100*	24 h	Cell viability	[23]
AFB-1	HepG2	10	QUER, ISH	10, 25*, 50	48 h	Resepct to ROS, GSH, and cell viability	[9]

To sum up this section, contradictory results have been shown for QUER cytoprotection tested in different cellular models. On the one hand, pre-treatment of 24 h with QUER (10 μM) was able to protect hPBMC, HepG2 and Vero cells from OTA-induced cytotoxicity (10-20 μM), demonstrating positive effects for cell viability, oxidative stress and ROS production, apoptosis, chromosomal aberrations, DNA damage and genotoxicity, calcium release, NF-κB induction/expression, and antiinflammatory effects [16-18]. However, non observed cytoprotective effect against OTA (20 μM) was observed by QUER treatment (50 mM) in HepG2 cells [20], even reporting negative effects with increased cytotoxicity or bioavailability in HepG2 and Caco-2 cells, respectively [21,24]. These effects could be explained by the interaction that QUER and OTA might have on the level of intestinal transporters after their *in vitro* co-incubation significantly increasing OTA absorption and cellular accumulation, which may result in a higher systemic availability of OTA and the possible adverse effects associated to this mycotoxin [25].

On the other hand, QUER pre-treatment (5 μM) demostrated positive cytoprotective effects against ZEN (25-200 μM) and its metabolites α-ZOL, β-ZOL (100-400 μM) for ER stress, unfolded protein response activaton, and apoptosis in HCT116 and HEK293 cells [7,22]. Pre-treatment (2 h) with QUER (1-10 μM) evidenced positive effects for ROS production and apoptosis, ER stress, LPO, and DNA fragmentation triggered by PAT (15-20 μM) after 24 h exposure in HEK293 and HCT116 cells [5]. Positive effects for ROS production, GSH levels, and viability was observed by QUER (25 μM) pretreatment against AFB1 (10 μM) induced-toxicity after 48 h expsoure in HepG2 cells [9]. QUER (3-100 μM) co-treatment demonstrated positive cytoprotective effects with

viability increasement in CHO-K1 cells simultaneously exposed to ENs (0.5-15 μM), or BEA, DON and T-2 (single compound; 0.03-2.6 μM) during 24 h [2,23].

Contrary to these findings, non observed cytoprotective effects of QUER (3.125-100 μM) against AOH and AME (3.125-100 μM) were reported when they were exposed simultaneously in Caco-2 cells after 24 and 48 h. Cells did not show any sign of cell proliferation, stimulation neither cytoprotection, maybe because QUER protective effects strongly depend on the presence, the concentration, and the interaction between other compounds [1].

3. QUER suplementation *in vivo*

QUER showed promising *in vitro* results that made researchers move up to *in vivo* experiments. The safety of QUER has been reviewed based on results of numerous genotoxicity and mutagenicity animal and human studies, a weight of evidence that supported the safety of QUER for food addition [24]. Recently, transcriptome analyses in liver and small intestine from QUER supplemented mice (~350 mg/kg bw/day for 12 weeks) were performed showing neither hepatotoxicity nor up-regulation of genotoxic related pathways by studing serum alanine and aspartate aminotransferases levels, microarray pathways analysis, and DNA damage related genes [25].

Lesser extent research has been made with regard to QUER protective effect against mycotoxins *in vivo* toxicity; however some experimental animals including mouse, rat, and rabbit have been used for this purpose. The biochemical mechanisms associated with the effects of QUER on AFB1-mediated liver damage in mice were investigated, showing that oral supplementation with QUER decreased serum lactate dehydrogenase levels,

increased hepatic GSH levels and SOD activity, and reduced LPO in both the liver and kidney. However, QUER (15, 30 or 45 mg/kg body weight; every 3 days for 45 days) did not show a significant reduction on serum levels of alkaline phosphate, alanine aminotransferase and aspartate aminotransferase that were increased in AFB1-treated mice. HPLC analysis revealed that QUER in plasma is mainly present as glucoronides and/or sulfates of QUER. It is suggested that QUER does not directly protect against AFB1-mediated liver damage *in vivo* but exerts a partial role in promoting antioxidative defense systems and inhibiting LPO [9].

The cellular events involved in citrinin (CIT) toxicity was evaluated in mouse skin specifically focused on CIT-induced ROS and its relationship with DNA damage leading to apoptosis. Moreover, the potential prevention of CIT-induced dermal toxicity by topical treatment with several bio-antioxidants including QUER, butylated hydroxyanisole and α-TOC was assessed. Single topical application caused significant enhancement of ROS, cell cycle arrest at the G0/G1 phase and G2/M phase along with the induction of apoptosis, expression of p53, p21/waf1; Bax/Bcl2 ratio and cytochome c release, activities of caspase-9 and 3, as well as increased poly(ADP-ribose) polymerase cleavage. However, topical treatment of with QUER (10 μmol/100 μl), butylated hydroxyanisole (55 μmol/100 μl), and α-TOC (40 μmol/100 μl), abolished CIT induced oxidative stress, cell cycle arrest, and apoptosis, demonstrating the direct involvement of ROS in CIT-induced toxicological manifestations in mouse skin, as well as the protector effect of these compounds [26].

The effect of QUER chronic application and a single dose was compared against T-2 toxin on cell proliferation and apoptosis using rabbit

ovary as experimental model. Cell proliferation was decreased on rabbit ovarian cells after a single dose of T-2 toxin (0.08 mg/kg bw) however, chronic exposure to QUER (1 mg/kg bw; 90 days, 3 times per week) resulted in increased cell proliferation and reduced cell apoptosis, indicating the potential of QUER to attenuate T-2 toxin-induced proliferation arrest [27].

Regarding the binding site, it is well known that natural flavonoids can also bind to human serum albumin (HSA) at the same binding site as OTA does (site I, subdomain IIA). It has been suggested that reducing the bound fraction of OTA speeds up its elimination rate with a potential decrease in its toxicity. In this line, the competitive binding properties of several flavonoid aglycones, including QUER, was examined with a fluorescence polarization-based approach, showing that flavonols and flavones were able to remove OTA from HSA, while flavanones and isoflavonoids were ineffective competitors. Moreover, QUER and GAL were the most effective OTA competitors among the 13 studied flavonoid aglycones [28].

On the contrary, the influence of QUER on OTA toxicokinetics was evaluated in male rats concluding that QUER has no impact on its toxicokinetics *in vivo* based on the total excretion and tissue concentrations. No significant differences in fecal and urinary excretion of OTA and its metabolite OTα were found, as well as in OTA tissues concentration, when comparing the control and the QUER suplemented diet group (100 mg/kg for 6 days). Moreover, QUER supplementation had no effect ($P > 0.05$) on feed consumption, OTA-intake, water intake and body weight gain [15].

4. General considerations

Since mycotoxins are frequently found in food, a thorough knowledge on effective measures to counter and/or reduce their toxicity is an important aspect to be considered in food safety. Food commodities containing polyphenols could contribute to diminish the toxicological risk to humans that mycotoxins can induce. In this sense, dietary intake of antioxidants is a plausible and effective way to augment and fortify endogenous defence systems, since many of them can act as free radical scavengers resulting in cytoprotection. Many studies on the antioxidant activity of polyphenols have to be developed to understand the relationship between the structure of polyphenols and their radical-scavenging capability. The properties of QUER make it suitable to study cytoprotection against mycotoxin exposure, both *in vitro* and *in vivo*. Due to the specific chemical structure of QUER, it counteracts oxidative stress generated as a result of ROS, which contributes to the genesis of atherosclerosis, diabetes, ischemic heartdisease, heart failure, and hypertension [8]. Thus, consuming food rich in QUER could be considered as a great strategy to prevent mycotoxin induced toxicity [5].

However, to evidence beneficial effects on health throught food antioxidants intake from fruit, vegetables and beverages, it is mandatory that plasma concentrations of these compouds are sufficient to have a biological effect in the real situation. It was suggested that plasma concentrations of flavonoids can be reached to the levels that exert a biological effect on health. Due to the flavonoids metabolism performed by liver enzymes and intestinal microflora, free flavonoids from a normal diet do not persist in general circulation after ingestion [29]. These compounds in blood and tissues are mostly present at a conjugate form, and it is unclear whether the flavonoids

conjugates are the metabolites responsible for the beneficial effects of flavonoids.

5. Conclusions

QUER has demonstrated to be a powerful bioactive antioxidant compound mainly based on its ability to counteract oxidative stress by its free radical scavenging properties. There has been a growing interest in the possible health benefits of QUER. For instance, QUER seemed to protect against OTA cytotoxicity in liver (HepG2), blood (hPMBC), and Vero cell lines, but non protective effects -even pro-oxidant - were also shown for the same mycotoxin in HepG2 and Caco-2 cells.

In conclusion, a general protective activity was mostly reported for several mycotoxins such as PAT, AFB1, DON, T-2, ENs and BEA, pointing to QUER as a dietary antioxidant, which could reduce the effects of mycotoxins when are combined in food and feed. Nevertheless, more studies about QUER and its bioactive metabolites mechanism of action, as well as its bioavailability and bioaccessibility, are required.

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3.7 *In vitro* Study of Beauvericin and Enniatin B Effects on Human Lymphoblastoid Jurkat T-cell Model

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In vitro Study of Beauvericin and Enniatin B Effects on Human Lymphoblastoid Jurkat T-cell Model

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Abstract

Several mycotoxins exert their effect at immunological system. This is a fact since some of them have been classified as immunotoxic. Jurkat T-cells were used to determine mechanisms of inducing cytotoxicity with Beauvericin (BEA) and Enniatin B (ENN B). Both are no legislated mycotoxins compounds with increasing presence in feed and food. Concentration range studied was from 1 to 15 μM and exposure times were 24, 48 and 72h. BEA mycotoxin affected cell viability by increasing the percentage of apoptotic/necrotic cells in a highly manner than ENN B. IC_{50} values ranged from 3 to 7.5 μM for BEA while ENN B at 15 μM showed a decrease in viability between 21% and 29%. The percentage of apoptotic / necrotic cells increased for BEA more than for ENN B. Caspase-3&7 activation profile revealed different results depending on the mycotoxin tested, although both mycotoxins increased the number of cells activated by caspase-3&7. No differences in ROS production between treated-cells and post-exposure control for any of the mycotoxins studied were observed. Arrest in S phase for both mycotoxins was obtained. For DNA damage, BEA increased the DNA% in the tail by 18% and 20% for 3 and 5 μM, respectively with respect to the control, whereas ENN B for any of the concentrations showed significant changes. In summary, BEA mediates cytotoxicity through mitochondrial alterations involvement; while ENN B only at high concentrations and time assayed; BEA, overpass a no-return point of recovery and cell cycle is highly affected at doses and times assayed and apoptotic and apoptotic/necrotic cells increase; while for ENN B these effects were not such evident since according to all assays it seems to recover Jurkat T-cells. The results suggest that BEA and ENN B may be implicated in different toxic responses in Jurkat T-cells.

Keywords: Mycotoxins, flow cytometry, beauvericin, enniatin B, Jurkat-T cells, oxidative stress, DNA damage, cell cycle, caspase-3&7

1. Introduction

Beauvericin (BEA) and enniatin B (ENN B) are secondary metabolites synthesized by various toxigenic fungi, including several *Fusarium* species. Both have chemical structure of cyclic hexadepsipeptides and are able to grow in maize, wheat, rice and other commodities (Juan et al., 2016; Covarelli et al., 2015). The observed levels of contamination in food chain demonstrate that reduction of mycotoxin levels in food are needed to ensure food safety to humans and animals (EFSA, 2014; Juan et al., 2014).

Toxicological effects attributed to either BEA and ENN B have been studied in literature (Mallebrera et al., 2014, 2016; Properini et al., 2013a, 2013b; Juan-García et al., 2015), nevertheless a classification of toxic responses from the mechanistic point of view is difficult. BEA, as ionophore compound, increases ion permeability in biological membranes by forming a complex with essential cations (Ca^{2+}, Na^{+}, K^{+}) consequently affecting the ionic homeostasis (Kouri et al., 2003) and uncoupling the oxidative phosphorylation (Tonshin et al., 2010). Additionally, disruption in mitochondrial enzymatic activity and cell proliferation has been observed after BEA exposure, which can lead or be consequence of cell death (Mallebrera et al., 2016). ENN B has been described as phytotoxin, with a wide range of toxicological effects, such as antibacterial, antifungal, insecticidal, phytotoxic and cytotoxic properties (Kamyar et al., 2004). ENN B cytotoxicity involved early reactive oxygen species (ROS) generation that induced lipid peroxidation (LPO), apoptosis and necrosis via the mitochondrial pathway in Caco-2 cells (Prosperini et al., 2013a).

The number of *in vitro* studies reporting cytotoxic effects of ENNs on several cell types is high, as reviewed by Jestoi et al., (2008) (EFSA, 2014) and it has increased in the last years, but the lack of data of their effects at immunological system is a fact especially when several mycotoxins have been classified as immunotoxic (Milićević et al., 2010). Among that, literature are scare when searching for mycotoxins effect on Jurkat-T cells; in fact, only few mycotoxins have been tested on those cells as follows: aflatoxins AFB1 and AFM1 (Luongo et al., 2014), satratoxin H (Nielsen et al., 2009), deoxynivalenol (DON) (Katika et al., 2012; Taranu et al., 2010) and nivalenol (NIV) (Taranu et al., 2010), fumonisin B1 (FB1), alpha-zearalenol (alpha-ZEA) (Luongo et al., 2008, 2006), 4 beta-acetoxyscirpendiol (4-MAS), 15-acetoxyscirpenol (15-MAS), 4,15-diacetoxyscirpenol (4,15-DAS), and 30-acetyldiacetoxyscirpenol (TAS) (Lee et al., 2006). To notice that none emergent mycotoxin, neither ennitins (ENNs) nor BEA, have been studied *in vitro* in human malignant immunologic cells lines.

Cytotoxicity produced by toxicants can involve early ROS generation; an excess of which can lead to oxidative stress status, oxidized macromolecular structures, including membrane lipids, proteins and DNA, and cause cell cycle arrest and cell apoptosis (Ferrer et al., 2009). In the execution of apoptosis activation and cleavage of caspase-3 plays a central role (Jeruc et al., 2006). Caspases, also known as proteolytic enzymes, participate in a series of reactions that are triggered in response to pro-apoptotic signal and result in the cleavage of protein substrates, causing the disassembly of the cell. It has been demonstrated that mycotoxins induce mitochondrial-mediated apoptosis by release of cytochrome c and activation of caspase-3 and -9 in human colon

cancer cells (HCT116 and HT-29 DON treated) (Bensassi et al., 2012; Ma et al., 2012).

ENN B and BEA are highly present in food, at high levels and several toxic effects have been previously carried out in cell lines as revealed by recent publications above mentioned. Jurkat T-cells have been chosen in this study because are of human origin (human malignant cell line), easy to culture and its established reliability, as it keeps being used in a large variety of *in vitro* experiments involving immunotoxicology research (Schmeits et al., 2013).

This study intends to fill the gap of the potential modes of action (MOAs) of two mycotoxins, potentially classified as direct immunotoxicants (Shao et al., 2013), by assessing their effects on the human Jurkat-T cell *in vitro* through the following assays: viability, oxidative stress, cell cycle distribution, apoptosis-necrosis, caspase-3&7 activation and DNA damage.

2. Materials and methods

2.1. Reagents

The reagent grade chemicals and cell culture components used, Roswell Park Memorial Institute (RPMI) medium, penicillin, streptomycin, tetrazolium Bromide (MTT), phosphate buffer saline (PBS) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was from Cambrex Company (Belgium). Deionised water (resistivity <18 MΩ cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Methanol (MeOH) and glacial acetic acid were from VWR International (LLC, Pennsylvania, USA). Human recombinant annexin V-FITC conjugate, propidium iodide (PI), tetramethyl rhodamine methyl ester (TMRM), To-Pro®-3 iodide

and the protonophore carbonylcyanide p-trifluoromethoxyphenyl-hydrazone (FCCP) were from Invitrogen, (USA). CaspaTag™ Caspase -3/7 *In situ* assay kit, Fluorescein (FLICA Kit) from EMD Millipore Corporation (CA, USA) was used to detect active caspase.

BEA (purity: ≥97%, molecular weight: 654.9 g/mol) and ENN B (purity: ≥90%, molecular weight: 639.4 g/mol) were from Enzo Life Sciences, Switzerland. Stock solutions of BEA (1500 µM) and ENN B (1500 µM) were prepared in methanol and maintained at −20 °C.

2.2. Cell culture

Jurkat T-cells (a human T lymphoma cell line derived from an acute T cell leukaemia) were maintained in Roswell Park Memorial Institute 1640 (RPMI) medium supplemented with 100U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FCS inactivated. Incubation conditions were pH 7.4, 37 °C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma–Aldrich, St. Louis MO, USA).

2.3. Cell viability assay

For MTT assay, 3×10^4 cells/well were seeded in a 96-well/ plate. After 24, 48 and 72 h of exposure with concentrations of 1, 3, 5, 10 and 15 µM ENN B or BEA, each well received 100 µL of fresh medium containing 25 µL of MTT. After 4 h of incubation (37 °C in darkness), 50 µL of DMSO were added and after 30 min cytotoxicity was determined. The absorbance was measured at 570 nm using the ELISA plate reader Multiscan Ex (Thermo Scientific, MA, USA). Cell viability was expressed in percent relative to the solvent control (1%

MeOH). The concentrations resulting in 50% inhibition of live cells (IC_{50}) values were calculated from full dose–response curves. Three independent experiments were performed with eight replicates each.

2.4. Intracellular ROS generation

Early intracellular ROS production was monitored in Jurkat T-cells according to H_2-DCFDA probe. Dichlorofluorescein diacetate (DCFH-DA) is taken up by the cells, then deacetylated by intracellular esterases and the resulting product H_2-DCFDA is oxidized by ROS to the highly fluorescent dichlorofluorescein (DCF). Briefly, 3×10^4 cells/well were seeded in a 96-well black culture microplate loaded with 20 µM H_2-DCFDA in fresh medium for 20 min. Subsequently H_2-DCFDA was removed after centrifuging and exposed to 1.5, 3 and 5 µM of BEA or ENN B. Concentrations assayed here are correlated with those found in food. H_2O_2 was used as positive control, with cells being exposed to concentrations in the range of 0.2-5 mM. Increases in fluorescence were measured on a Wallace Victor², model 1420 multi-label counter (Perkin Elmer, Turku, Finland), at intervals up to 2h at excitation/emission wavelengths of 485/535 nm. Results are expressed as increase in fluorescence respect to control. Determinations were performed in two independent experiments with eight replicates each.

2.5. Cell cycle analysis

This assay was performed using Vindelov's PI staining solution previously described (Juan-García et al., 2013). PI is a DNA intercalating agent that only stains stochemietrally the DNA of cells in the late phases of cell death when the integrity of both, cellular and nuclear membranes is lost. Cell

proliferation and cell cycle distribution was performed using BD FACSCanto™ Flow Cytometer (Beckton-Dickinson, Italy) with FACSDiva software v 6.1.3 (BD Biosciences).

A total of 4.8×10^5 Jurkat T-cells/well were seeded in 6-well plates incubated with BEA or ENN B individually at 1.5, 3 and 5 μM as mentioned in section 2.4, for 24 and 48 h, for the staining procedure. Then, cells were incubated at 37 °C for 30 min with 860 μL of fresh medium containing 29 ng/mL of Vindelov's PI staining solution. Cell cycle analysis was carried out as described by Minervini et al. (2004), by rectangular fitting (CYLCHRED software, Beckton Dickinson, Milan, Italy) using 1024 channels which produced histograms with a single G0/G1 peak at channel 200 when DNA was diploid, an S-peak between channels 200 and 400 when DNA was replicating, a G2/M peak at channels 400 when DNA was tetraploid and a Sub-G0 peak (debris peak), between channels 100 and 200 when DNA was hypodiploid or damaged. The reduced coefficient of variation (CV) obtained in this study was the result of the high resolution reached by proper alignment. Cicloheximide (CLX), a known synthesis of proteins inhibitor that leads to cellular quiescence and cell death by apoptosis was used as positive control. Three independent experiments were performed for BEA and ENN B and at least 10,000 cells were analyzed for each sample.

2.6. Flow cytometry analysis of apoptosis/necrosis death pathway

The differential of population of apoptotic cells (early or late), necrosis and dead cells was identified by Annexin V-FITC/PI double staining (Vermes et al., 1995). Annexin V is a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidil serine (PS), and binds to cells exposing PS to the

extracellular side of the plasma membrane, whereas PI binds to the DNA of necrotic/dead cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Cells considered as viable are both Annexin V-FITC$^-$/PI$^-$; cells in early apoptosis (pro-apototic/apoptotic) cells are Annexin V-FITC$^+$/PI$^-$; cells in late apoptosis, that have completed the apoptotic and start the necrotic process (apoptotic/necrotic), are both annexin V-FITC$^+$/PI$^+$. For the annexin V-FITC assay, 4.8 × 10^5 cells/well were seeded in 6-well plates. After 24 and 48 h of exposure at 1.5, 3 and 5 μM of BEA and ENN B individually as in assay of section 2.4, cells were centrifuged, media containing the mycotoxins discarded and resuspended in 360 μL of Hepes Ca^{2+} buffer prepared as follows: 10 mM HEPES-NaOH (pH 7.4), 135 mM NaCl and 2.5 mM $CaCl_2$. After incubation at 4 °C for 30 min in the dark, 10,000 cells were acquired and analyzed by flow cytometry. Quadrant statistics were performed to determine viable cells, early apoptotic, late apoptotic and dead cells from the total population of cells. Jurkat T-cells treated with 1% MeOH in the media were considered as control. Three independent experiments were performed for both mycotoxins treatment.

2.7. Caspase-3 and 7 by FLICA kit

To detect active caspases, the methodology based on fluorochrome inhibitors of caspase (FLICA) was used. FLICA reagent had carboxyfluorescein-labeled fluoromethyl ketone peptide (FAM·DEVD-FMK) as inhibitor caspase-3&7 which produces a green fluorescence. When added to a population of cells, the FAM·DEVD-FMK probe enters each cell and covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labeled

reagent is retained within the cell, while any unbound reagent will diffuse out of the cell and is washed away. The green fluorescent signal is a direct measure of the amount of active caspase-3&7.

Cells (1.0×10^6 cells/mL) were incubated with above mentioned concentrations (section 2.4) of ENN B and BEA for 24 or 48 h at 37ºC prior to flow cytometry analysis. Briefly, FLICA reagent (FAM·DEVD-FMK) was added to cells prior to incubation for 1 h at 37ºC in 5% CO_2. After thorough washings, cells were twice centrifuged (238 g for 5 min at room temperature). Dual color staining with both PI and the FLICA reagent was prepared for flow cytometry analysis. Live cells, dead cells, caspase-negative, and caspase-positive cells can be detected by measuring fluorescein on the FL1 channel and red fluorescence (PI) on the FL2 channel as follows: caspase negative living cells (FL1-/PI-), necrotic membrane-compromised caspase-negative cells (FL-/PI+) and caspase-positive dead cells (FL1+/PI+). Assays were repeated on three separate experiments and at least 10,000 cells were analyzed for each sample.

2.8 Alkaline comet assay

The induction of DNA strand breaks was determined using the alkaline comet assay (pH > 13), according to the method described previously by Prosperini et al. (2013a). Briefly, 1 mL per well of Jurkat cells (6 x 10^5 cells/well) was added in 6 well-plates. Immediately, the following treatments were applied: negative control (C-) 1% methanol (MeOH), positive control (C+) etoposide 5 µM, BEA 1.5, 3 and 5 µM or ENN B 1.5, 3 and 5 µM (as ion section 2.4). Subsequently, the cells were suspended in pre-warmed LMA (0.5% PBS; 37ºC) and 80 mL of the suspension rapidly transferred to agarose precoated slides (1% PBS) and covered with a coverslip (24x36 mm). After

gelling for 10 min at 4ºC, the coverslip was gently removed and a second layer of 80 mL LMA was added and the gelling step was repeated. The slides were placed in a tank filled with lysis solution (2.5 M NaCl, 100 mM Na-EDTA, 10 mM Tris, 250 mM NaOH, 10% DMSO and 1% Triton X-100, freshly added) for 30 min at 4ºC. The lysis solution was then removed, washed with neutralization buffer (0.4 M Tris, pH 7.5) and incubated in fresh electrophoresis buffer (300 mM NaOH, 1 mM Na-EDTA) for 20 min at room temperature to allow the DNA to unwind. Electrophoresis was then carried out at room temperature in fresh electrophoresis buffer for 40 min (25 V, 300 mA). After electrophoresis, slides were gently washed once for 5 min in fresh neutralization buffer. After drying overnight at RT, slides were stained with 500 µL of PI (20 µg/mL) and covered with a coverslip. Slides were visualized under a fluorescence microscope (Leica DM), equipped with camera (Leica DFC450C) to capture imagines. A minimum of 50 individual cells were randomly selected from each slide and analyzed with the aid of the Automatic Comet Assay by TriTek CometScoreTM freeware (http://autocomet.com/index.php?id=cometscore).

The DNA damage in Jurkat cells was expressed as a percentage of the total DNA content in the tail based on the total fluorescence and single cells with damaged DNA appeared as comets.

2.9. Statistical analysis of data

Statistical analysis of data was carried out using the statistical software package IBM SPSS Statistic 22.0 (SPSS, Chicago, Il, USA). Data were expressed as mean ± SEM (standard error of the mean) of independent experiments and compared to control. The statistical analysis of the results was performed by

student's T-test for paired samples. $p \leq 0.05$ was considered statistically significant.

COMET assay data from three different experiments for each mycotoxin were analyzed for normality using Kolmogorov-Smirnov test with Lilliefors correction. As data were non-parametric, Hypothesis testing and Bonferroni post-hoc test were applied. The *p value* equal to or smaller than 0.05 it was considered significant; $p \leq 0.05$ (*), $p \leq 0.01$ (**), $p \leq 0.001$ (***) indicate a significant difference from the negative control.

3.-Results

3.1. Cell viability

In order to evaluate the viability of Jurkat T-cells exposed to ENN B and BEA the MTT assay was used. Cells exposed to BEA mycotoxin revealed a decrease in cell viability in a time and concentration-dependent manner. The results obtained in Figure 1 indicate that cells treated with BEA showed at 24h an IC_{50}= 7.5μM; at 48h an IC_{50}=5 μM and at 72h an IC_{50}=3 μM. For ENN B it was observed that at the highest concentration tested (15 μM) the greatest significant differences compared to control sample were found in the decrease of viability in the order of 21 %, 23 % and 29 % for 24, 48 and 72h respectively. At 10 μM a decrease in cell viability was observed at 48h (9%).

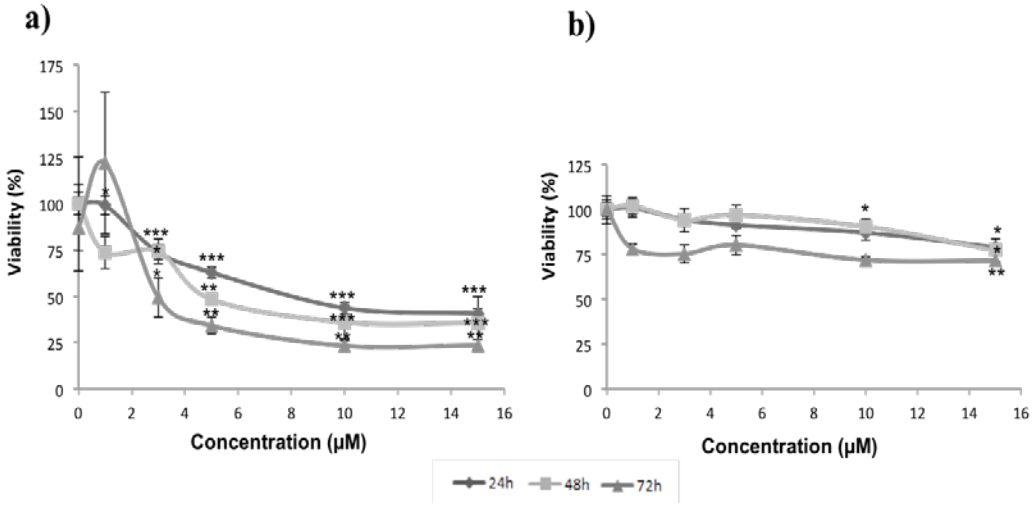

Figure 1. Cell viability (%) in Jurkat T-Cells exposed to BEA (a) and ENN B (b) by MTT assay after 24h, 48h and 72h. Data are expressed as mean ± SEM (n = 3). **p ≤ 0.05, **p ≤ 0.01 and ***p≤0.000* indicate significant differences from the control.

3.2. Intracellular ROS production

The ability of BEA and ENN B to produce ROS and consequent oxidative damage was evaluated. To determine the changes in the redox status in response to mycotoxins, Jurkat T-cells were exposed to different concentrations of both mycotoxins from 0 to 120 min and at 24 and 48 hours (Fig. 2). Results obtained demonstrated that Jurkat T-cells treated with BEA or ENN B did not show any significant variation in the production of oxidizing species depending on the time or concentrations of exposure when compared to the basal rate.

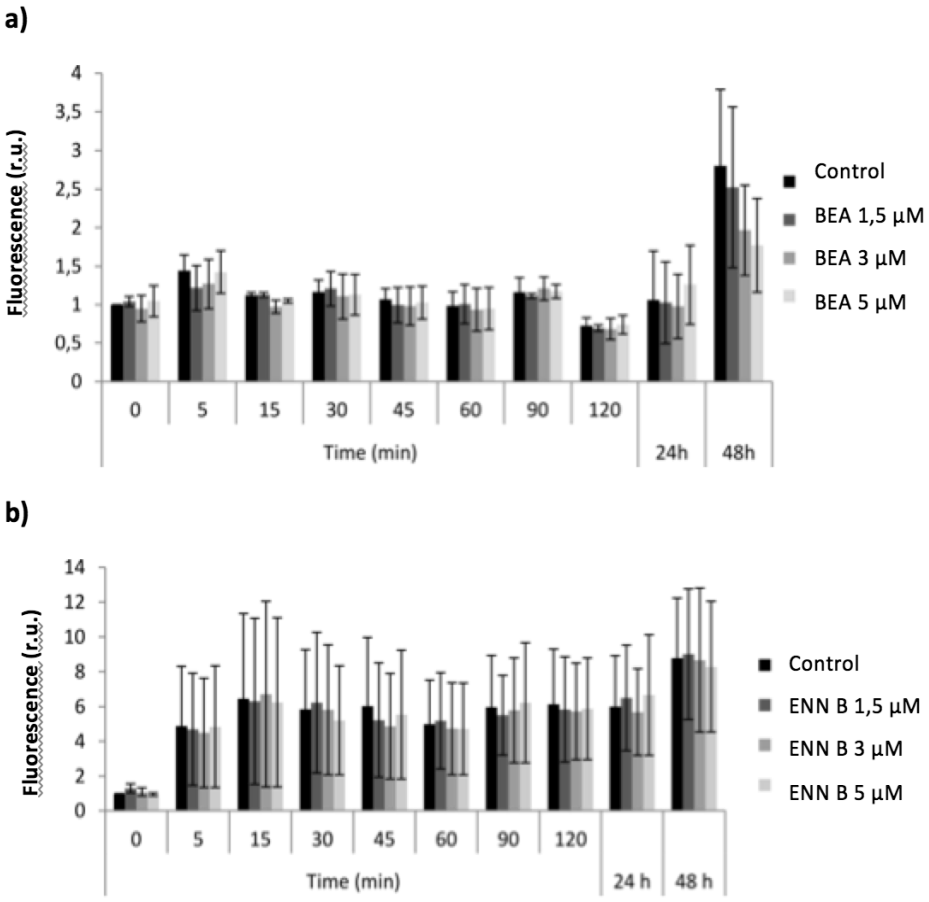

Figure 2. Time dependence of ROS-induced fluorescence in Jurkat T-cells exposed to BEA (a) and ENN B (b). Jurkat T-cells were loaded with H_2-DCFDA for 20 min in 96-well plates (30,000 cells per well) and then exposed to BEA or ENN B (1.5, 3 and 5 µM) or control (1% MeOH). Fluorescence of oxidized DCF was followed by emission at 535 nm and the excitation of 485 nm. Data are expressed as mean ± SEM (three inter-day experiments with 24 replicates each).

3.3. Cell cycle analysis

Analysis of DNA content by flow cytometry provides a cell cycle perturbation. Indeed, time-course analysis carried out on Jurkat T-cells showed cell cycle perturbation following exposure to ENN B and BEA toxin.

No modification was recorded in Jurkat T-cells following treatment with 1.5 µM BEA after incubation for 24 and 48 h (Figure 3.a). BEA exposure to 3 and 5 µM after 24h revealed a statistical significant decrease of G2/M phase ($p \leq 0.01$) accompanied by an increase of phase S percentage of cells ($p \leq 0.05$) compared to the control (Figure 3.a1). After 48 h of 3 µM BEA exposure, the percentage of Jurkat T-cells in the G0/G1 and G2/M phases were lower than the control ($p \leq 0.01$ and $p \leq 0.05$, respectively) while the S phase was significantly higher compared to control ($p \leq 0.05$); similar effect in G2/M phase was observed at 5 µM ($p \leq 0.05$) (Figure 3.a2).

Regarding ENN B treatment, a decrease in the percentage of number of cells in the G0/G1 and G2/M phases was observed after 24h exposure to 3 and 5 µM as compared to the control, with an increase in cells in S phase; however, this behave was inverted at 1.5 µM (Figure 3.b1). After 48 h of exposure similar effect was observed in Jurkat T-cells (Figure 3.b2).

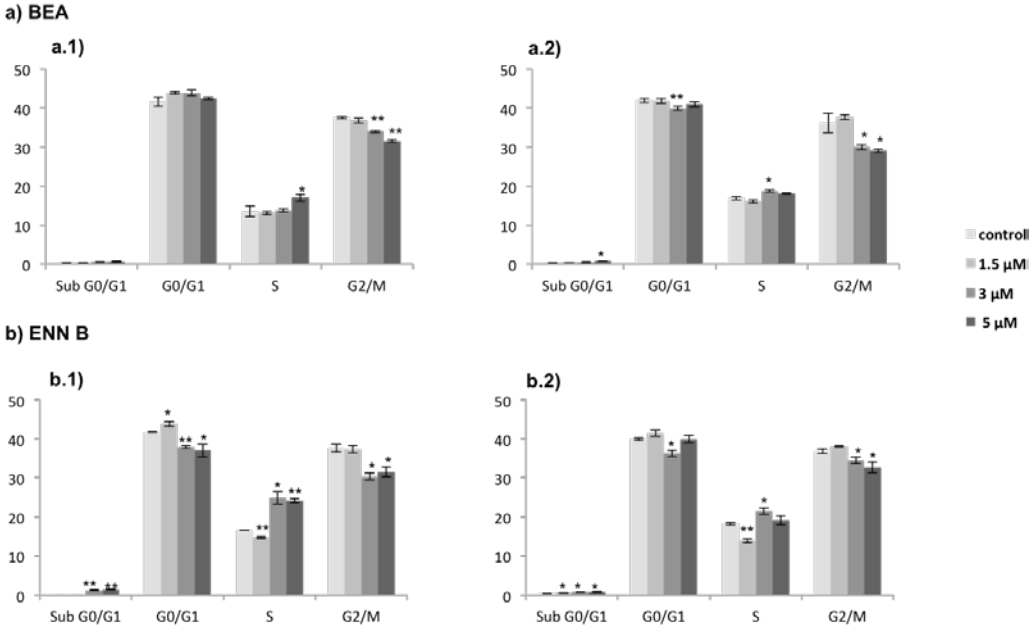

Figure 3. Cell cycle distribution in Jurkat T-cells exposed to 1.5, 3 and 5 µM BEA (a) and ENNB (b) after 24h (a.1 and b.1) and 48h (b.1 and b.2). Data are expressed as mean ± SEM (n=3). *$p \leq 0.05$ indicates significant differences from the control; *$p \leq 0.05$; **p ≤ 0.01; ***p ≤ 0.001, compared to control.

3.4. Apoptosis/necrosis death pathway assay

The early apoptotic, apoptotic/necrotic and necrotic cells induced by 1.5, 3 and 5 µM BEA and ENN B after 24 and 48 h of exposure in Jurkat T-cells is shown in Figure 4. Firstly, after 24 h of exposure, at 1.5 and 5 µM BEA only early apoptosis cells decreased statistically significant compared to control (Figure 4a). However, after 48 h of BEA exposure, all cell states were significantly increased from control at all concentrations except apoptotic/necrotic at 1.5 µM.

In relation to ENN B treatment, no significant differences were found at 24h for apoptotic/necrotic at any concentration tested; while early apoptotic cells decreased respect to the control ($p \leq 0.001$) at all concentrations tested. In opposition, at 48h of exposure all doses at all phases were significantly higher as compared to control (Figure 4b). Specifically, early apoptotic cells reached the highest percentage at the lowest concentration assayed (20 % at 1.5 μM, respect to the control) while at higher concentrations this population decreased (15 %, respect to the control); conversely apoptotic/necrotic cells reached the highest percentage at the highest concentration assayed 17 % respect to the control at 3 and 5 μM) while at lowest concentrations assayed this was the lowest (4 % respect to the control).

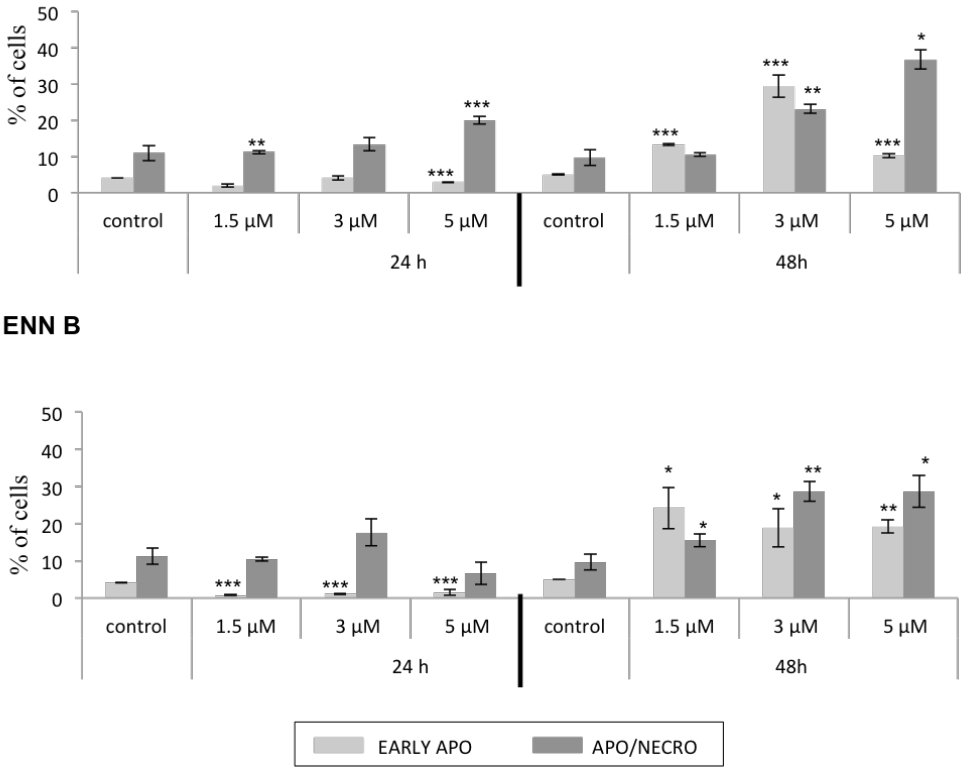

Figure 4. Changes in the percent distribution of Jurkat-T death cells after exposure to different concentrations (1.5 and 3 µM) of different mycotoxins (ENN B and BEA) and different incubation periods (24 and 48h). a) Percentage of early apoptotic Jurkat T cells treated with BEA at 1.5, 3 and 5 µM; b) percentage of apoptotic/necrotic for Jurkat T cells treated with ENN B at 1.5, 3 and 5 µM. Data are the mean ±SEM of three replicates. **p<0.05*, ***p≤0.01* and ****p≤0.001* compared to control.

3.5. FLICA kit (caspase-3&7)

The approach to detect active caspases *in situ* is based on the entry of fluorochrome inhibitor of caspase (FLICA) inside the cell. In other words, on the covalent link of FLICA inhibitor carboxyfluorescein-labeled DEVD-FMK to a

reactive cystine residue that resides on the large subunit of the activated caspase-3&7 with following inhibition of further enzymatic activity (Darzynkiewicz et al., 2002). By analyzing the biparametric data on Figure 5, it allowed distinguishing the caspase negative living cells (FL1-/PI-), the necrotic membrane-compromised caspase-negative cells (FL-/PI+), and the caspase-positive dead cells (FL1+/PI+), previously mentioned (section 2.5). Live cells decreased in a time-dependent manner for BEA respect to the control, ranging from 88% to 83% at 24h and from 49 to 31% at 48h (Figure 5a); while for ENN B this decrease was both time and dose-dependent manner ranging from 84% to 44% at 24h and from 91% to 21% at 48h (Figure 5b). Necrotic cells increased in a time and dose-dependent manner for BEA and ENN B respect to the control, ranging from 3% to 8% and from 10% to 15% for 24h and 48h, respectively for BEA and, from 4% to 23% and from 2% to 35% for 24h and 48h, respectively for ENN B. And finally, caspase positive dead cells for BEA increased in a dose-dependent manner from 10% to 6% and from 56% to 36% for 24 and 48h, respectively; while for ENN B increased in a dose-dependent manner from 8% to 32% and from 5% to 42% for 24 and 48h, respectively (Figure 5).

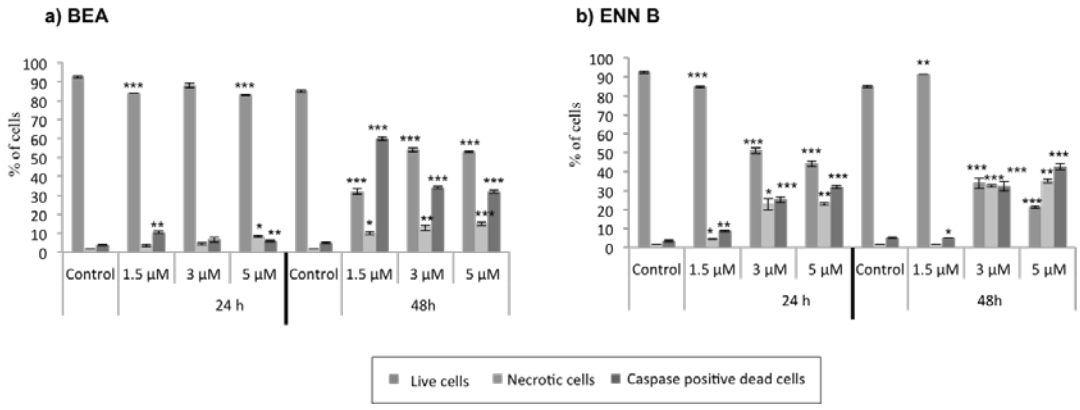

Figure 5. Caspase-3&7 activation on Jurkat T-cells induced (FAM,DEVD-FMK) after 1.5, 3 and 5 µM BEA (A) and ENN B (B) for 24 and 48 h. Data are expressed as mean ± SEM. (n=3)$* p < 0.05; ** p < 0.01; *** p < 0.001$, as compared to control at the same time point.

3.6. COMET assay

Results obtained indicated that after 24h of exposure the two highest concentrations tested using BEA (3 and 5 µM) produced a significant increase in the % of DNA in the tail compared to the negative control (Figure 6). The percentage of DNA in the tail significantly increased by 18% and 20% after 3 and 5 µM BEA exposure, respectively, compared to the negative control. On the contrary, none of the ENN B concentrations tested during 24h in Jurkat T-cells produced changes in the percentage of DNA in tail.

Bonferroni's multiple comparison indicates that the % DNA in tail was significantly higher ($p \leq 0.001$) after treatment of Jurkat T-cells with 3 and 5 µM AOH when compared to the MeOH treatment (Figure 6).

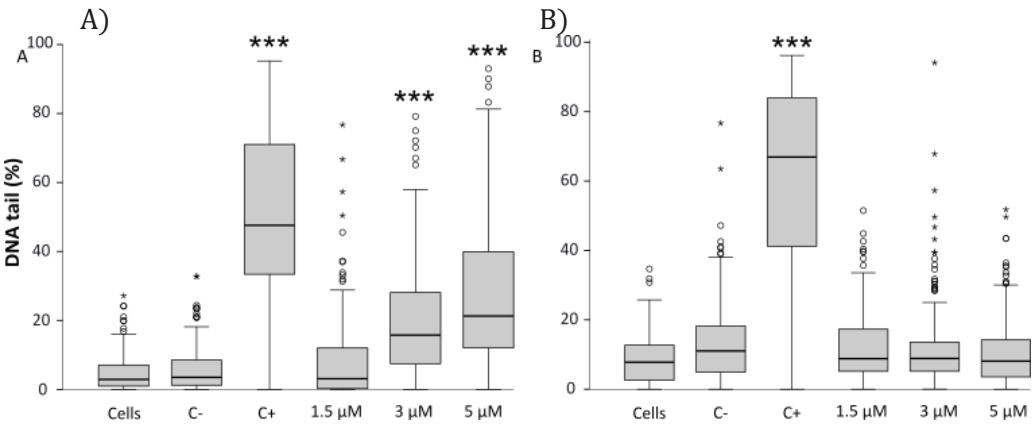

Figure 6. The percentage of DNA in the tail of Jurkat T-cells after 24h exposure to (A) BEA and (B) ENN B. (***) $p \leq 0.05$, significantly different from negative control.

4.-Discussion

In this study, a range of experiments has been used to discriminate different mechanisms of BEA or ENN B-mediated toxicity in Jurkat T-cell lines.

Cytotoxic effect of BEA and ENN B was measured by the reduction of formazan by mitochondrial dehydrogenases of living cells through the MTT assay. Results in this study are connected with those obtained by other authors, whose results varied depending on the periods of time, the cell line and the medium components. Accordingly, the IC_{50} of BEA in H4IIE hepatoma cells was 1 μM while in human colon carcinoma Caco-2 cells reached up to 20.6 ± 6.9 μM (Prosperini et al., 2013a; Waetjen et al., 2014). CHO-K1 cells BEA-treated performed by Ferrer et al., (2009) reached IC_{50} value of 12.08 ± 1.10 μM after 24 h of exposure while Kamyar et al., (2004) after 24, 48 and 72 h of BEA exposure obtained an IC_{50} of 10.7 ± 3.7, 2.5 ± 3.3 and 2.2 ± 3.3 μM, respectively. Cytotoxicity values obtained with BEA on Jurkat T-cells are closer to those obtained with CHO-K1 cells than Caco-2 or H4IIE cells; which indicate similar sensibility with those cells decreasing cellular proliferation. On the other hand, although no IC_{50} values were reached for ENN B at the concentrations tested on Jurkat T-cells, results are in accordance to those obtained on Caco-2 cells by Prosperini et al. (2013b) where IC_{50} values were >15 μM for 24h and 48h and also similar to those obtained for several cell lines as: HepG2, HT-29, H4IIE and CHO-K1 (Meca et al., 2011; Lu et al., 2013). Testing higher concentrations compromised solubility and to test a far realistic situation since these mycotoxins have not been found in food commodities at such higher concentrations (Juan et al., 2014, 2016). Although other authors have associated cytotoxicity with ROS production, this cannot be set in this study since ROS measurement was not statistically significant different respect

to the control at any time or dose assayed (Figure 2). So according to MTT assay, BEA mycotoxins might have higher mitochondrial involvement in Jurkat T-cells viability.

It has been postulated that neutral red (NR) method is the most sensitive method for calculating IC_{50} for ENNs on Caco-2 cells (Prosperini et al., 2013b) based on lysosomal damage. Plasmatic membrane of the lysosomes is attacked more easily as compared to the mitochondrial one as occurs with MTT assay and suggesting that ENNs might be related to lysosomal damage. However, these differences were not found for the MTT method when BEA was assayed on HepG2 cells (Meca et al., 2011).

Few studies of cytotoxicity effects on Jurkat-T cells by mycotoxins have been published. Literature reports that trichothecenes type A and type B have been studied by Nasri et al., (2006); acetoxyscirpenol (ASM) mycotoxins by Lee et al., (2006) and, DAS mycotoxin by Jun et al., (2007). However, stress oxidation studies through ROS measurement are widespread. ROS are mediators of intracellular signaling cascades of cell death and its production induce several changes at cellular level. Intracellular ROS production obtained by Mallebrera et al., (2015) on CHO-K1 cells exposed to 1 and 5 μM of BEA increased significantly in time and concentration dependent manner (1.3-folds of control). Moreover, Prosperini et al (2013a) evidenced significant production of ROS (1.4-fold higher than the control) immediately in Caco-2 cells after BEA exposure (1.5 and 3.0 μM); as well as for ENN B (3.0 μM, 2.6-fold higher than control) (Prosperini et al., 2013a). In those studies ROS production was associated as a cause or consequence of mitochondrial alterations. In our study, neither BEA nor ENN B on Jurkat T-cells is related to this (Figure 2)

suggesting that toxic mechanism depends on the cell type for BEA and ENN B mycotoxins.

Cell cycle arrest is a transient condition that may be induced by several factors and generally cells spontaneously revert from this conditions restoring their proliferation rate or becoming apoptotic (or necrotic) (Juan-García et al., 2013). Although ENNs are capable to disturb the normal progression of proliferating cells (Dornetshuber et al., 2007; Juan-García et al., 2013), in this study cytometric cell cycle analysis permitted to notice that in Jurkat T-cells BEA suffered strongest changes than ENN B (Figure 3); which agreed with results obtained in cell viability (Figure 1). While ENN B produced higher number of changes in cell cycle distribution than BEA, compared to the control, alteration produced by BEA interfere more dramatically in the cell viability as revealed by the obtained IC_{50} values (Figure 1 and Figure 3). A reason related to this could be the reach of a cell step-status of not recovering when Jurkat T-cells are exposed to BEA, associated to an overpassing no-return point and associated to its ionophoric activity linked to the cell homeostasis (a characteristic of hexadepsipeptidic mycotoxins) (Tonshin et al., 2010). Interestingly, S arrest was induced for both mycotoxins and a decrease in the cells percentage of G2/M stage at the highest doses had a stronger end effect for BEA than for ENNB (Figure 3). Conversely, in the literature it is described that BEA inhibits CHO-K1 and Caco-2 cell proliferation by arresting (increasing) cells in G2/M (Mallebrera et al., 2016; Prosperini et al., 2013b). Furthermore, ENNs (1.5–10 μM) have similar behavior to BEA in human epithelial carcinoma-derived (KB-3-1) cell line (Dornetshuber et al., 2007) and in Caco-2 cells (Prosperini et al., 2013b). Other studies interrupting the cell cycle in Jurkat T-cells reported that DAS stops the cell cycle in G1 and G2/M phases (Jun et al.,

2007); in J774A.1 murine macrophages AFB1-treated a significant increase of the S phase accompanied by a decrease in G0/G1 phase cell population (Bianco et al., 2012) while in MOLT-4 and IM-9 cells treated with T-2 toxin produced an increase of debris percentage in a time dependent manner (Minervini et al., 2005). All of them studied the interruption of cell cycle with death cell pathway of apoptosis, necrosis (or both). Considering that a disturbance of the normal progression of the cell cycle is also affected by unrepaired DNA damaged and that these cells can undergo apoptotic or necrotic. In this study, it was investigated the apoptotic and necrotic cell death pathway through flow cytometry and caspase-3&7 and DNA damage through COMET assay.

Apoptosis induction has been detected on Jurkat-T cells for ASM mycotoxins (Lee et al., 2006), DON (Katika et al., 2012) and DAS (Nasri et al., 2006). Conversely, necrosis induction in Jurkat-T cells has been detected for T-2 toxin (Nasri et al., 2006) and α-Zearalenol (Luongo et al., 2006). In this study, exposure of Jurkat T-cells to BEA or ENN B induced increases in early apoptotic or apoptotic/necrotic cells (Figure 4) but BEA increased apoptotic/necrotic cells population more severely than ENN B in a dose and time-dependent manner. Apoptosis and apoptosis/necrosis simultaneously can be caused as a consequence that under the same stimulus, features of both populations may coexist in the same cells. In an assay carried out in CHO-K1 cells, no time or dose dependent trend was found when studying BEA (Mallebrera et al., 2016); while Juan-García et al., (2013) tested ENN B in HepG2 cells and a time-dependent decrease in apoptotic/necrotic cells was observed and, on Caco-2 cells Prosperini et al. (2013a) obtained an increase in early apoptotic cells at low times assayed (24 and 48h) whereas at high time (72h) necrosis was observed. In those previous published reports, a step further for studying this

singularity was carried out by associating cell death pathway with ROS production and alterations in mitochondrial membrane potential. In this study due to the lack of ROS production statistically different from the control for any mycotoxin studied and the results of cell viability, it was studied the execution of apoptosis by measuring the activation of caspase-3&7, which play a central role in such mechanism.

Caspases are a family of cysteine proteases that normally exist as proenzymes and, are activated upon cleavage during apoptosis (Kumar, 2004). An active caspase consists of two large and two small subunits that form two heterodimers, which are associated in a tetramer. Caspases enzymes specifically recognize a 4 or 5 amino acid sequence on the target substrate, which necessarily includes an aspartic acid residue. This residue is the target for cleavage, which occurs at the carbonyl end of the aspartic acid residue. In this study, it was shown that Jurkat T-cells treated with BEA or ENN B underwent typical cascades of apoptotic cell death (Figures 4 and 5) by increasing caspase-3&7 activation. Comparing these results with literature it is revealed that sometimes, anti-proliferative effect is associated to caspase-3&7 activation; coinciding with other authors when a significant time-dose increase of caspase-3&7 activity on Jurkat-T cells has been obtained for α-ZEA (0-80 μM, 24h) (Luongo et al., 2006), NIV and DON (0.0625-8 μM) (Severino et al., 2006; Katika et al., 2015).

It is known that Ca^{+2} is involved in the activation of several caspases and subsequent DNA degradation and, that one of the major caspase-3&7 substrates is poly (ADP-ribose) polymerase (PARP) cleavage (Negase et al., 2001). In our study where FAM·DEVD-FMK was used as a reagent to detect apoptosis with caspase-3&7 measurement, BEA and ENN B revealed caspase-

3&7 activation (Figure 5) indicating also typical cascades of apoptotic cell death. The cause or consequence associated to this could be related to alteration in ionic homeostasis as ionophors and consequently the intracellular calcium which it is simultaneously related to the involvement of Ca^{+2} caspase-activation and subsequent DNA degradation (Nagase et al., 2001; Jow et al., 2004). Our results will complete the cell death pathway for BEA which is associated to an increase of caspase-3&7 activation (Jow et al., 2004, Negase et al., 2001); specifically with caspase positive dead cells population and correlated with MTT results in a dose and time-dependent manner (Figure 1 and 5). However, for ENN B this correlation is less evident and a specific cell population is not possible to be pointed, suggesting a possible intent of stabilization in cell proliferation and cell death during Jurkat T-cells treatment as well as in the ionophoric activity for the doses and times assayed.

Finally, effect on DNA through the Comet assay was carried out and enforced these findings. BEA genotoxicity has been tested in Caco-2 cells (Prosperini et al., 2013b), pK15 cell line and human leucocytes (whole blood culture) (Klarić et al., 2010). Using the alkaline comet assay, at 24h of exposure it showed similar genotoxicity in PK15 and human leucocytes to BEA than Jurkat T-cells but a 10 times lower concentration was used, 0.5 μM. On the contrary, Caco-2 cells only presented genotoxicity compared to control when exposed to 12 μM BEA during 24h.

ENN B has been already tested for genotoxicity using other methods (Ames test and micronucleous) but also Comet assay using another cell line (V79 cells) (Behm et al., 2009; Föllmann et al. 2009). None of these assays revealed a significant genotoxic potential of ENN B, a similar outcome to the data obtained using Jurkat T-cells. Moreover, there are *in vitro* studies which

suggest that the cytotoxic effect of ENN B is rather due to other mechanisms than genotoxicity (Behm et al., 2009; Gammelsrud et al., 2012).

In conclusion, these results demonstrate that BEA and ENN B generate several toxicological consequences in Jurkat T-cells as well as different mechanism-mediated toxicity. BEA mediates cytotoxicity through mitochondrial alterations involvement; while ENN B only at high concentrations and time assayed; BEA, overpass a no-return point of recovery and cell cycle is highly affected at doses and times assayed and apoptotic and apoptotic/necrotic cells increase; while for ENN B these effects were not such evident since according to all assays it seems to recover Jurkat T-cells. Caspase-3&7 activation and its activity profile were different depending on the mycotoxin tested, although both mycotoxins enhanced the number of caspase activated cells; and finally DNA damage reveals that BEA is involved in genotoxicity effects at high concentrations while this cannot be pointed for ENN B at concentrations tested.

Altogether, BEA and ENN B exercise cytotoxicity over Jurkat T-cells triggering different mechanisms. So that, further studies focused in highly specific in mechanism of action not investigated here, need to be further studied.

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3.8 *In Vivo* Toxicity Studies of *Fusarium* Mycotoxins in the Last Decade: A Review

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In Vivo Toxicity Studies of Fusarium Mycotoxins in the Last Decade: A Review

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Abstract

This review summarizes the information regarding the in vivo studies of *Fusarium* mycotoxins in the last decade. The most common studies are classified as subacute toxicity, subchronic toxicity, acute toxicity, toxicokinetic studies, and teratogenicity, in order of importance. The most used animals in *in vivo* studies are pigs, rats, chickens, and mice. Fumonisin B1, deoxynivalenol, zearalenone, nivalenol, and T-2 toxin are the most studied fusarotoxins. Studies with combinations of mycotoxins are also frequent, deoxynivalenol generally being one of them. The predominant route of administration is oral administered mostly in the form of naturally contaminated feed. Other administration routes also used are intraperitoneal, intravenous, and subcutaneous. *In vivo* research on *Fusarium* mycotoxins has increased since 2010 highlighting the need for such studies in the field of food and feed safety.

1. Introduction

1.1. *Fusarium* genera

The genus *Fusarium* is a large fungal form genus that is more than hundreds of years old and globally one of the most important genera of fungi. Its species, which invade agriculturally important grains, are probably the most prevalent toxin-producing fungi of the northern temperate regions, and they are commonly found in cereals grown in America, Europe, and Asia (Tiemann et al., 2006). Most members of the genus produce –under favorable environmental conditions– an array of secondary metabolites which vary widely in chemical form. A number of the secondary metabolites are important, as mycotoxins, that are toxic and/or carcinogenic to humans and

animals and may have a role in plant disease. These mycotoxins are commonly found in cereals, food, and feed, and in other animal products daily consumed. They should be regulated in commercial and international trade. Mycotoxins possess biological activities that have been shown in many different studies and may represent a problem to both human and animal health.

The diseases that *Fusarium* species cause, the toxins they produce, and the general social impact on agricultural communities are an ongoing problem (Summerell and Leslie, 2011). *Fusarium* mycotoxins are endowed with both acute and chronic toxic effects and have been shown to cause a broad variety of toxic effects in animals. The consequences of ingestion of these fungal compounds vary from acute overt diseases with high morbidity and death to chronic disease, decreased resistance to pathogens, and reduced animal productivity. However, the major problem associated with animal feed contaminated with mycotoxins is not acute disease episodes, but rather the ingestion of low levels of toxins, which may cause an array of metabolic physiologic and immunologic disturbances. Symptoms related to mycotoxicosis can occur at very low toxin concentrations, even below the detection limits for the current analysis methods, and clinical symptoms are in many cases not very pronounced (Kanora and Maes, 2009). In addition, as it is a common practice to use multiple grain sources in animal diets, the risk of exposure to several mycotoxins increases with diet complexity (Grenier and Applegate, 2013).

Knowledge of the effects of mycotoxins is expanding rapidly, mainly because of the development of novel analytical techniques that facilitate the study of these compounds (Kanora and Maes, 2009). Mycotoxin research into effects on intestinal functions has made substantial progress in recent years.

The intestinal epithelium is the major site for the effects of mycotoxin-contaminated material even at low levels of contamination. The intestinal tract is the first barrier against ingested antigens, including mycotoxins and pathogenic bacteria. Following ingestion of mycotoxin-contaminated food enterocytes may be exposed to high concentrations of toxins. A role of food associated mycotoxins in the induction or persistence of human chronic intestinal inflammatory diseases has also been suspected. Studies focusing on the influence of food-derived antigens on intestinal morphology, as an indicator of animal health, are common but there are few publications on the effects of chronic exposure to a mycotoxin co-contaminated diet (Loureiro-Bracarense et al., 2012). Studies on the effect of these compounds on the gastrointestinal tract are limited. Studying the occurrence of any given mycotoxin alone provides incomplete information about the risk associated with the respective feedstuff. Compound feed is particularly vulnerable to multiple contaminations as it typically contains a mixture of several raw materials. *Fusarium* mycotoxins in general are often found to occur together in contaminated cereals. In most cases, the resulting toxic effects will be additive combinations of the mycotoxins' individual toxicities but synergistic interactions have been observed (Streit et al., 2012).

Spontaneous outbreaks of *Fusarium* mycotoxicosis have been reported in Europe, Asia, Africa, New Zealand, and South America. Moreover, chronic intake of these mycotoxins is reported on a regular and more widespread basis due to their global occurrence (Cortinovis et al., 2013).

1.2. *Fusarium* mycotoxins

From the large variety of known mycotoxins, the major *Fusarium* mycotoxins are besides aflatoxins (AFs) the most prevalent and harmful mycotoxins to animal productivity, and responsible for extensive and recurring economic damage. These toxins inflict loss to farmers and reduce the value of contaminated feeds (Grenier and Applegate, 2013). *Fusarium* species produce three of the most important classes of mycotoxins with respect to animal health and production: fumonisins (FBs), zearalenone (ZEN), and trichothecenes (deoxynivalenol (DON), nivalenol (NIV), T-2, and HT-2 toxins), but *Fusarium* genera also produce emerging mycotoxins such as fusaproliferin (FUS), beauvericin (BEA), enniatins (ENNs), and moniliformin (MON), which are more recently discovered and less studied (Summerell and Leslie, 2011).

1.2.1. Trichothecenes

Trichothecenes are the main and the chemically most diverse chemical group of the three major classes of *Fusarium* mycotoxins (Summerell and Leslie, 2011). They represent a large family of chemically related toxins produced by fungi in taxonomically unrelated genera such as *Fusarium* *Myrothecium* and *Stachybotrys*, and present a potential threat to animal health throughout the world (Li et al., 2011). The broad family of trichothecenes is extremely prevalent and their molecular weights range between 200 and 500 Da. The trichothecenes family includes over 200 toxins with a sesquiterpenoid structure with or without a macrocyclic ester or an ester–ether bridge between C-4 and C-15. They contain a common 12,13-epoxytrichothecene group responsible for their cytotoxicity, and a 9,10 double

bond with various side chain substitutions. They have been classified into four groups (Types A, B, C, and D) based on the substitution pattern of the tricyclic 12,13 epoxytrichothec- 9-ene (EPT) core structure. Types A, B, and C can be differentiated based on the substitution at the C-8 position. The non-macrocyclic trichothecenes constitute two groups: Type A, that has a hydroxyl ester or non-oxygen substitution group at C-8, including T-2 toxin, HT-2 toxin, neosolaniol, and diacetoxyscirpenol, while the Type B group contains a keto (carbonyl) function at C-8 and includes fusarenon-X, nivalenol (NIV), and deoxynivalenol (DON), and its 3-acetyl and 15-acetyl derivatives. *Fusarium* Type B trichothecenes typically have a C-7 hydroxyl group but this structural feature is not present in other genera. The number and position of the hydroxyl and acetyl-ester groups can influence the relative toxicity within eukaryotic cells. Type C trichothecenes have a C-7/C-8 epxide (crotocin), and type D trichothecenes have an additional ring linking the C-4 and C-15 positions (roridin A verrucarin A satratoxin H) (McCormick et al. ,2011; Pinton and Oswald, 2014).

Trichothecenes are small amphipathic molecules that can move passively across cell membranes. They are easily absorbed via the integumentary and gastrointestinal systems allowing for a rapid effect of ingested trichothecenes on rapidly proliferating tissues (Pinton and Oswald, 2014). Trichothecenes are toxic to animals and its exposure has been linked to reproductive disorders in domestic animals (Cortinovis et al., 2013). Because of their effects on the immune system, the exposure of trichothecenes could predispose humans and animals to infectious disease, especially for sensitive human populations like young children, immuno-depressed people, and old people (Gouze et al., 2007).

Exposure to these toxins can cause feed refusal, immunological problems, vomiting skin, dermatitis, and hemorrhagic lesions. They are also phytotoxic and can cause chlorosis inhibition of root elongation and dwarfism, and act as a virulence factor in wheat head scab (McCormick et al., 2011). The adverse effects of trichothecenes include emesis, nausea, anorexia, growth retardation, neuroendocrine changes, and immunosuppression. In humans there is a body of evidence suggesting that trichothecenes cause acute illness and are frequently associated with outbreaks of gastroenteritis. At the molecular level trichothecenes display multiple inhibitory effects on the primary metabolism of eukaryotic cells including the inhibition of proteins, DNA, and RNA synthesis. This impairment leads to the alteration in cell proliferation in tissue with high rates of cell turnover such as intestinal epithelial cells. Thus, intestinal epithelial cells are especially sensitive to trichothecenes and their exposure to these toxins may induce toxicity (Alassane-Kpembi et al., 2013). Exposure to DON and other *Fusarium* mycotoxins generally exacerbates infections with parasites, bacteria, and viruses across a wide range of animal host species.Well-known examples include coccidiosis in poultry, salmonellosis in pigs and mice colibacillosis in pigs necrotic enteritis in poultry enteric septicemia of catfish, swine respiratory disease, aspergillosis in poultry and rabbits, reovirus infection in mice, and Porcine Reproductive and Respiratory Syndrome Virus infection in pigs. On the other hand, T-2 toxin has been shown to markedly decrease the colonization capacity of Salmonella in the pig intestine. Although the impact of the exposure of humans to *Fusarium* toxins on infectious diseases is less well known, extrapolation from animal models suggests possible exacerbation of,

for instance, colibacillosis and salmonellosis in humans, as well (Antonissen et al. 2014).

1.2.1.1. Deoxynivalenol

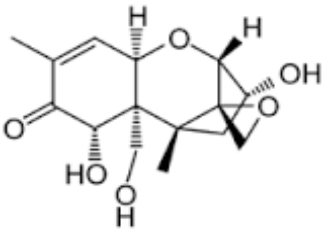

Figure 1. Molecular structure of DON

DON (Fig. 1) is considered as one of the most important trichothecenes found in all kinds of grains, such as wheat, rye, barley and oats. Due to its widespread occurrence and the potential for economic losses, DON is much more widely regulated than T-2 even though human or animal intoxications with either DON or NIV are much less likely to be fatal than those with T-2 (Summerell and Leslie, 2011). Although the adverse impacts of DON on immune function have been documented in experimental animals it is not completely known how DON modulates the immune responses (Awad et al., 2013). Toxicity of DON relies on its ability to cross the biological barriers and to affect the functions and viability of the cells forming such organ systems (Maresca, 2013). At the cellular level, DON interferes with the active site of peptidyl transferase on ribosomes and inhibits protein synthesis. Further, binding of DON to the ribosome in eukaryotic cells triggers a "ribotoxic stress response", which involves phosphorylation of the mitogen-activated protein kinases (MAPKs). MAPK activation modulates the expression of genes associated with the immune response, chemotaxis, inflammation and apoptosis (Grenier et al., 2011). The ingestion of DON has been associated with alterations of the intestinal, immune, endocrine and nervous systems, thus leading, in cases of acute exposure, to illnesses characterized by vomiting, anorexia, abdominal pain,

diarrhea, malnutrition, headache and dizziness (Maresca, 2013). The impact of DON on the immune system ranges from immunosuppression to immunostimulation, according to its concentration, duration and time of exposure. The No-Observed-Adverse-Effect Level (NOAEL) of DON in mice is lower than 45 μg/kg bw/day (Kouadio et al., 2013). As DON is one of the most prevalent and hazardous food-associated mycotoxins, a provisional maximum tolerable daily intake (PMTDI) of 1 μg/kg of body weight (bw) per day has been proposed by the Joint FAO/WHO Expert Committee on Food Additives (JECFA, 2011). The legislation sets a maximum level of 1.25 mg/kg of DON in cereals other than durum wheat, oats and corn; and 1.75 mg/kg of DON in durum wheat, oats and corn (except the one used for wet milling) (Prieto et al., 2005). Humans are significantly exposed to DON and its derivatives and this represents a risk to human health based on the presence of a low margin between the doses of DON affecting cell functions and the amounts of DON that may be consumed in relation to its PMTDI. The human risk concerns mainly the intestinal and immune systems and the brain; DON effects on the endocrine system are unlikely to be observed in humans exposed to DON at doses close to its PMTDI (Maresca, 2013). According to current research, DON should not be classified as a carcinogen to human beings (Ma and Guo, 2008).

1.2.1.2. Nivalenol

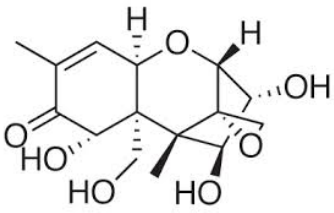

Figure 2. Molecular structure of NIV

NIV (Fig. 2) usually coexists with DON in nature. It has been described as causing feed refusal, decrease in weight gain, increase in serum alkaline phosphatase activity, elevation of serum IgA level, and it can alter both serum

biochemical parameters and hepatic drug metabolizing activities. DON and NIV share highly similar chemical structures and the only difference between them is a single oxygen atom at the 4 position in the trichothecene structure. As expected, they reportedly also share many toxicological properties, such as the inhibition of cell proliferation, induction of interleukin-8 secretion, and the involvement of stress-activated MAPKs and nuclear factor κB in the signal transduction pathways of toxicities. The difference between carbon monoxide and carbon dioxide is only a single oxygen atom as in the case of DON and NIV, nonetheless the toxicities of these carbon oxides differ totally. Although both toxins share highly similar chemical structures, there are evident differences in their toxicities that are crucial for synergism (Nagashima and Nakagawa, 2014). NIV was more potent than DON in human promyelocytic leukemia cell line HL60, human lymphoblastic leukemia cell line MOLT-4, and rat aortic myoblast cell line A-10. NIV exhibited almost equivalent potency than DON in human hepatoblastoma cell line HepG2. If both toxins exert their toxicities through the same mechanism, one should be more potent than the other, regardless of cell types (Nagashima and Nakagawa, 2014). There is a lack of data regarding the effects of low oral doses of NIV in animal models. Gouze et al. (2007) described the NOAEL of NIV in mice as 1.774 mg/kg bw, corresponding to an exposure to 5 mg/kg contaminated feed. Although very few studies describe the effects of chronic exposure of NIV at low doses, several countries have established regulations on trichothecenes where only low limits (0.5 to 2 mg/kg) of contamination in cereals are tolerated (Gouze et al., 2007).

1.2.1.3. T-2 and HT-2 toxins

T-2 toxin (Fig. 3) is the most common trichothecene mycotoxin belonging to type A and one of the most acutely toxic members of the trichothecene family. Although it is less frequently detected compared to the other trichothecenes, T-2 has received much attention because it is one of the most toxic mycotoxins present in wheat, rye, maize and soybeans (Seeboth et al., 2012). Over the years, there have been many reports from different regions of the world describing the association of T-2 with damage to agriculture and its toxic effects in animals. A recent report from the European Union (EU) stated that T-2 toxin was a common contaminant in cereal samples from EU member states, and therefore EU members were at risk of dietary exposure to *Fusarium* toxins (Prieto et al., 2005).

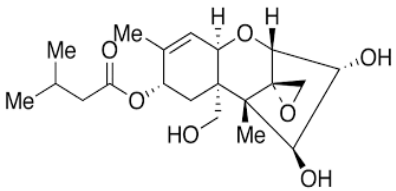

Figure 3. Molecular structure of T-2 toxin

The major effect of T-2 toxin is inhibition of protein synthesis, which leads to secondary disruption of DNA and RNA synthesis. T-2 toxin causes a large range of toxic effects in animals, such as weight loss, emesis, diarrhea, lethargy, inhibition of immunity, necrosis, damage of cartilaginous tissues, decreases in blood cell and leukocyte count, hemorrhage, reduction in plasma glucose and pathological changes in the liver and stomach. It is associated with an increased infection rate, alimentary toxic aleukia, DNA damage, induction of apoptosis and death. Acute toxicity of T-2 was studied in rats, mice, guinea pigs, and pigeons resulting from different routes of exposure, including intravenous (IV), intragastric, subcutaneous (SC), intraperitoneal (IP), and intratracheal. T-2 mycotoxicosis or 'moldy corn disease' in pigs is characterized by multiple hemorrhages on the serosa of the

liver, stomach and esophagus (at necropsy). T-2 also has an important impact on reproductive performance in pigs (Kanora and Maes, 2009). Exposure to T-2 toxin is associated with leukopenia and cell depletion in lymphoid organs and inhibition of erythropoiesis in bone marrow and the spleen. Furthermore, T-2 toxin reduces lymphocyte proliferative response and disturbs the maturation process of dendritic cells suggesting its immunosuppressant potency. Indeed, it was previously shown that exposure to T-2 suppresses the immune response to systemic infections by bacterial infection such as *Salmonella typhimurium*, *Listeria monocytogenes*, *Mycobacterium bovis*, *Babesia microti*. Respiratory immune defences are also compromised by T-2 exposure (Seeboth et al., 2012). Although the metabolites of T-2 toxin and their associated structures are well-known, the toxicity of these compounds to animals and humans is not clearly understood because of the limited scientific investigations describing their toxicity in animals. Also, some of the T-2 and HT-2 toxic effects observed in animals may affect humans. The mechanisms responsible for T-2 apoptosis and its metabolites are still unknown (Li et al., 2011).

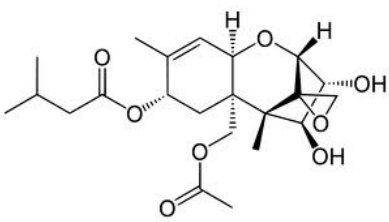

Figure 4. Molecular structure of HT-2 toxin

HT-2 toxin (Fig. 4) has been identified as the major metabolite of T-2 toxin both *in vivo* and *in vitro*, and the toxicity of this material is quite similar to that of the T-2 toxin (Yang et al., 2013b). HT-2 toxin (a deacetylated metabolite of T-2 toxin) is formed in microbial transformation via deacetylation reaction. This reaction is performed by several intestinal microorganisms in different animals. T-2 toxin is rapidly metabolized to the HT-2 toxin, and the

toxicity of T-2 may therefore be attributed in part to the efficiency of HT-2 formation (Yang et al., 2013b).

Recently, the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain established a group TDI of 100 ng/kg body weight for the sum of HT-2 and T-2 toxins. The report included occurrence results on HT-2 and T-2 toxins obtained from 22 European countries, which showed that the highest mean concentrations for the sum of these toxins were observed in grains and grain milling products, especially in oats and oat products (EFSA, 2011).

1.2.2. Zearalenone

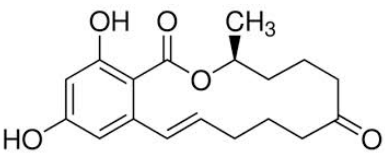

Figure 5. Molecular structure of ZEN

ZEN (Fig. 5), along with FBs and DON, is considered to be the most important exponent of the *Fusarium* mycotoxins with regard to animal health implications and associated economic loss (Borutovaa et al., 2012). Many of the strains that produce ZEN also produce one or more of the trichothecenes (Summerell and Leslie, 2011). There are multiple studies showing the co-occurrence of ZEN with other mycotoxins. The co-occurrence of ZEN with FBs and DON is very frequent and indicates that these mycotoxins might be involved in a wide range of synergistic and additive interactions. ZEN is considered, after DON, the second most common mycotoxin commonly found in unprocessed maize kernel samples but rarely detected in other cereal samples (Streit et al., 2012). Given that the same growing conditions that favor AF formation also favor FB production and that corn is always a prevalent host is an important issue,

which means that corn can be contaminated simultaneously with AFs, FBs, trichothecenes and ZEN (Borutovaa et al., 2012).

Clinically observed symptoms of ZEN exposure may not only be associated with the actual toxin concentration in a given feed batch but may also be induced or modulated as the result of previous exposure. This is of practical relevance, because the 'historical exposure' of an animal is usually unknown but should be recognized as a possible contributor to disease expression (Fink-Gremmels and Malekinejad, 2007). ZEN is biologically potent but hardly toxic (Tiemann and Dänicke, 2007). The major target of this mycotoxin is the reproductive system because of the structural similarity of ZEN and some of its metabolites with the estrogen hormones. ZEN competitively binds to estrogen receptors and causes alterations in the reproductive tract of laboratory mice, rats, guinea pigs, hamsters and rabbits and domestic animals and it is able to induce liver lesions with subsequent development of hepatocarcinoma and alterations of some enzymatic parameters of the hepatic function in rats, rabbits and gilts. ZEN has been shown to be immunotoxic and hepatotoxic and nephrotoxic and an enhancer of lipid peroxidation (Pistol et al., 2014).

Several *in vivo* studies report the toxicological effect of ZEN on the reproductive system, including alterations in the reproductive tract, uterus enlargement, decreased fertility, increased embryolethal resorptions, reduced litter size, and changes in the serum levels of progesterone and estradiol in laboratory animals (Koraichi et al., 2012). ZEN has been reported to produce a variety of adverse health effects in farm animals. It has estrogenic activity and this is reflected by alterations of fertility and reproduction in pigs, which are considered the most sensitive animal species (Fink-Gremmels and

Malekinejad, 2007; Tiemann and Dänicke, 2007). Swine can become effectively sterile if the level of ZEN in the feed is sufficiently high (Kanora and Maes, 2009). In humans, a few studies show a relationship between central precocious puberty or the incidence of endometrial adenocarcinomas and ZEN concentration in serum, tissue and foodstuffs (Koraichi et al., 2012). The results of the diverse interactions of ZEN are manifested by functional and morphological alterations of the reproductive organs (Duca et al., 2012). ZEN is considered to be a risk factor from both public health and agricultural perspectives (Pistol et al., 2014). ZEN levels are regulated in international trade, depending on the end product into which the grain being tested will be incorporated (Summerell and Leslie, 2011). European legislation establishes maximum permissible limits of 0.1, 0.2, 0.075, 0.2, 0.05 and 0.02 mg/kg ZEN in unprocessed cereals other than maize; unprocessed maize; cereals intended for direct human consumption; maize intended for direct human consumption; bread, pastries, biscuits, cereal snacks and breakfast cereals; processed cereal-based foods for infants and young children (Commission Regulation/EC, 2006).

1.2.3. Fumonisins

Fumonisins (Figs. 6-8) are a group of mycotoxins that are non-fluorescent, water-soluble and polar. At least 15 related fumonisin compounds have been identified so far (Ahangarkani et al., 2014). Fumonisins are divided into four categories: fumonisins A (A1, A2 and A3), fumonisins B (B1, B2 and B3), fumonisins C (C4, C3 and C1) and fumonisins P (P1, P2 and P3), but only groups B and A are presumed important (Ahangarkani et al., 2014). From the plethora of different fumonisin analogs, FB1, FB2 and FB3

forms account for the bulk of the fumonisins observed under field conditions (Summerell and Leslie, 2011). These toxins are diesters of propane-1, 2, 3-tricarboxylic acid (TCA) and have long-chain aminopolyol backbones. FB1 is the most significant fumonisin in terms of toxicity and occurrence. FB1 (2S-amino-12S, 16R-dimethyl-3S, 5R, 10R, 14S, 15R-pentahydroxyeicosane) is chemically described as polyhydroxy alkyl amine that is esterified on C14 and C15 with 2 molecules of tricarballylic acid and it contains 1024 different stereoisomers. Biological activity of FB1 depends on their stereochemistries and an unsubstituted primary amine group. As the alcohol part of polyhydric in fumonisins is similar to the amino alcohol complex in sphingosine (So), this toxin interferes with the biosynthesis of sphingolipids and So products (Ahangarkani et al., 2014).

Symptoms induced by FBs are unusually broad and include neural tube defects in newborns, brain lesions in horses, lung edema in swine and cancer in experimental animals. FBs are not mutagenic, but are cancer promoters (Summerell and Leslie, 2011). The European Union has legislated maximum permissible levels for FBs in maize and derived foods (Commission Regulation/EC, 2006). For example, they have established a number of 2 mg/kg FBs in unprocessed maize. In 2003 the International Agency for Research on Cancer (IARC) designated FB1 in Group 2B as 'possibly carcinogenic to humans' (IARC 1993) (Scott, 2012).

FB1 is the most abundant and the most toxic FB and an important contaminant of maize and maize based products, with exposures in some parts of the world exceeding the PMTDI of 2 μg/kg bw (Mueller et al., 2012). There is a record of only one outbreak of foodborne disease connected with

exposure to FB1. FB1 is a neurodegenerative mycotoxin and alters membrane functions (Domijan, 2012).

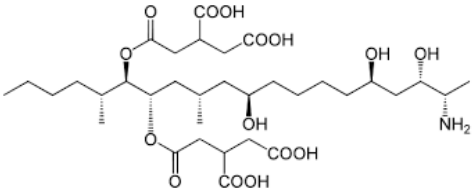

Figure 6: Molecular structure of FB1

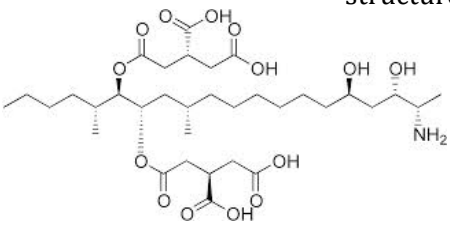

Figure 7: Molecular structure of FB2

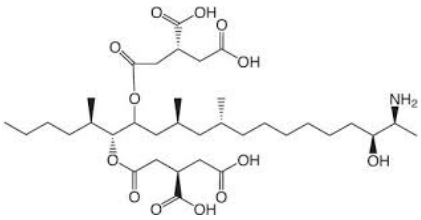

Figure 8: Molecular structure of FB3

FB1 interferes with myelin synthesis and causes leukoencephalomalacia (ELEM), liver necrosis and eventual death in horses. Several researchers showed that FBs are poisonous for pigs, chickens and other farm animals (Ahangarkani et al., 2014). Ingestion of FB1-contaminated feed causes pulmonary edema in pigs and is nephrotoxic and hepatotoxic in rats and mice (Scott, 2012). FBs are also involved in a handful of diseases such as liver cancer (in rats), hemorrhage (in rabbits' brain) and nephrotoxicity in some animals (Ahangarkani et al., 2014). FB1 is also carcinogenic, hepatotoxic, nephrotoxic and embryotoxic in laboratory animals (Scott, 2012). In rodent toxicity studies, the liver and kidney were identified as the main target organs of FB1 toxicity, whereby renal carcinogenicity of FB1 in rats is of greatest concern to human health. FBs are associated with apoptosis,

esophageal cancer and neural tube defects in humans (Ahangarkani et al., 2014; Scott, 2012).

Mechanisms of toxicity for fumonisins are complex. FB1 is structurally similar to the sphingolipids So and sphinganine (Sa). In high concentrations FB1 inhibits ceramide synthase (Sa and So N-acyltransferase) which is an important enzyme in the *de novo* pathway of sphingolipid biosynthesis. Ceramide synthase inhibition leads to reduced levels of ceramide and to the accumulation of Sa and So. These free sphingoid bases are proapoptotic, cytotoxic and growth inhibitors (Lallès et al., 2010). The resulting increases in serum and tissue concentrations of the sphingoid bases, particularly Sa and its 1-phosphate (Sa 1-P) metabolite, have been repeatedly correlated with FB toxicity in animals (Voss et al., 2013). Correlation between ceramide synthase inhibition and apoptosis, also mitosis and Sa and So accumulation in liver and kidney cells, is the first microscopic evidence of exposure to FBs. Recent data suggest that the accumulation of sphingoid can inhibit protein kinase Akt that is a signal factor for mitochondrial apoptosis. This enzyme is responsible for the phosphorylation of protein BCL-2 that is an anti-apoptosis protein. Inactivation of ceramide synthase increases free Sa and So, sphingosine 1-phosphate (So 1-P) and decreases complex sphingolipids such as ceramides, sphingomyelin, ganglioside and glycosphingolipids in tissues, blood and urine. FBs impair complex sphingolipids that are involved in synthesis and transport. Also inactivation of ceramide synthase impairs metabolism of arachidonic acid and reduces re-acylation of So. FBs block sphingolipid biosynthesis and lead to degeneration of the sphingolipid-rich tissues. Since sphingolipids are a key factor in various aspects of cell regulation, sphingolipid metabolism disorder may form a suitable groundwork for

toxicity. Sphingolipids are membrane lipids and by controlling some membrane proteins they play an important role in the regulation of cell membranes. These proteins are vital for maintaining the membrane structure, cell communications, cell interactions, cell morphology, extracellular interactions (such as cell–matrix and cell–cell adhesion) and cell differentiation, adjusting of growth factors, carcinogenicity and apoptosis. Also, sphingolipids act as the secondary messenger in signal transduction pathways (Ahangarkani et al., 2014).

1.2.4. Emerging mycotoxins

There is little information on emerging mycotoxins and this hinders the establishment of regulations and the development of a comprehensive monitoring plan capable of assessing risk and defining the limits of acceptability of these compounds. For some years the emerging mycotoxins have been considered less important because of the low probability of acute toxicity. However, they have a high prevalence in food products, sometimes even in high concentrations. Although this group represents only a small part of the total number of mycotoxins, they should be considered and also become part of further toxicology studies, allowing an intensive monitoring and control of risk in order to provide an adequate level of safety for consumers (Jestoi, 2008; Ruiz et al., 2011).

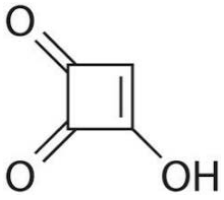

Figure 9. Molecular structure of MON

1.2.4.1. Moniliformin

The cytotoxicity of MON (Fig. 9) is usually quite low except in lymphocytes skeletomyocytes and cardiomyocytes. However, the effective

concentration levels observed in *in vitro* studies suggest that the toxic effect of MON is much more severe at cell level than *in vivo* (Jestoi, 2008). Data from *in vivo* toxicity of MON show significant differences between species, showing greater sensitivity in birds and mink. In general, greater toxicity is observed *in vivo* after IP or IV administration compared with oral administration. This suggests that there may be some system of deactivation or detoxification of MON. However, as with other emerging mycotoxins, toxicokinetics of MON is still unknown. The effects of MON obtained from tests *in vivo* include only studies with the purified toxin. The molecular mechanism of action of MON is still obscure. Because of the structural similarity to pyruvate, MON affects energy metabolism via the inhibition of mitochondrial pyruvate and a-ketoglutarate oxidation during the Krebs cycle. MON is also thought to inhibit other metabolic pathways involving pyruvate. It has been reported that MON inhibits the free radical scavenging enzymes glutathione peroxidase and reductase in rat heart homogenate, possibly having an additive impact on cardiotoxicity (Jonsson et al., 2013).

1.2.4.2. Beauvericin

BEA (Fig. 10) is a cyclic hexadepsipeptide which shows antimicrobial and anti-tumor activities. BEA has a strong insecticidal activity against a broad spectrum of insects, and a strong antibacterial activity against human, animal, and plant pathogenic bacteria, with no selectivity between Gram-positive and Gram-negative bacteria. The

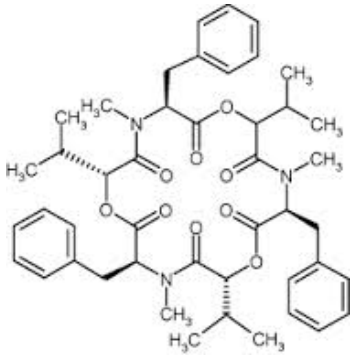

Figure 10. Molecular structure of BEA

antiviral activity of BEA has also been detected. Moreover, BEA shows cytotoxic, ionophoric, apoptotic, and immunosuppressive activity. It is the most potent specific inhibitor of cholesterol acyltransferase (Klari., et al. 2006; Wang and Xu, 2012).

BEA increases the permeability of biological membranes by forming a complex with essential cations (Ca2+ Na+ K+) and/or cation-selective channels in lipid membranes, which affect cell homeostasis, and the uncoupling of oxidative phosphorylation (Ruiz et al., 2011).

1.2.4.3. Enniatins

ENNs have antimicrobial, anthelmintic, insecticidal, antifungal, herbicide, phytotoxic, and cytotoxic potential activity. The main toxic action of ENNs is related to their ionophoric properties, through which ENNs are capable of promoting the formation of stable lipophilic complexes with essential cations mono and divalent (Ca2+ Na+ K+), and their transport through biological membranes, disrupting normal physiological concentrations. ENNs change the monovalent ion transport across membranes and disrupt the ionic selectivity of cell walls. This effect is particularly debilitating in mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation (Manyes et al., 2014). But recent studies have found that the mode of action of ENNs is much more complex acting as enzyme inhibitors. ENNs inhibit acyl coenzyme A: cholesterol acyl transferase (ACAT), and 30, 50-cyclo-nucleotide phosphodiesterase enzymes, causing mitochondrial dysfunction and the inhibition of multidrug resistance associated protein-1 (ABCG2) and P glycoprotein (ABCB1) efflux pumps (Juan et al., 2014; Prosperini et al., 2012; Ruiz et al., 2011)

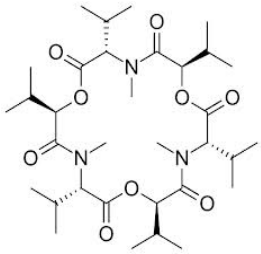

Figure 13. Molecular structure of ENN B

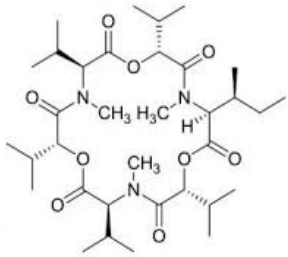

Figure 14. Molecular structure of ENN B1

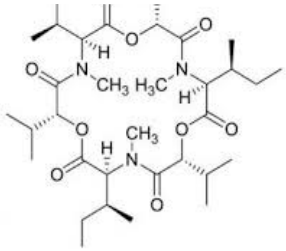

Figure 11. Molecular structure of ENN A

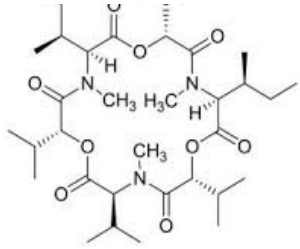

Figure 12. Molecular structure of ENN A1

1.2.4.4. Fusaproliferin

FUS (Fig. 15) is a sesterterpene toxic to *Artemia salina*, insect cells and human B lymphocytes and has teratogenic and pathogenic effects on chicken embryos. The LC50 of FUS to *Artemia salina* is 53 µmol/L (equivalent to 24 µg toxin/mL) and its cytotoxic concentration 50% to the lepidopteran *Spodoptera frugiperda* cell line SF-9 is 70 µmol/L and to the human nonneoplastic B-lymphocyte cell line IARC/LCL 171 is 55 µmol/L (Santini et al., 2012).

Its biosynthesis can be considered similar to that of retigeranic acid. This mycotoxin is produced by species of *Fusarium section Liseola* such as *Fusarium proliferatum* and *Fusarium subglutinans*. *Fusarium*-strains that

produce FUS usually produce also deacetyl–FUS in a ratio of 3:1; however, the deacetylated form shows only a limited toxicological activity compared to FUS (Marín et al., 2013). The chemical nature of FUS and the presence of methyl substituents do not allow the location of hydrophilic and hydrophobic parts on both sides of the plane determined by the two rings forming FUS (Jestoi, 2008; Santini et al., 2009).

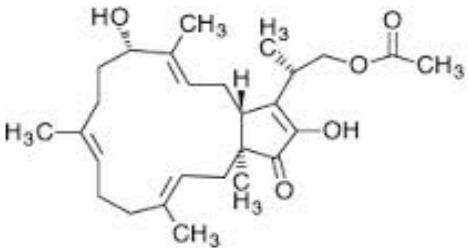

Figure 15. Molecular structure of FUS

1.3. *In vivo* studies

In vitro toxicity studies can provide useful information, but the complexity of living organisms requires research on animals to extrapolate reliable results to the human body. The *in vitro* preliminary data need to be contrasted with *in vivo* models, which represent the closest approach to the body on which you want to know the toxic effects.

Currently no generally accepted alternative methods are available for replacing repeated-dose *in vivo* testing. Although other alternative models have been developed there is no real substitute for the use of laboratory animals. They have not yet led to suitable *in vitro* replacements for the most animal usage in toxicologic studies. This failure to develop complete substitutes for intact living organisms used in research is likely due to the

complex interactions that exist at organ, cellular and subcellular levels. A suitable replacement for animals will have to reliably predict biological phenomena, including, being at least as good and consistent a model for risk assessment in humans as animals. Such systems have to be extensively validated and accepted by regulatory bodies as suitable substitutes (White et al., 2008). Complete replacement of animal usage in these areas represents an enormous scientific and technical challenge. Animal test results often represent the only means by which toxicity in humans can be effectively predicted (Prieto et al., 2005). The *in vivo* investigations provide information on the effects in whole animals, whereas cell specific answers result from *in vitro* investigations. For example, the summarized results reviewed by Tiemann et al. (2006) about the toxicological data on the effects of ZEN and DON on different parameters relating to reproductive and non-reproductive organs in female pigs indicated that *in vitro* studies with porcine cell cultures only partially agree with that of *in vivo* experiments. An explanation could be the fact that sometimes the cells *in vitro* react more sensitively than those *in vivo*, because a direct interaction of chemical substances with the plasma membrane exists which may alter the membrane structure and function.

Animal species differ in their susceptibility to mycotoxins. For example, as far as DON is concerned, the animal species can be ranked in the following order: pigs > mice > rats > poultry ≈ ruminants (Pinton and Oswald, 2014). The effects of DON and other trichothecenes type B have been investigated in different species, including man, laboratory animals, poultry and pigs. Among other species, pig react most sensitively to the exposure to trichothecenes and are particularly interesting as a target species for mycotoxins. Pigs are of particular concern for at least two reasons: (i) due to

the cereal-rich diet, pigs can be exposed to a high level of toxins; and (ii) the pig is one of the most sensitive species. In addition, swine are physiologically similar to humans and because of the similarities in the intestinal tract, pigs can be considered as a good model for humans (Seeboth et al., 2012; Tiemann et al., 2006). Moreover, swine are the target of several pathogenic agents responsible for many respiratory diseases such as Influenza virus, porcine reproductive and respiratory syndromes, or bacteria as Haemophilus parasuis. Then, mycotoxins, especially T-2 toxin, could play a determining role in lowering the immune response of pigs to these bacterial and viral infections (Pinton and Oswald, 2014; Seeboth et al., 2012). Pigs are one of the most sensitive species with regard to their response to DON-contaminated feed and thus they are the best model for studying the effects of DON intake on the human and animal intestine or immune system (Wu et al., 2013). Swine are more sensitive to DON than other species, in part because of differences in the metabolism of DON (Loureiro-Bracarense et al., 2012). In the pig, DON is rapidly and efficiently absorbed, most probably in the upper part of the small intestine, and is mainly excreted in the urine, with no accumulation in tissues (Waché et al., 2009). Moreover, pigs are generally considered to be the most sensitive animal species to ZEN and its metabolites (Pistol et al., 2014). In the case of FB1 male pigs have higher immune sensitivity as compared to female pigs (Lallès et al., 2010). In this species, several complementary approaches have been developed to investigate the effects of DON and other trichothecenes on the intestine. *In vivo* trials and cell culture models were used to study the long-term exposure to mycotoxins (Pinton and Oswald, 2014).

2. Data resource

2.1. Database

A broad search strategy was used to capture abstracts in the databases Web of Science (WOS) and PUBMED. Abstracts were screened to include only study types containing relevant information and the studies of interest were identified. The search was performed using the following keywords and topics to find specific literature: *Fusarium* mycotoxins *in vivo*, trichothecenes *in vivo*, deoxynivalenol *in vivo*, nivalenol *in vivo*, T-2 toxin *in vivo*, HT-2 toxin *in vivo*, zearalenone *in vivo*, fumonisins *in vivo*, enniatins *in vivo*, beauvericin *in vivo*, moniliformin *in vivo*, fusaproliferin *in vivo*.

The search was refined between the years 2003 and 2014 to focus on recent literature and frame the studies in the last decade. From the publications identified relevant data were found in 123 publications among which 92 were classified as *in vivo* studies about *Fusarium* mycotoxins. The information was double-checked to select bibliographies of relevant literature and data are summarized herein.

2.2. Criteria selection for *in vivo* studies

Articles obtained in the systematic search were evaluated to discard unnecessary, incomplete or irrelevant literature. Criteria to be met were fixed in order to obtain a set of unified and comparable global information. The criteria selection established were: (1) to have the description of the type, age, sex and number of the animals used in the test; (2) to have the description of the dose, exposure time and application routine; (3) to have the description of the diets and its analysis in case of suspected contamination; (4) to have the

negative or positive control; (5) to have the description of the methodology and animal procedures.

2.3. Criteria for classification of *in vivo* studies

A selected study that met the criteria was classified as a function of the trial duration and the main objective. The classification criteria along with a short description and examples of OECD guides are shown in Table 1.

Table 1. Study type duration objective and examples of OECD guidelines.

	Duration	Objective	OECD Guidelines
Acute Toxicity	1-14 days	Single high dose exposure or multiple exposures in a short space of time.	*Acute Oral Toxicity – Acute Toxic Class Method OECD/OCDE 423 2001b*
Subacute Toxicity	15-28 days	Diary exposure that cause toxicity but not lethality.	*Repeated Dose 28-Day Oral Toxicity Study OECD/OCDE 407 2008*
Subchronic Toxicity	28 day-3 months	Diary long-term exposure that cause toxicity but not lethality.	*Repeated Dose 90-day Oral Toxicity Study in rodents OECD/OCDE 408 1998a* *Repeated Dose 90-day Oral Toxicity Study in non-rodents OECD/OCDE 409 1998b*
Chronic Toxicity	6-12 months	Diary exposure during very long-term toxicity	*Chronic Toxicity Studies OECD/OCDE 452 2009b*
Carcinogenicity	18-24 months	Tumor-promoting effect by affecting growth factors or activated oncogenes	*Carcinogenicity Studies OECD/OCDE 451 2009a*
Teratogenicity		Abnormalities of physiological development	*One Generation Reproduction Toxicity Test 415 1983a* *Two Generation Reproduction Toxicity Test 416 1983b* *Fish Embryo Acute Toxicity (FET) Test 236 2013*
Studies on Reproductive		Reproduction Fertility and Natal Developmental Toxicity	*Reproduction/Developmental Toxicity Screening Test 421 1995* *Prenatal Developmental Toxicity Study 414 2001a*
Toxicokinetic	In conjunction with subacute/ subchronic	Pharmacokinetic profile and persistence and potential accumulation	*Toxicokinetic OECD/OCDE 417 Adopted: 22 July 2010*
Method Optimization		Test the method effectiveness	*Environment Health and Safety Publications Series on Testing and Assessment OECD/OCDE 50 2005*

3. Results of data analysis

The importance of the fusarotoxins has been increasing in the last years. The increasing interest in the study of *Fusarium* mycotoxins since 2010 is shown in the large number of articles and reviews published about them. From the 18 reviews analyzed, 78% (14 references) date from the last 4 years. In this decade, the year when the most articles about *Fusarium* mycotoxin *in vivo* were published was 2013, followed by 2012 and 2006/07/14. An increasing trend seems to be continuing this year 2014 with 7 articles already published (Fig. 16).

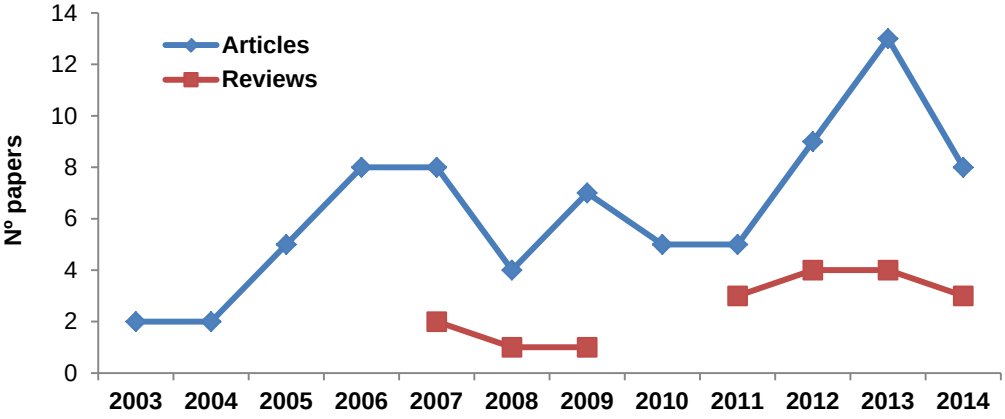

Figure 16. Chronologic diagram of the articles and reviews published between the years 2003 and 2014.

From these reviews, 65% are focused on one mycotoxin and the remaining 35% on combinations of various mycotoxins, mainly the combination DON/ZEN as shown in Table 2. The most individually reviewed mycotoxin is DON (29%), followed by FBs (24%), and finally ZEN (6%), and T-2 toxin (6%) (Fig. 17). All the information about the reviews is shown in Table 2.

Table 2. Reviews about *in vivo* studies of *Fusarium* mycotoxins: mycotoxins reviewed, titles and references

Mycotoxin	Title	References
DON	Mini-review of studies on the carcinogenicity of deoxynivalenol.	Ma and Guo 2008
DON	The toxicity of *Fusarium* mycotoxin deoxynivalenol in poultry feeding.	Awad et al. 2012
DON	The toxicological impacts of the *Fusarium* mycotoxin deoxynivalenol in poultry flocks with special reference to immunotoxicity.	Awad et al. 2013
DON	From the gut to the brain: journey and pathophysiological effects of the food-associated trichothecene mycotoxin deoxynivalenol.	Maresca 2013
DON	Effect of deoxynivalenol and other type b trichothecenes on the intestine: a review.	Pinton and Oswald. 2014
FB	Fumonisin B1 and the kidney: modes of action for renal tumor formation by fumonisin B1 in rodents.	Mueller et al. 2012
FB1	Fumonisin B1: a neurotoxic mycotoxin.	Domijan 2012
FBs	Recent research on fumonisins: a review.	Scott 2012
FBs	A review on incidence and toxicity of fumonisins.	Ahangarkani et al. 2014
ZEN	Clinical effects and biochemical mechanisms associated with exposure to the mycoestrogen zearalenone.	Fink-Gremmels and Malekinejad 2007
T-2	T-2 toxin a trichothecene mycotoxin: review of toxicity metabolism and analytical methods.	Li et al. 2011
ZEN/DON	*In vivo* and *in vitro* effects of the mycotoxins zearalenone and deoxynivalenol on different non-reproductive and reproductive organs in female pigs-a review.	Tiemann et al. 2006
DON T-2; ZEN FBs	The impact of fusarium mycotoxins on human and animal host susceptibility to infectious diseases.	Antonissen et al. 2014
ZEN DON T-2 AFs	The role of mycotoxins in pig reproduction: a review.	Kanora and Maes 2009
ZEN DON T-2 FB1	*Fusarium* mycotoxins: effects on reproductive function in domestic animals-a review.	Cortinovis et al. 2013
ZEN DON T-2 FBs AFs OTA	Modulation of intestinal functions following mycotoxin ingestion: meta-analysis of published experiments in animals.	Grenier and Applegate 2013
TCTs	Trichothecenes: from simple to complex mycotoxins.	McCormick et al. 2011

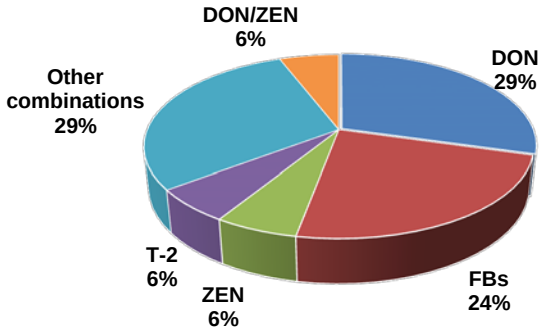

Figure 17. Percentage of reviews according to the studied mycotoxin.

In all cases, DON and ZEN are one of the components in the reviewed mycotoxin combinations, confirming the concern for these mycotoxins due to their prevalence and toxicity, demonstrated in several studies. DON is one of the most prevalent and hazardous food associated mycotoxins (Maresca, 2013) and ZEN is one of the most common mycotoxins worldwide (Schwartz et al., 2010). These studies also underline the increased risk after simultaneous exposure to several mycotoxins in a single food matrix due to the possible interactions of the components. The risk of exposure to several mycotoxins increases with diet complexity (Grenier and Applegate, 2013).

Regarding the articles analyzed the most studied are major *Fusarium* mycotoxins (71%) that correspond with those legislated mycotoxins (FBs, DON, ZEN, T-2, HT-2 and NIV) or combinations of these (22%). However, only 7% are focused on emerging mycotoxins (BEA, ENNs, MON and FUS) (Fig. 18). From the mainly studied mycotoxins, 34% correspond with FBs followed by DON (19%), ZEN (12%), T-2/HT-2 toxin (5%) and NIV (1%) (Fig. 18).

In the same way as was observed in the case of the reviews, in the great majority DON is one of the components of the mycotoxin combinations.

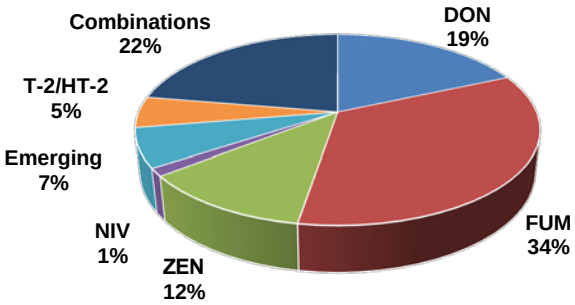

Figure 18. Percentage of articles according to the studied mycotoxin.

For laboratory animals pigs, (piglet, landrace, weaned, barrow) and rats (Sprague-Dawley, Wistar, Fisher) are the most used animals (40 and 31%, respectively) followed by broiler chickens (14%), and mice (8%). Other animals used are fish (zebrafish, carp), horses, turkeys, and rabbits (New Zealand). Finally, a few studies combine the use of laboratory animals with the analysis of human samples (Fig. 19).

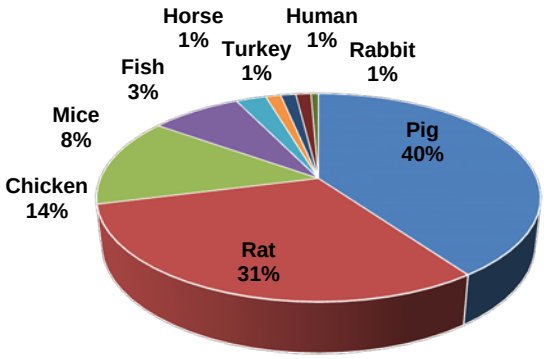

Figure 19. Percentage of studies according to the animal species used.

After the classification of the studies it is shown (Fig. 20) that the most common studies are of short-medium duration term, such as subacute toxicity (40%), subchronic toxicity (24%) and acute toxicity (14%). Toxicokinetic

studies are also frequent (14%). Finally, it is shown that long term studies on *Fusarium*mycotoxins, such as chronic toxicity and carcinogenicity are scarce possibly due to its complexity. To our knowledge, only 3 teratogenicity studies (4%) about *Fusarium* mycotoxins are available.

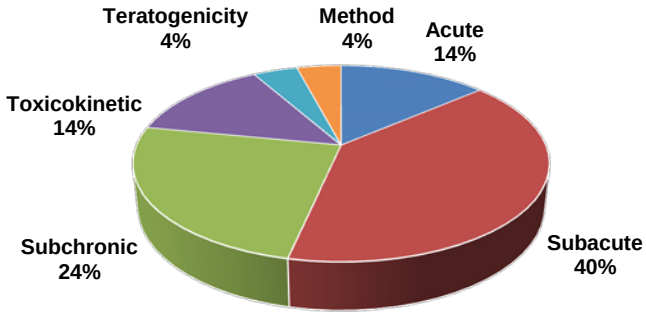

Figure 20. Percentage of the studies according to the assay duration and main objective.

As shown in Fig. 21, the predominant route of administration is oral (80%) administered mostly in the form of naturally contaminated feed (57%) compared with oral bolus (23%) administration. Other administration routes also used are IP, IV and SC (8, 4 and 3%, respectively) and administration in a two-way cross-over design IV/oral or IP/oral (4 and 3%, respectively).

Finally, an important fact is that only the 48% of articles express the mycotoxin administered dose based on body weight of the animal, while the remaining 51% refers to the amount of mycotoxins present in the administered feed (or in water in the case of one study in zebra fish).

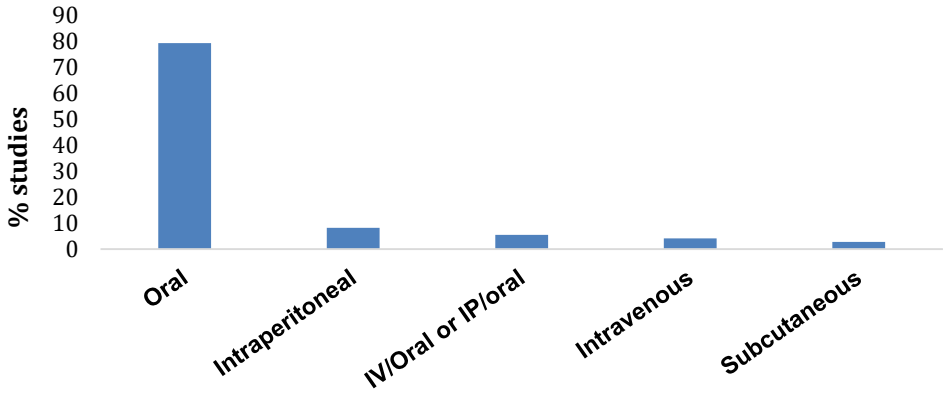

Figure 21. Most common administration routes used.

3.1. Acute toxicity

For acute toxicity studies, the predominantly studied mycotoxin is FB1 followed by ZEN. Also the emerging mycotoxin MON and the mycotoxin combination DON/ZEN have been studied. These types of studies focused on a single high dose exposure in a short space of time, the most used laboratory animals are rats and mice, followed to a lesser extent by rabbits. The mycotoxin administration was both oral and IP. The summary of acute toxicity studies is shown in Table 3.

3.1.1. Fumonisins

FB1 administered orally to rabbits as a single dose of 31.5 mg/kg bw presented high acute toxicity interfering with body and liver weight, altering serum biochemical parameters, although there were no visible pathological abnormalities (Orsi et al., 2009). FB1 causes DNA lesions in the kidney of experimental animals before affecting the enzymatic activity of catalase (CAT) and the concentration of protein carbonyls and malondialdehyde (MDA). It is

shown that the metabolism of sphingolipids may have an important role in the DNA damage caused by FB1 (Domijan et al., 2008). The hepatotoxic mechanism of FB1 in mice does not involve peroxisome proliferator activated receptor α-mediated signaling pathways. The specific sequence of events linking the inhibition of ceramide synthase by FBs to altered gene expression, apoptosis, mitosis, and overt hepatotoxicity in rodents remains to be elucidated (Voss et al., 2006). Apoptosis in rat liver appears, in a time- and dose-dependent manner, 24 hours after a single oral dose of 5 μg FB1/kg bw. Apoptosis occurs before DNA damage suggesting that FB1-induced apoptosis is not primarily caused by DNA damage. Mitotic figures seen at low doses of FB1 indicate that regenerative processes could be involved in its carcinogenesis as they increase DNA replicates (Domijan et al., 2008). The NOAEL of FB1 in mice is lower than 110 μg/kg bw/day (Kouadio et al., 2013).

Three of these acute toxicity studies are in conjunction with toxicokinetic and metabolic assays and show that FB1 was detected in feces at a high mean concentration with a peak of elimination 24 h after its administration, demonstrating that enterohepatic circulation is the main route of elimination of the mycotoxin in rabbits. The mean concentration of excreted FB1 in urine 12 h after administration was low (Orsi et al., 2009). FB1 and FB2 are metabolized by microsomes *in vitro* and (at least) FB1 by rats *in vivo* to acylated derivatives that resemble ceramide in structure (Seiferlein et al., 2007).

3.1.2. Zearalenone

Acute ZEN treatment in rat leads to noticeable variations of mRNA expression of the proteins involved in detoxification of xenobiotics and those involved in their biotransformation. These variations are rather different from those observed upon acute rat treatment with classical CYP inducers: phenobarbital, dexamethasone, b-naphthoflavone, or clofibrate (Duca et al., 2012).

ZEN induces chromosome aberrations in bone marrow cells of mice proceeding by binding to estrogen receptors and steroid receptors, which permits its translocation to the nucleus prior to its chromosome-damaging effects. 17-Estradiol and progesterone as well as vitamin E prevent the ZEN-induced chromosome aberrations by very similar mechanisms. The lethal dose 50% (LD50) in mice is 500 mg/kg bw (Ouanes et al., 2005).

3.1.3. Moniliformin

The emerging mycotoxin MON is acutely toxic to rats with a rather narrow range of toxicity and it can be classified into category 2 (LD50 cut-off value 25 mg/kg bw), according to the Globally Harmonized System for the Classification of Chemicals. The clinical observations included muscular weakness, respiratory distress and heart muscle damage. Pathological findings confirmed that heart is the main target tissue of acute MON toxicity. The study is complemented by a toxicokinetic assay showing that a significant proportion (about 38%) of the administered MON was rapidly excreted in urine in less than 6 h. However, the toxicokinetics of the majority of the administered dose still requires clarification, as the total excretion was only close to 42% (Jonsson et al., 2013).

Table 3. Acute toxicity studies of *Fusarium* mycotoxins: animal species, number of animals, studied mycotoxins, other substances administered, application routines, doses administered, exposure time, and reference.

Acute toxicity							
Species	**Animals**	**Mycotoxin**	**Other subst.**	**Application routine**	**Doses**	**Exposure time**	**Reference**
Rabbit	n=18	FB1		Oral	31.5 mg/kg bw	Admin. + 7 days	Orsi et al. 2009
Rat	n=11	FB1		IP	253 558 1120 mg/kg bw	5 days	Seiferlein et al. 2007
Rat	n=20	FB1		IP	0.5 mg/kg bw	2 /7days	Domijan et al. 2007
Rat	n=66	FB1		IP	5 50 500 µg/kg bw	2 days	Domijan et al. 2008
Mice	n=20	FB1	Peroxisome proliferator (WY) phenobarbital dexamethasone b-naphtoflavone clofibrate.	Oral	FB1:300 mg/kg feed WY:500 mg/kg feed	7 days	Voss et al. 2006
Rat	n=18	ZEN		IP	25 mg/kg bw	1 days	Duca et al. 2012
Mice	n=54	ZEN		IP	2-40 mg/kg bw	24h/ 48h/ 72h	Ouanes et al. 2005
Rat	n=24	T-2		Oral	3 mg/kg feed		Yang et al. 2013a
Rat	n=5	MON		Oral	5 10 25 40 50 mg/kg bw	Admin. + 14 days	Jonsson et al. 2013
Mice	n=40	DON FB1		Oral	DON:45 µg/kg bw FB1:110 µg/kg bw	7 days	Kouadio et al. 2013

3.2. Repeated-dose toxicity studies

3.2.1. Subacute toxicity

The most studied mycotoxin in subacute studies is FB1, as in the case of acute toxicity. Other single mycotoxins studied were DON, ZEN, T-2 toxin and NIV. Also the emerging mycotoxin ENN A and mycotoxin combinations such as DON/T-2, DON/ZEN and DON/FB1 were studied. In these trials based on a diary exposure during a short space of time that caused toxicity, but not lethality, the most used animals were pigs and rats, followed by chickens, mice and horses. The administration was oral in the great majority but a few subcutaneous studies have been performed. The summary of the subacute toxicity studies is shown in Tables 4 and 5.

Table 4. Subacute toxicity studies of FB1: animal species, number of animals, other substances administered, application routines, doses administered, exposure time, and reference.

Subacute toxicity							
Specie	**Animals**	**Mycotoxin**	**Other subst.**	**Application routine**	**Doses**	**Exposure time**	**Reference**
Pig	n= 20	FB1		Cont. feed	0.99 and 1.99 mg/Kg bw	28 days	Marin et al. 2006
Pig	n=25	FB1		Oral	1 mg/Kg bw/day	10 day	Devriendt et al. 2009
Pig	n=16	FB1		Cont. feed Special T-cannula	FB1: 45 mg/kg feed FB2: 8.6 mg/kg feed FB3: 4.6 mg/kg feed	10 days	Fodor et al. 2008
Pig	n=22	FB1		Oral Cont. feed	1.5 mg/kg bw/day 8 mg/kg feed	7 days 28 days	Taranu et al. 2005
Pig	n=nd	FB1		Oral Special T-cannula	2-22 mg/kg bw	10 days 22 days	Fodor et al. 2007
Pig	n=36	FB1		Cont. feed	1.5 mg/kg bw	9 days	Lallès et al. 2010
Pig	n=18	FB1/ HFB1		Oral	2.02 mg FB1 or HFB1/kg bw/day	14 days	Grenier et al. 2012
Rat	n=30 n=20	FB1		Oral	Prolonged exposure: 5 and 10 mg/kg bw Single dose: 5 mg/kg bw	21 day Single dose	Direito et al. 2009

Rat	n=60	FB1	Royal jelly	Cont.feed	200mg/kg feed	3 weeks	El-Nekeety et al. 2007
Rat	n=90	FB1	Diethylnitros amine (DEN)	Cont.feed. Drink fluid tea	FB1: 250 mg/kg diet DEN: 200 mg/kg bw	21 days	Marnewick et al. 2009
Rat	n=20	FB1		Cont. feed	92.2-132.2 mg/kg feed	2 weeks	Voss et al. 2003
Rat	n=35	FB1		Cont. feed	Untreated 1.8-4.2 mg/kg feed Treated 0.08-0.37 mg/kg feed	3 weeks	Voss et al. 2013
Rat	n=56	FB1		Cont.feed	1.19-9.08 mg/kg feed	3 weeks	Burns et al. 2008
Rat	n=36	FB1 FB2 FB3		Cont. feed	13.5- 88.6 mg/kg feed	10 days	Riley et al. 2006

Table 5. Subacute toxicity studies of Fusarium mycotoxins: animal species, number of animals, studied mycotoxins, other substances administered, application routines, doses administered, exposure time, and references.

Subacute toxicity							
Specie	**Animals**	**Mycotoxin**	**Other subst.**	**Application routine**	**Doses**	**Exposure time**	**Reference**
Pig	n=40	DON	Arginine, Glutamine	Cont. feed	DON: 6 mg/kg feed	28 days (7d aa treatment +21d DON)	Wu et al. 2013
Chicken	n=32	DON		Cont. feed	DON: 7.5 mg/kg feed (FB1:0.7; FB2:0.2; FB3:0.2 mg/Kg feed)	3 weeks	Osselaere et al. 2013a
Pig	n=4	DON/DG3		Oral and IV	D3G: 116 mg/kg bw DON: 75 mg/kg bw D3G: 15.5 mg/kg bw (iv)	13 days	Nagl et al. 2014
Pig	n=40	ZEN	Genistein, Resveratrol, Bisphenol A	Cont. feed	250 mg/kg feed	18 days	Pistol et al. 2014
Mice	n= 45	ZEN		SC	0.5-10 µg/kg/day	21 days	Nikaido et al. 2004
Chicken	n=80	T-2		Cont. feed	8.1 mg/kg feed	21 days	Dvorska et al. 2007
Chicken	n=50	T-2		Cont. diet	0.5-13.5 mg/kg feed.	17 days	Rezar et al. 2007
Mice	n=60	NIV		Oral (3 days/week)	0.014-8.87 mg/kg bw	4 weeks	Gouze et al. 2007

Rat	n=10	EN A		Cont. feed	20.91 mg/Kg bw	28 days	Manyes et al. 2014
Rat	n=10	EN A		Cont. feed	465 mg/kg feed	28 days	Juan et al. 2014
Pig	n=40	DON/ZEN	Sodium metabisulfite (SBS)	Cont. feed	DON: 0.084-2.312 mg/kg feed ZEN: 0.006-0.017 mg/kg feed	28 days	Dänicke et al. 2010
Horse	n=6	ZEN DON		Cont. feed	ZEN:1 mg/Kg feed; DON:12 mg/Kg feed	18 days	Songsermsakul et al. 2011
Pig	n=48	T-2 DON	Vitamin E	Cont. feed	T-2: 3 mg/kg feed DON: 4 mg/kg feed Vit E: 100 mg/kg	14 days	Frankic et al. 2008
Pig	n=24	T-2 DON	Doxycycline paromomycin (oral bolus)	Cont. feed	111 μg/kg feed 813 μg/kg feed	7 days 13 days	Goossens et al. 2012
Chicken	n=50	T-2 DON	Nucleotides (nc)	Cont. feed	DON: 10 mg/kg feed T-2: 10 mg/kg feed nc: 2g/kg feed	17 days	Frankic et al.2006

3.2.1.1. Fumonisins

High oral doses (50 mg/kg) of FB1 administered for 19 or 22 days to pigs do not induce clinical signs of disease, feed refusal or reduction of the feed intake. In contrast, pathological alterations were found after the treatment, primarily in the lung and liver. Even 10 days after the cessation of toxin feeding marked pathological signs were experienced. The highest FB1 concentrations were found in the liver and kidneys. However, FB1 was not detected or found in very low concentrations in muscle and fat samples (Fodor et al., 2007).

After 10 days of FB1 exposure in pigs (45 mg/kg) pulmonary edema was developed in all of the animals and pathological changes in the liver, heart, kidneys and hyperplasia in the spleen were observed. The maximum FB1 concentration content was found in the liver (Fodor et al., 2008). Rats receiving FB1 (200 mg/kg) for three weeks showed severe histological and histochemical changes in the liver and kidney tissues. FB1 ingestion decreased body weight gain, feed intake, glutathione peroxidase (GPX) and SOD whereas it increased ALT, AST, triglycerides, cholesterol, HDL, LDL, creatinine and uric acid levels (El-Nekeety et al., 2007).

Ingestion of FB1-contaminated feed (1–2 mg/kg bw) during 28 days significantly decreased weight gain in male pigs but had no effect in females. No sex-related difference was observed in biochemical parameters, but a higher level of creatinine was noted in toxin-treated animals. FB1 altered the immune response in a sex-specific manner. The magnitude of the FB1-induced immunosuppression is highly dependent on sex with males being more susceptible than females (Marin et al., 2006).

The ingestion of FB1 alters cytokine production and decreases the vaccinal antibody response that could have important consequences in

humans and animals (Taranu et al., 2005). FB1 ingestion leads to an impaired function of intestinal antigen presenting cells (Devriendt et al., 2009). FB1 induces hepatotoxicity as indicated by the level of several biochemical analytes and the expression of inflammatory cytokines, the impaired morphology of the different segments of the small intestine, or the reduced villi height and modified intestinal cytokine expression (Grenier et al., 2012).

Prolonged exposure to FB1 caused an increase in Sa levels in urine, serum and liver, especially in rats treated with higher doses of FB1 (10 mg/kg). Urine was found to be a good material for the determination of the Sa/So ratio in male Wistar rats compared to liver and serum since higher urinary levels are observed with increasing doses (Direito et al., 2009). There was a time- and dose-dependent increase in Sa in both liver and kidney, which was closely correlated with the tissue concentration of FB1 and histopathologic findings. The concentration of FB1 in liver and kidney that first elicited an increase in Sa was similar in both tissues; however, over time, the kidney accumulated significantly more FB1 and total Sa compared to the liver. The relative sensitivity of male Sprague-Dawley rat kidney and liver is most likely a consequence of differences in the mechanisms responsible for both FB1 uptake/clearance and Sa metabolism (Riley and Voss, 2006).

Repeated consumption of FB1 (1.5 mg/kg bw) drastically enhanced colonic levels of aB crystallin and COX-1, with milder increases in other stress proteins along the gastrointestinal tract of pigs. The colon is an important target for FB1-induced stress responses (Lallès et al., 2010).

3.2.1.1.1. Hydrolyzed products of FB1

Inactivating fumonisins with bases – such as nixtamalization or treatment with alkaline solvent – increases the pH level and causes cleavage

of side chains of tricarballylic acid and removes TCA groups in C14 and C15 producing hydrolyzed fumonisins. Fumonisin concentrations are reduced by hydrolysis of one or both of the molecule's TCA groups, yielding the partially hydrolyzed FB1 (PHFB1) and FB2 (PHFB2), or hydrolyzed FB1 (HFB1) and FB2 (HFB2), respectively (De Girolamo et al., 2014). Hydrolyzed fumonisins were detected only from the B-group (HFB1, HFB2 and HFB3) (Ahangarkani et al., 2014).

FB1 has been shown to be degraded through thermal (baking, extrusion, and frying) and alkali processing (Park et al., 2013). Some publications have indicated that FBs bound to food components such as proteins, sugars, and starch might be present in heat-processed corn foods. As the binding with food matrix changes the FB structure in such a way that the amino group essential for detection by derivatization is blocked, bound FBs are not detectable by conventional HPLC analysis, so that FB1 would be considered destroyed by the heat.

It is possible to find N-acylated FBs conjugated with fatty acids from frying oils in alkali-processed corn foods (Park et al., 2013). Data on the toxicity of HFBs and PHFBs are still unclarified and refer only to that of HFB1 (De Girolamo et al., 2014). Some N-acylated HFB1 derivatives were found not only to be more toxic in a mammalian cell line *in vitro* than FB1, but also might be expected to accumulate in body fat (Park et al., 2013). HFB1 is less toxic than FB1 in cell culture but different studies showed the *in vivo* toxicity of HFB1 (Ahangarkani et al., 2014).

Several studies indicated that nixtamalized culture materials of *F. proliferatum*and *Fusarium verticillioides* in which only hydrolyzed fumonisins were detected remained toxic when fed to rats. Other studies showed that HFB1 induced anomalies in cultured rat embryos, including

neural tube defects, suggesting that HFB1, like FB1, might induce anomalies *in vivo* when tested in a sensitive animal model. By contrast, in mice fed purified HFB1, no hepatotoxicity or pathological changes were detected. Moreover, HFB1 was not carcinogenic in rats and did not affect fetal development in either mice or rats, whether given intraperitoneally or orally. Other studies demonstrate that HFB1 does not cause intestinal or hepatic toxicity in the sensitive pig model and only slightly disrupts sphingolipids metabolism. All these findings indicate that conflicting data still exist on the toxicity of HFB1 (De Girolamo et al., 2014).

It is possible that bound fumonisins can be released in the gastrointestinal tract and reconvert into the parent toxin during digestion, by microflora, and may be hydrolyzed to partially or hydrolyzed FBs and increase the possibility of exposure to these toxins after ingestion, adding to the toxicity of the food or feedstuff by a mechanism similar to free FBs (Ahangarkani et al., 2014).

The intestinal microbiota of pigs can transform the intact FB1 to PHFB1 and HFB1, more hydrophobic molecules with more effective absorption (Szabo-Fodor et al., 2008). From the two metabolites PHFB1 has priority during the metabolic process. The conversion of FB1 to HFB1 is notable even despite its little amount (Fodor et al., 2008).

In order to determine the FB1 absorption from the feed a special T-cannula was implanted into the distal part of pigs' ileum. After determining the intact FB1 moieties, a strongly negative balance was found in the total toxin amount, when taking the moieties excreted in the urine and feces into account. This was even true considering the literature data, namely the low (0–6%) bioavailability of FB1 (Fodor et al., 2007). The mean accumulative absorption rate of all animals tested for intact FB1 and its metabolites formed

in small intestine (till the end of the ileum) is 4%. There was no significant correlation between the daily FB1 intake and the absorption of the toxin (Fodor et al., 2008).

From the point of view of human exposure, special attention should be paid to HFB1 appearing in edible tissues even 10 days after the dosage of the non-contaminated feed because there is a lack of information about the effect of this metabolite consumed in low concentrations over a long-term period. HFB1 did not trigger hepatotoxicity, did not impair intestinal morphology, did not cause intestinal toxicity in the sensitive pig model and slightly modified the intestinal immune response and disrupted sphingolipids metabolism. Therefore conversion to HFB1 could be a good strategy to reduce FB1 exposure (Grenier et al., 2012). FB1 is poorly absorbed, but rapidly eliminated in weaned piglets. Humans do not ingest notable amounts of FB1 with raw food materials originating from pigs (Szabo-Fodor et al., 2008). The accumulative absorption of FB1 in pigs was 3.9%–0.7%. In the thymus, FB1 conversion into HFB1 and PHFB1 were 1.0 and 3.9%, respectively. The degree of metabolism in feces was variable, although the main product was PHFB1, with very small amounts of the HFB1 moiety being recovered. In the investigated tissues (lung, liver, kidney, brain, muscle, and fat) the FB1 conversion to HFB1 and PHFB1 was 30 and 20%, respectively (Fodor et al., 2008).

3.2.1.1.2. Nixtamalization

The process of nixtamalization is a widely used food processing method which involves alkaline treatment of maize prior to cooking and significantly reduces the amount of bioavailable FB1, which is cleaved to HFB1, and reduces *in vivo*toxicity of whole kernel corn. After feeding rats for

up to three weeks with equivalent weights of the nixtamalizated products, un-nixtamalizated *Fusarium*culture material, corn or nixtamalized corn it is suggested that mycotoxin–corn matrix interactions during nixtamalization reduce the bioavailability and toxicity of FB1. Therefore nixtamalization is an effective alkaline treatment method for reducing FB exposures from corn-based foods (Burns et al., 2008).

After feeding rats for 2 weeks at high or low doses of treated materials and untreated culture material-spiked cornmeal it was shown that the toxic response to the untreated spiked cornmeal and the treated products included decreased body weight gain (high-dose only), decreased kidney weight, and microscopic kidney and liver lesions of the type caused by FBs. After determining FB concentration, it is shown that baking and frying had no significant effect on the biological activity or concentration of FBs in these corn-based products (Voss et al., 2003).

After feeding rats with diets containing low, mid or high levels of untreated or alkaline treated FB1-contaminated corn for 3 weeks, apoptotic kidney lesions were not found in the low and medium contamination level treated groups. Lesions in the group fed with the high treated level (0.37 mg/kg) were minimal and less severe than those found in the rats fed with low, mid and high untreated levels (1.8–4.2 mg/kg). Furthermore, significantly increased Sa and So concentrations indicative of FB1 exposure were found in the kidneys of the rats given untreated FB1-contaminated corn for 3 weeks. Concentrations were also elevated, but to a lesser extent, in rats fed with high level of treated contaminated corn. FB1 concentrations in the treated diets were markedly reduced compared with their untreated counterparts so nixtamalization is an effective method for reducing the potential toxicity of FB1 contaminated corn (Voss et al., 2013).

3.2.1.1.3. Other substances.

Co-treatment with FBs plus royal jelly resulted in a significant improvement in all the tested parameters and the histological and histochemical pictures of the liver and kidney. These improvements were pronounced in animals fed FBs-contaminated diet plus the highest dose of royal jelly (150 mg/kg bw) that has protective effects against FBs toxicity and this protection was dose-dependent (El-Nekeety et al., 2007).

Various teas administered to rats differently affected the clinical chemical parameters associated with liver and kidney FB1-damage. Green tea enhanced the FB1-induced reduction of the oxygen radical absorbance capacity, while fermented herbal teas and unfermented honey bush significantly decreased FB1-induced lipid peroxidation in the liver. The teas exhibited varying effects on FB1-induced changes in the activities of CAT, GPX, glutathione reductase (GR) as well as the glutathione (GSH) status. Fermentation seems to reduce the protective effect of herbal teas. Differences in the major polyphenolic components and certain FB1/polyphenolic/tissue interactions may explain the varying effects of the different teas on the oxidative parameters, hepatotoxic effects and sphingolipid metabolism promotion in rat liver (Marnewick et al., 2009).

3.2.1.2. Deoxynivalenol

After feeding pigs with DON-contaminated diet (6 mg/kg) for 21 days combined with amino acid treatments it was shown that dietary supplementation with arginine and glutamine alleviated the impairment induced by DON stress and immune relevant cytokines in growing pigs. Dietary supplementation with amino acids significantly reduced anorexic effects during the feeding period (Wu et al., 2013).

Feeding DON affects the gut wall morphology of both the duodenum and jejunum of broiler chickens acting in a very specific way on the intestinal barrier. Increased intestinal barrier permeability after chronic exposure to DON may lead to intestinal inflammation. The mechanism of action of DON can be different depending on the investigated target organ. The mycotoxin adsorbing agent illite-ambrosite clay does not cause direct damage or irritation. However, feeding this clay mineral in combination with DON may result in higher concentrations of the mycotoxin in more distal parts of the small intestine, resulting in damage of the intestinal barrier (Osselaere et al., 2013a).

After oral application of DON (75 mg/kg bw) and deoxynivalenol-3-β-D-glucoside (D3G), 85% and 40% of the given dose were detected in urine, respectively. The majority of orally administered D3G was excreted in the form of DON, deoxynivalenol-15-GlcA (DON-15-GlcA), deepoxy-deoxynivalenol (DOM-1) and deoxynivalenol-3-glucuronide (DON-3-GlcA), while urinary D3G accounted for only 2.7%. In feces, just trace amounts of metabolites were found. IV administered D3G (15.5 mg/kg bw) was almost exclusively excreted in unmetabolized form via urine. Data indicate that ingested D3G is nearly completely hydrolyzed in pigs, but only partially absorbed. Cleavage predominantly occurs in the digestive tract, while the toxin seems to be rather stable after systemic absorption. Compared to DON, the proportion of urinary excreted metabolites after oral D3G administration was reduced by a factor of 2, approximately. It seems D3G is less bioavailable than its parent toxin in pigs and therefore of lower toxicological relevance. However, the bioavailability of D3G in pigs may increase after chronic exposure via feed, as has already been shown for DON. D3G may exhibit biological activity on its own (Nagl et al., 2014).

3.2.1.3. Zearalenone

ZEN caused severe hepatic immunosuppression (significant reduction in the expression and protein concentrations of several markers of pro- and anti-inflammatory processes) by inhibiting the gene expression and protein concentrations which might have important consequences during an infection process (Pistol et al., 2014).

Maternal exposure to xenoestrogens accelerated puberty onset (vaginal opening) and increased the length of the estrous cycle; mice treated with genistein, resveratrol, bisphenol A or diethylstilbestrol (DES) spent more time in diestrus, and ZEN-treated mice spent more time in estrus. High-dose genistein and resveratrol, and low- and high-dose bisphenol A and DES, had transient effects on the reproductive tract and mammary glands, whereas high-dose ZEN induced prolonged effects. Mammary gland growth was accelerated in ZEN- and bisphenol A-treated mice with *corpora lutea* at 4 weeks of age, whereas mammary gland growth was suppressed in ZEN-treated mice lacking *corpora lutea* from 8 to 16 weeks of age (Nikaido et al., 2004).

3.2.1.4. T-2 and HT-2 toxin

Inclusion of T-2 toxin in chickens' diet was associated with significant decreases in the concentrations of selenium (Se), α-tocopherol, total carotenoids, ascorbic acid, and reduced GSH in the liver, as well as a decrease in the hepatic activity of Se-dependent glutathione peroxidase (Se-GSH-Px). A combination of modified glucomannan mycotoxin binder (GMA) with organic Se was shown to provide further protection against toxin-induced antioxidant depletion and lipid peroxidation in the chicken liver. The data indicate a major protective effect of the mycotoxin-binder in combination with organic Se

against the detrimental consequences T-2 toxin-contaminated feed consumption by growing chickens (Dvorska et al., 2007).

A concentration of 4.5 mg/kg feed or higher decreased feed consumption and consecutively weight gain of the animals. Low concentrations (up to 1.5 mg/kg feed) did not provoke DNA fragmentation in spleen leukocytes. The significant elevation of DNA damage in leukocytes was observed only at a concentration of 13.5 mg/kg (Rezar et al., 2007). Indeed hepatotoxicity appeared in mice treated with a five-fold higher oral dose of 8.87 mg/kg bw. Such exposure levels appear to be by far higher than the maximal natural occurrence measured in European cereals, known to range from 0.34 to 1.86 mg/kg (Gouze et al., 2007).

3.2.1.5. Enniatins

No adverse effects were found in treated rats at an ENN A concentration of 20.91 mg/kg bw/day, tested during a 28-day experiment. ENN A quantitation in biological fluids ranged from 1.50 to 9.00 mg/kg, whereas in the gastrointestinal organs the ENN A concentration was between 2.50 and 23.00 mg/kg. The high ENN A concentration found in jejunum liquid and tissue points to them as an absorption area. Finally, two ENN A degradation products were identified in duodenum, jejunum and colon content, probably produced by gut microflora (Manyes et al., 2014). ENN A was non-detectable in urine and feces from treated rats during the 28 days of exposure time. Its presence in serum over time might be the cause of alterations reflected in lymphocyte phenotyping. The observed changes of lymphocytes in peripheral blood may not alter immune system function in normal life, but in infectious conditions, reduced lymphocytes could lessen humoral and innate immunity. Peripheral blood lymphocytes surface antigen

expression and immune status in treated rats were probably impaired by ENN A (Juan et al., 2014).

3.2.1.6. Mycotoxin combinations

The relative differences between the DON concentrations in other physiological specimens (muscle, liver, kidney, bile and urine) in piglets fed DON/ZEN contaminated feed and the DON/ZEN contaminated diet with sodium metabisulfite (SBS) were comparable with the blood DON concentration differences. ZEN could only be detected in bile and urine where their levels were not influenced by the SBS treatment (Dänicke et al., 2010).

When horses were fed with DON/ZEN-contaminated oats for 18 days, ß-zearalenol (ß-ZOL) was detected in plasma at high levels on day 10 of the study. ß-ZOL and a-zearalenol (α-ZOL) were the major metabolites in urine. ZEN, α-ZOL and ß-ZOL were predominantly found in feces. ZEN could also be detected in urine and feces. The degree of glucuronidation was established in all sample types, approximately 100% in urine and plasma. A low level of glucuronidation (4–15%) was found in feces samples. The results indicate the main conversion of ZEN into ß-ZOL in the horse (Songsermsakul et al., 2013).

T-2 toxin and DON (10 mg/kg feed) induced DNA fragmentation in chicken spleen leukocytes and supplementation with nucleotides reduced the amount of damage only when added to T-2 toxin. Dietary nucleotides have the potency to reduce the extent of DNA damage induced by the action of T-2 toxin in immune cells. This underlines their possible beneficial effect on the immune system in mycotoxin intoxication (Frankic et al., 2006). DON and T-2 toxin are genotoxic to pig lymphocytes at concentrations of 4 mg/kg feed and 3 mg/kg feed, respectively. Oxidative pathways may be only of minor importance in mycotoxin-induced DNA fragmentation. Vitamin E

supplementation partially reduced the risk of DNA damage in immune cells due to the action of DON and T-2 toxin and partially increased synthesis of IgG, which was impaired by T-2 toxin. Enhancement of antioxidant status with vitamin E in the case of DON and T-2 toxin intoxication can be beneficial for retaining lymphocyte DNA integrity (Frankic et al., 2008).

3.2.2. Subchronic toxicity

In subchronic toxicity studies DON is the most studied mycotoxin followed by FB1 and ZEN. Mycotoxin combinations are also frequent as DON/ZEN and DON/ZEN/NIV/FB1.

Fusarium mycotoxin–non *Fusarium* mycotoxin combinations are also common in subchronic studies as FB1/AFs and DON/AFs. The most used animals in subchronic toxicity studies were pigs and chickens, followed by rats, fish and turkeys. In these long-term exposure studies mycotoxin administration was oral except in one IV study. The summary of subchronic toxicity studies is shown in Table 6.

Table 6. Subchronic toxicity studies of Fusarium mycotoxins: animal species, number of animals, studied mycotoxins, other substances administered, application routines, doses administered, exposure time and references.

Subchronic toxicity							
Specie	**Animals**	**Mycotoxin**	**Other subst.**	**Application routine**	**Doses**	**Exposure time**	**Reference**
Pig	n=24	DON	LPS	IV	DON: 100 μg/kg bw/h LPS: 7.5 μg/kg bw/h	37 days	Kullik et al. 2013
	n=12			Cont. feed	31 mg/kg feed		
Pig	n=10	DON		Cont. feed	2.2-2.9 mg/kg feed	11 weeks	Nossol et al. 2013
Pig	n=12	DON		Cont. feed	0,05-0,215 μg/kg bw	5, 6 and 11 weeks	Bimczok et al. 2007
Pig	n=24	DON		Cont. feed	2.8 mg/kg feed	4 weeks	Waché et al. 2009
Chicken	n=40	DON	Microbial feed addit. (Mycofix)	Cont. feed	10 mg/kg feed	5 weeks	Ghareeb et al. 2014
Chicken	n=40	DON		Cont. feed	10 mg/kg feed	5 weeks	Ghareeb et al. 2013
Chicken	n=40	DON	Microbial feed addit. (Mycofix)	Cont. feed	10 mg/kg feed	5 weeks	Awad et al. 2014
Turkey poults	n=12	DON	GMA	Cont. feed	4.0-6.5 mg/kg feed	12 weeks	Devreese et al. 2014
Fish	n=32	FB1		Cont. feed	10 mg/kg feed	42 days	Kovacic et al. 2009
Chicken	n=50	FB1		Cont. feed	10 mg/kg feed	38 days	Todorova et al. 2011

Pig	n=24	DON/FB1		Cont. feed	DON: 3 mg/kg feed FB1: 6 mg/kg feed	5weeks	Loureiro-Bracarense et al. 2012
Pig	n=24	DON/FB1		Cont. feed	DON: 3 mg/kg feed FB: 6 mg/kg feed	5 weeks	Grenier et al. 2011
Rat	n=24	FB1/AF		Cont. feed	FB1: 980; FB1:742 (+AFB1:299) μg/Kg bw	90 days	Theumer et al. 2010
Rat	n=18	FB1/AF		Cont. feed	FB1: 865 μg/Kg bw (+AFB1= 350)	90 days	Theumer et al. 2003
Fish	n=20	ZEN		Nominal exposure	100-3200 ng/L water	21d exp.+42d reproduct.	Schwartz et al.2010
Pig	n=36	DON, ZEN		Cont. feed	DON: 210-9570 μg/kg feed ZEN: 4-358 μg/kg feed	35 days	Tiemann et al. 2006
Pig	n=36	DON/ZEN		Cont. feed	DON: 3.6-104.2 μg/kg bw ZEN: 0.1-6.3 μg/kg bw	35 days	Alm et al. 2006
Pig	n=225	DON/AF	Feed additives	Cont. feed	DON:1100 μg/kg feed (AFs:150 μg/kg feed)	42 days	Weaver et al. 2013
Pig	n=80 n=48	DON/ZEN/ NIV/FBs	Aluminiosilicate	Cont. feed	DON:8.6 mg/kg feed ZEN:1.2 mg/kg feed NIV: 2,5 mg/kg feed FBs= 2.8 mg/kg feed	36 days	Doll et al. 2005

3.2.2.1. Deoxynivalenol

There was no evidence that feeding DON (3.1 mg/kg feed) for 37 days had influence on the induced sub-acute stage of sepsis in pigs. DON had neither an impact on *in vivo* synthesis of albumin and fibrinogen nor on protein synthesis and metabolic activity of porcine peripheral blood mononuclear cells (PBMC) and cytokine concentration after dietary exposure to 3.1 mg/kg feed or infusion (100 µg DON/kg bw) to pigs. The dose and dosing regimen of DON seems to be insufficient to alter the investigated immune parameters, both when DON was given alone or in combination with lipopolysaccharides (LPS) (Kullik et al., 2013).

DON affects the composition of the basement membrane affecting the frequency of pores of the jejunum (increased pore number) but not in the ileum. DON may change the composition of the basement membrane by influencing the regulation of important genes of the basement membrane in intestinal epithelial cells and reduces the production of laminin. Low and physiological concentrations of DON (comparable to blood level in DON-fed pigs) show identical results compared with high concentrations (Nossol et al., 2013).

Subchronic dietary exposure to DON (5, 6 and 11 weeks) in pigs resulted in the modulation of dendritic cell function, which might contribute to the immunosuppressive effects after DON feeding (Bimczok et al., 2007). DON reduced the level of plasma tumor necrosis factor (TNF-a) and down-regulated the relative mRNA expression, which indicates the ability of DON to inhibit protein synthesis. DON could have an effect on the innate immune response which can impair the resistance of chickens to infectious diseases and consequently increase the susceptibility of the host to infection.

Furthermore, a microbial feed additive has the ability to modulate DON effects on plasma levels of TNF-a and on the relative expression of mRNA in the intestinal epithelium of broiler chickens (Ghareeb et al., 2013).

DON is cytotoxic and genotoxic to the chicken intestinal and immune cells. Feeding of broilers with DON-contaminated feed (10 mg/kg) for 5 weeks resulted in lipid peroxidation and oxidative stress of jejunal cells and damage to the DNA of blood lymphocytes (Awad et al., 2014). It increased the underlying fearfulness and physiological stress responses of broilers (Ghareeb et al., 2014). Feeding DON-contaminated diets (4–6.5 mg/kg) for 12 weeks to turkey poults altered some non-specific parameters such as growth rate, plasma biochemistry profile, duodenal villus height and apparent villus surface area in the duodenum (Devreese et al., 2014a).

Consumption of DON-contaminated feed (2.8 mg/kg) in pigs for 4 weeks significantly reduced animal weight gain during the first week but had a moderate effect on cultivable bacteria in the intestine. Changes in the intestinal microflora were observed in DON exposed animals, suggesting an impact of this toxin on the dynamics of intestinal bacteria communities. The mechanism of action of DON on eukaryotic cells is well documented but the effect of this toxin on the intestinal microbiota is largely unknown (Waché et al., 2009).

3.2.2.2. Fumonisins

Subchronic experimental toxicosis has been reported in fish (carp) receiving FB1-contaminated feed (10 mg/kg) for 42 days. After staining with hemalaun–eosin, histology of the fish brain revealed vacuolated, degenerated, or necrotic neural cells, scattered around damaged blood capillaries and in the

periventricular area. Although FB1 is a hydrophilic molecule it may pass the blood–brain barrier of young carp and have a toxic effect on neuronal cells. Therefore, chronic environmental exposure in early life may play a major role in the development of neurodegenerative disorders later in life (Kovacic et al., 2009).

FB1 causes *in vivo* suppression of development, differentiation, and function of the lymphoid cells and the stromal epithelial reticular cells, and activates the processes leading to cell death in organs of the immune system of chickens. This will inevitably cause disruption in cellular and humoral immune competence and may also affect certain endocrine functions of the thymus (Todorova et al., 2011).

An increase of malondialdehyde (MDA) levels (CAT and SOD activities) and DNA damage was found in rats fed for 90 days with AFs, FB1 and AFs/FB1-contaminated feed (FB1: 740–980, AFB1: 300 μg/kg). An indirect genotoxic action of FB1 on mammalian cells could be related somehow to the previously reported immunotoxic effects induced by AFs and FBs, individually or as mixtures. Such genetic lesions could be mediated by the oxidative stress induced by the toxins and the subsequent widespread oxidation of biomolecules including DNA (Theumer et al., 2010). Animals fed with a mixture of toxins (AFs/FB1) showed decreased body weight. Different immunobiological effects were produced by a mixture of mycotoxins in comparison to the individual action of the same toxins. The models of subchronic and chronic intoxication produced by mixtures of mycotoxins could add better information with respect to the pathogenesis of mycotoxicoses that occur naturally (Theumer et al., 2003).

Chronic ingestion of contaminated diets (DON: 3 mg/kg, or FBs: 6 mg/kg, or both toxins) in pigs induced morphological and histological changes, as shown by the atrophy and fusion of villi, the decreased villi height and cell proliferation in the jejunum, and by the reduced number of goblet cells and lymphocytes. The expression levels of several cytokines were significantly up-regulated in the ileum or the jejunum at the end of the experiment. The ingestion of contaminated diets reduced the expression of the adherent junction protein E-cadherin and the tight junction protein occludin in the intestine. When animals were fed with a co-contaminated diet (DON and FBs), several types of interactions were observed depending on the parameters and segments assessed: synergistic (immune cells); additive (cytokines and junction protein expression); less than additive (histological lesions and cytokine expression); antagonistic (immune cells and cytokine expression). There is strong evidence that chronic ingestion of low doses of mycotoxins alters the intestine, and thus may predispose animals to infections by enteric pathogens. The effects on the immune response were higher when fed in combination (DON and FBs) rather than individually, demonstrating that ingestion of multi-contaminated diet induces greater histopathological lesions and higher immune suppression than ingestion of mono-contaminated diets (Loureiro-Bracarense et al., 2012).

3.2.2.3. Zearalenone

Exposure of zebrafish to ZEN (100–3200 ng/L water) for 21 days reduced relative spawning frequency and relative fecundity in relation to the 21-day pre-exposure period. Exposure to ZEN did not affect fertility, hatch, embryo survival or gonad morphology of zebrafish. ZEN possesses a moderate

estrogenic potency *in vitro* and it exhibits a comparably strong effect on induction of vitellogenin and reproduction *in vivo* following waterborne short-term exposure. This indicates that ZEN might contribute to the overall estrogenic activity in the environment and could therefore pose a risk for wild fish in their natural habitat (Schwartz et al., 2010).

3.2.2.4. Mycotoxin combinations

After 5 weeks of feeding DON/ZEN-contaminated feed (9.57 and 0.358 mg/kg, respectively) significant effects on the spleen in gilts can be mostly observed as a decreased cellular immune response to concanavalin A in splenocytes and appearance of a dysfunction in the spleen, which can be observed as hemosiderosis in the absence of clinical signs, especially in pigs fed with higher concentrations. Pathophysiology attributable to DON/ZEN in pigs can be important in understanding whether human exposure to DON/ZEN might have unfavorable effects because swine are physiologically similar to humans and are widely used as models for human disease (Tiemann et al., 2006).

Oocyte quality is significantly reduced by feeding of *Fusarium*-toxins to gilts. Feeding pigs with DON- and ZEN-contaminated feed at high concentrations for 35 days was associated with oocyte degeneration and reduced meiotic competence of compact cumulus oocyte complexes after *in vitro* maturation. The reproductive failure associated with ingestion of both mycotoxins may be due to an effect on oocyte quality (Alm et al., 2006).

AFs and DON can be harmful to pigs when chronically consumed at moderate concentrations of contamination causing liver damage. Even consuming moderate concentrations mycotoxins caused impacted growth

performance and alterations to the immune system resulting in damage to internal organs (Weaver et al., 2013).

Feeding contaminated diet (8.6 mg/kg DON, 1.2 mg/kg ZEN) to pigs for 42 days resulted in a significant decrease in performance of the piglets, increased relative uterus weight and altered serum parameters. Both DON and ZEN dose-related toxic effects were demonstrated (Doll et al., 2005).

3.2.2.5. Additives as detoxifying agents

The use of additives with protective properties against mycotoxins and addition of non-nutritive adsorbing agents, microorganisms or biomolecules capable of reducing the bioavailability of mycotoxins (mycotoxin-detoxifying agents) is increasing. Two main categories of feed additives can be defined; adsorbing agents that decrease mycotoxin bioavailability, and biological agents (bacteria, yeast, fungi, protozoa and enzymes) which degrade mycotoxins decreasing their potential toxicity. Despite the many publications on biological transformation of mycotoxins by microorganisms, their application in practice in detoxification of animal feeds has been limited. This may be due to lack of information about mechanisms of transformation, toxicity of transformation products, effects of the transformation reactions on nutritional values of the feeds, and safety toward animals (Boudergue et al., 2009).

The microbial feed additive Mycofix Select has potential ability to prevent DNA damage induced by DON and reduce the risk of DNA damage in immune cells, which underlines their possible beneficial effect on the immune system in DON intoxication (Awad et al., 2014). Addition of the microbial feed additive, a commercial antidote for DON mycotoxin, was effective in

overcoming DON effects on fearfulness and growth parameters of broilers (Ghareeb et al., 2014).

The adsorbing agent GMA was partially effective in preventing the immune suppression effects of DON, and was able to counteract the negative effects on duodenal morphometry. The beneficial effects of GMA may be due to mechanisms in addition to DON adsorption in the gut (Devreese et al., 2014a).

The addition of feed additives (a clay additive, a clay and dried yeast additive and a clay and yeast culture additive) can lessen the negative effects of AFs and DON on the immune system and internal organs, subsequently improving the health of the pig; therefore, they may be beneficial at reducing mycotoxin effects. Both clay and yeast materials helped to minimize immune and inflammatory challenges, as measured by the immune parameters. The benefits of these additives are variable, and the results are not clear as to which materials provide a stronger protection from the mycotoxins (Weaver et al., 2013).

The addition of aluminosilicate (AS) as a detoxifying agent was not effective in preventing the absorption of mycotoxins ZEN and DON. A tendency for decreased feed intake, significantly altered serum parameters and a tendency for decreased serum tocopherol levels were attributed to the supplementation (Doll et al., 2005).

3.3. Toxicokinetics/ADME studies

Toxicokinetics/absorption, distribution, metabolism and excretion (ADME) studies, focused on the determination of the toxicokinetic profile, mycotoxin persistence and accumulation potential, were mainly focused on

DON, FBs, ZEN, and the emerging mycotoxins BEA and ENNs. Also mycotoxin combinations such as DON/T-2 and DON/T-2/ZEN/OTA have been studied. The most used laboratory animal was the pig followed by rat and chicken. In one of these trials pig and chicken were used, allowing evaluation of the variations between species because of their wide variations in their responses to toxicological compounds. There is great variation in how toxicological compounds are absorbed and metabolized by different species and the differences between species and the amount and type of enzymes possessed hinder extrapolation of the data from these animal assays to humans.

The mycotoxin administration was oral, IV, and oral/IV in a two-way cross-over design. The summary of toxicokinetic studies is shown in Table 7.

Table 7. Toxicokinetic/Bioavailability/ADME studies of Fusarium mycotoxins: animal species, number of animals, studied mycotoxins, application routines, doses administered, exposure time and references.

Toxicokinetic / Bioavailability / ADME						
Species	**Animals**	**Mycotoxin**	**Application routine**	**Doses**	**Exposure time/sample**	**Reference**
Rats	n=6	DON, D3G	Oral	DON: 2 mg/kg bw D3G: 3.1 mg/kg bw	1,8 and 15 days	Nagl et al. 2012
Pig	n=16	DON	*Kinetic chronic* Contaminated feed	69 mg/kg bw	5-8 weeks	Goyarts and Danike 2006
			Kinetic acute Oral /IV	77 mg/kg bw 53 mg/kg bw	Single bolus	
	n=11		*Balance chronic*	163 mg/kg bw	4-6 weeks	
Pig	n=16	FB1	Contaminated feed	37 mg/kg bw/day	10 days	Fordor et al. 2008
Pig	n=16	FB1, FB2, FB3	Contaminated feed	FB1: 45 mg/kg feed FB2: 8.6 mg/kg feed FB3: 4.6 mg/kg feed	10 days admin. +10 days elimin.	Szabó-Fodor et al. 2008
Pig	n=10	ZEN	IV	10 mg/kg bw	14 days	Dänicke et al. 2005
Pig	n=5	EN B1	IV and Oral (two-way cross-over)	0.05 mg/kg bw	Plasma	Devreese et al. 2014b
Pig	n=1	BEA, ENN A, ENN A1, ENN B, ENN B1	Oral	0.05mg/kg bw	Plasma	Devreese et al. 2013

Chicken	n=24	DON, T-2, ZEN	IV and Oral (two-way cross-over)	DON: 0.75 mg/kg bw T-2: 0.02 mg/kg bw ZEN: 0.3 mg/kg bw	72h	Osselaere et al. 2013b
	Chicken n=2	T-2, HT-2	IV	0.15 µg/kg bw	Plasma	
	Pig n=1	DON	Oral	0.05 mg/kg bw	Plasma	
Chiken Pig	Chicken n=2 (fasted and non-fasted)	DON	Oral	0.015 mg/kg bw	Plasma	De Baere et al. 2011
	Chicken n=12	DON	Contaminated feed	5.5-9.5 mg/kg feed	21 days	
Rat	n=24	T-2	Oral	3 mg/kg feed	Urine Feces	Yang et al. 2013

3.3.1. Deoxynivalenol

In balance studies radiolabeled doses are given to intact or surgically prepared animals and samples including blood, bile, urine, feces and expired air are collected to determine the processing of the toxic-related material and to investigate its absorption and possible retention. A balance study was carried out by Goyarts and Dänicke (2006) to collect further metabolic data, to determine a quantitative DON balance over a longer period of time (5 days) after chronic exposure and to estimate systemic DON absorption based on its urinary excretion. After feeding pigs with control or DON-contaminated diets (163 mg/kg bw) for 5–6 weeks a quantitative collection of urine and feces was carried out. DON excretion was significantly influenced by dietary treatment: concentrations of DON, but not its metabolite de-epoxy-DON, in urine and feces were significantly increased after feeding the DON-contaminated diet. In the control and DON-contaminated group, 61.8% and 49.7% of the ingested DON was eliminated in the urine which was the main route of excretion, whereas just 4.8% and 2.5% of the DON intake of control and DON diet was excreted in the feces. De-epoxy-DON was only detected in excrements of pigs fed the DON-contaminated diets over 6 weeks whereas it was not detected after 4 weeks of DON exposure or in the control group. Occurrence of de-epoxy-DON was accompanied by a superproportional reduced excretion of the parent toxin. The mean excretion of de-epoxy-DON in pigs fed a DON-contaminated diet was approximately 10 times higher in the feces than with the urine, whereby de-epoxy-DON was the predominant compound in feces (81–92% of total metabolites). Total recovery of the ingested DON as parent toxin and its metabolite de-epoxy-DON in feces and urine was 66.6% and 54.0% for the control and DON-contaminated group, respectively.

The results of the DON balance study confirmed the assumption that not all animals are able to detoxify DON to the metabolite de-epoxy-DON and this metabolism occurs primarily in the large intestine, where it is unlikely that absorption proceeds. Therefore, the ability to de-epoxidate does not seem to contribute to a substantial detoxification of DON. Furthermore, it was shown that quantitative urinary recovery of DON can be considered as an indicator for its systemic absorption as it approximates the bioavailability as estimated by the kinetic study.

Oral exposure of a diet contaminated naturally with DON resulted in a rapid absorption, a high distribution and low metabolism. More than 50% of DON was absorbed from naturally contaminated feedstuffs and could be recovered in the serum of pigs by the AUC-method and in urine employing the quantitative balance technique. This could explain, at least in part, the high susceptibility of pigs to DON in contrast to other species, e.g., ruminants. However, the effects of glucuronide conjugation of DON on toxicity and excretion behavior in pigs have to be clarified, assuming a high comparability of digestion and excretion in humans. Therefore, oral administered DON was quickly absorbed to an extent of over 50%, highly distributed and only poorly metabolized. Although DON is poorly detoxified, it is rapidly excreted and is not found in remarkable concentrations in serum after 24 h (Goyarts and Dänicke, 2006).

D3G is partly bioavailable in rats. However, the majority of administered D3G was cleaved during digestion and subsequently excreted in feces. Thus, D3G present in food and feed seems to have a significantly lower toxic equivalency compared to DON (Nagl et al., 2012).

3.3.2. Zearalenone

Terminal elimination half-life of ZEN was reduced in pigs of control group injected with ZEN, to 1.1 h when entero-hepatic cycling of ZEN was disrupted for 12 h. The maximum ZEN concentration in plasma of pigs with an induced entero-hepatic cycling of ZEN was found at 2.73 h after the bolus was given to their counterparts. It seems that substantial proportions of ZEN and α-ZOL are re-cycled via entero-hepatic re-circulation. Recovery of ZEN and α-ZOL expressed as percentage of the ZEN-bolus was characterized by a saturation kinetics in urine and duodenal digesta after 72 h. Fecal excretion started to increase steeply after 48 h and still continued to increase after 72 h. Fourteen days after the bolus injection, ZEN and α-ZOL concentrations in bile, liver and urine were lower than the detection limits of the applied method. Within this period of time a massive single bolus of ZEN is nearly completely eliminated from the body (Dänicke et al., 2005).

3.3.3. Enniatins and beauvericin

There is a big difference in oral absorption between the different ENNs analogs. After oral administration (0.05 mg/kg bw) to one pig, ENN B seems to have the highest oral absorption, followed by ENN B1, A1, A and finally BEA (Devreese et al., 2014b).

3.3.4. Mycotoxin combinations

DON has a low absolute oral bioavailability (19.3%) in broiler chickens. For ZEN and T-2 no plasma levels above the limit of quantification (LOQ) were observed after an oral bolus (ZEN: 0.3; T-2: 0.02 mg/kg bw). After

IV administration, T-2 had the shortest elimination half-life (3.9 min), followed by DON (27.9 min) and ZEN (31.8 min).

Oral bolus models respecting the maximum recommended levels in feed to test the efficacy of mycotoxin-detoxifying agents *in vivo* cannot be applied for the mycotoxins T-2 and ZEN, due to their low absolute oral bioavailability in broiler chickens. Administration of higher doses can offer a possibility, but can lead to higher risks for acute mycotoxicosis. On the other hand, plasma or blood concentrations of the tested parent toxins and their main reported metabolites as stated by the EFSA (2011) cannot be used as biomarkers to test the efficacy of mycotoxin detoxifiers in broilers, when maximum recommended levels in feed are respected (Osselaere et al., 2013b).

The main metabolic pathways of T-2 toxin were hydrolysis, hydroxylation, and de-epoxidation. One novel metabolic pathway of T-2 toxin, hydroxylation at C-9 position was demonstrated as a novel metabolic pathway. Several metabolites of hydroxylation at C-7 of T-2 toxin were detected in male Wistar rats, but they were not found in female rats nor in *in vitro* systems derived from Wistar rats (Yang et al., 2013a). T-2 toxin concentrations above LOQ could only be detected at 2 min after administration due to the low dose administered or the fast elimination (De Baere et al., 2011).

3.4. Teratogenicity

There were only three teratogenicity studies on *Fusarium* mycototoxins published in the last decade. The mycotoxins studied (FB1 and ZEN) were administered orally, IP, and SC to mice and rats. The summary of teratogenic studies is shown in Table 8.

3.4.1. Fumonisins

These studies showed that HFB1 decreased feed consumption and weight gain of pregnant rats but it was less toxic than FB1. HFB1 produced no dose-related nephrotoxic or hepatotoxic effects seen after FB1 treatment. HFB1 was not teratogenic, and did not retard fetal development. The NOAEL for fetal toxicity was 120 mg/kg, and the NOAEL for maternal toxicity was 15 mg/kg based on the significant decrease in body weight gain at 30 mg/kg/day. Although the decreased body weight gain was temporary and the body weight gain rebounded after treatment, there was nevertheless a significant decrease at 30 mg/kg/day on gestation day 0–17. HFB1 reduced female body weight gain, but was less toxic than FB1. HFB1 was not teratogenic, did not affect tissue sphingolipid ratios, did not alter reproduction or the development of fetuses, and produced no dose-related histopathological effects in females (Collins et al., 2006).

Maternal FB1 administration (20 mg/kg of bw) during early gestation resulted in 79% neural tube defects in exposed fetuses. Maternal FB1 exposure altered sphingolipid metabolism and folate concentrations in mice, resulting in a dose-dependent increase in neural tube defects that could be prevented when adequate folate levels were maintained (Gelineau-van Waes et al., 2005).

3.4.2. Zearalenone

ZEN exposure (1 mg/kg/day for 15 days) could impact maternal and fetal exposure to transporter substrates and influence fetus development through nuclear receptor modulation (Koraichi et al., 2012).

3.5. Method development

There are applied studies focused on method development and validation to determine mycotoxins in which the method usefulness is finally tested with a few animals.

These method validation studies have been achieved with ZEN, DON and the mycotoxin combination DON/ZEN/T-2/OTA and have been used in samples from chicken and pig, rat and human, and pig, respectively. In these trials the mycotoxin administration was oral, IV and oral/IV. The summary of method development studies is shown in Table 9.

3.5.1. Deoxynivalenol

DON and deoxynivalenol glucuronides were found in both human and rat urine, whereas de-epoxy-DON and its glucuronides conjugate were only detected in rat urine. DON urinary levels in the range 0.003–0.008 g/mL were detected in healthy human subjects, whereas DON and de-epoxynivalenol levels between 1.9–4.9 μg/mL and 1.6–5.9 μg/mL, respectively were found in rat urine. Differences in the urinary metabolite profile in human and rat have been shown (Lattanzio et al., 2011).

Table 8. Teratogenicity studies of Fusarium mycotoxins: animal species, number of animals, studied mycotoxins, application routines, doses administered, exposure time, and references.

Teratogenicity						
Species	**Animals**	**Mycotoxin**	**Application routine**	**Doses**	**Exposure time**	**References**
Mice (pregnant)	n = 20	FB1	IP	5, 10, 15, 20 mg/kg bw	Gestation time	Gelineau-van Waes et al. 2005
Rats (pregnant)	n = 34	HFB1 (FB1)	Oral	15, 30, 60, 90, 120 µg/kg bw	17 days	Collins et al. 2006
Rats (pregnant)	n = 22	ZEN	SC	1 mg/kg/day	15 days	Koraichi et al. 2012

Table 9. Method development studies of Fusarium mycotoxins: animal species, number of animals, studied mycotoxins, application routines, doses administered, analyzed sample, and references.

Method development						
Species	**Animals**	**Mycotoxin**	**Application routine**	**Doses**	**Exposure time/sample**	**References**
Chicken Pig	n = 8 n = 1	ZEN	IV and Oral	0.3 mg/kg bw	Plasma	De Baere et al., 2012
			IV	0.0075 mg/kg bw	Plasma	
Pig	n = 6	DON, T-2, ZEN, OTA	Single Oral gavage	0.05 mg/kg bw	Plasma	Devreese et al 2012
Rat (Wistar) Human	n = 8 n = 2	DON	Contaminated feed	3.6 mg/kg bw	Urine Urine	Lattanzio et al., 2011

4. Conclusions

This review shows that there is an increased interest in the study of *Fusarium*mycotoxins, especially since 2010. The most reviewed articles focus on the major *Fusarium* mycotoxins; however, only a few publications are about emerging mycotoxins. The most researched *Fusarium* mycotoxins are DON and FB1 followed by ZEN and T-2 toxin. The most common animals used for *Fusarium* mycotoxins *in vivo* studies are pig and rat, followed by chicken and mice. The most widely used trial types are tests of subacute, subchronic and acute toxicity, followed by toxicokinetics studies and teratogenicity. In the great majority the main route of administration is oral, mainly through contaminated feed and in some cases by oral bolus. Only in about half of the studies the administered dose is expressed as a function of animal body weight, while the remainder relates to the amount of mycotoxins present in the feed.

In acute toxicity studies the predominant studies involved mycotoxin FB1 followed by ZEN and the most used laboratory animals are rat and mice followed by the rabbit using mycotoxin administration both oral and IP.

In subacute studies the most studied mycotoxin is FB1, as in the case of acute toxicity. Other single mycotoxins studied were DON, ZEN, T-2 toxin and NIV. Also the emerging mycotoxin EN A and mycotoxin combinations as DON/T-2, DON/ZEN and DON/FB1 were studied. The most used animals were the pig and rat, followed by chicken, mice and horse. In this case the administration was oral in the great majority of assays but there are a few subcutaneous studies. DON is the most studied mycotoxin in subchronic toxicity studies followed by FB1 and ZEN. Mycotoxin combinations are also frequent as DON/ZEN and DON/ZEN/NIV/FB1. *Fusarium* mycotoxin-

non *Fusarium* mycotoxin combinations are also common in subchronic studies as FB1/AFs and DON/AFs. The most used animals were pig and chicken followed by rat, fish and turkey. In these long-term exposure studies the mycotoxin administration was oral except in one IV study. There were only three teratogenicity studies on *Fusarium* mycotoxins published in the last decade studying FB1 and ZEN. Toxicokinetics/ADME studies, focused on the determination of the toxicokinetic profile, mycotoxin persistence and potential accumulation, were mainly focused in DON, FBs, ZEN, and the emerging mycotoxins BEA and ENNs. The most used laboratory animal was pig followed by rat and chicken.

In summary it can be concluded that the lack of uniformity complicates the overall analysis of the results of different studies because it makes objective comparison between different trials very difficult, as is comparing the effects between species and, ultimately, to extrapolation of the results to humans, which is the ultimate goal of these studies in laboratory animals. Moreover, there is a needed to increase the *in vivo* studies of emerging *Fusarium* mycotoxins not yet legislated on which the EFSA is collecting data.

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3.9 Quantitation of Enniatins in Biological Samples of Wistar Rats after Oral Administration by LC-MS/MS.

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Quantitation of Enniatins in Biological Samples of Wistar Rats after Oral Administration by LC-MS/MS

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Abstract

The emerging *Fusarium* mycotoxins enniatins (ENNs) have diverse biological properties, mainly due to their ionophoric activity, and represent a potential risk to human and animal health since they are commonly found in food and feed. *In vivo* toxicity studies are scarce and limited to the major mycotoxins. Until now, any method for the simultaneous analysis of these compounds in plasma, serum and feces from rat has been reported. A method for the extraction and determination of ENNs A, A1, B and B1 from Wistar rat samples by liquid chromatography tandem mass spectrometry has been developed. The method was successfully validated with satisfactory recoveries (70% to 106%), good intraday (<10%) and interday (<20%) precision, expressed as relative standard deviation, and good linearity between limits of quantitation (LOQ) and 100 times LOQ. Limits of detection (LOD) and LOQ were ≤1ng/ml and ≤10ng/ml, respectively. The validated method was applied for the analysis of biological Wistar rat samples that were administered a single oral dose of a mixture of ENNs (1.03-2.16 mg/kg body weigh). Blood, urine and feces samples, collected every 2 hours during the 8 hours duration of the experiment, were analysed. The administered concentrations did not cause observable adverse effects on the animals. ENNs concentrations detected in serum and urine were below LOQs. The four ENNs were detected in feces reaching the maximum concentration at 6h after administration.

1. Introduction

Mycotoxins, as toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, are common contaminants of food and feed. Intake of high doses of mycotoxins may lead to acute mycotoxicoses, which are characterized by well-described clinical signs (Antonissen et al., 2014). Mycotoxins differ in structure, which explains the great variation of symptoms. The toxicological effects produced by mycotxins can range from reproductive disorders, hepato- and nephrotoxicity, impaired immunity or dermal effects, to severe health-associated risks, including cancer and neurological disorders (Bhat et al., 2010; Anfossi et al., 2010). *Fusarium* is one of the main mycotoxin producing fungi and their mycotoxins are endowed with both acute and chronic aspects of toxicity (Afsah-Hejri et al., 2013; Cortinovis et al., 2013). Among the mycotoxins produced by *Fusarium* spp., are included the enniatins (ENNs), beauvericin (BEA), moniliformin (MON) and fusaproliferin (FUS), which are a group of bioactive compounds called emerging mycotoxins or minor *Fusarium* mycotoxins (Serrano et al., 2012). There is only limited data available about these metabolites, not only due to their late recognition but especially because of the late understanding of their role as mycotoxins (Nazari et al., 2015). Scientific information about *in vivo* toxicity of the minor *Fusarium* mycotoxins is scarce (Santini et al., 2012; Escrivá et al., 2015). The emerging mycotoxins ENNs are cyclic hexadepsipeptides formed by alternating of the D-a-hydroxy-isovaleric acid (HyLv) and different N-methylamino acid residues as valine (Val) and isoleucine (Ile) (Serrano et al., 2013). Because of the widespread presence of these emerging mycotoxins in cereals and their diverse toxic effects, ENNs might pose a health risk to humans and animals (Hu et al., 2014). The most

well-known ENNs reported as natural contaminants are ENN A, A1, B and B1. They have shown cytotoxic and apoptotic activity. ENNs are specific inhibitors of acyl-coenzyme A cholesterol acyltransferase and cationophoric compounds capable of transporting cations through the cell membrane, leading to toxic actions by an altered membrane potential and thereby disturbing the intracellular ionic homeostasis (Marín et al., 2013; Nazari et al., 2015). Furthermore, their possible subclinical effects are of greater importance as these may lead to subacute effects such as feed refusal, weight loss, reduced animal productivity, and increased susceptibility to infectious disease (Juan et al., 2014). The undeniable evidence of BEA and ENNs toxicity, as well as the presence of high concentrations of these bioactive compounds in several types of food products, had persuaded the European Food Safety Authority (EFSA) to release an opinion on the risks to human and animal health related to the presence of BEA and ENNs in food and feed (Manzini et al., 2015), but given the lack of relevant toxicity data, a risk assessment was not possible (EFSA 2014). Owing to relevant *in vivo* toxicity data is needed to perform a human risk assessment, the EFSA is still collecting data about these emerging mycotoxins. Figure 1 shows chemical structures, molecular formula and CAS (Chemical Abstracts Service) number of ENNs A, A1, B and B1.

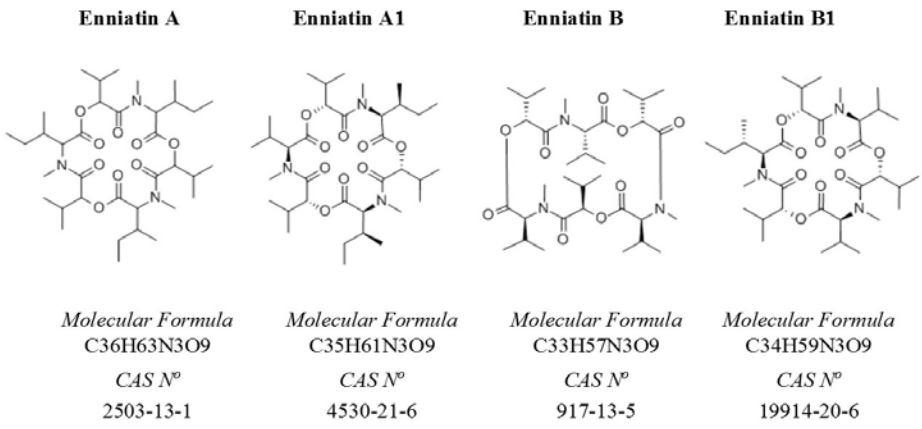

Figure 1. Molecular name, chemical structures, molecular formula and CAS number of ENNs A, A1, B and B1.

In recent years, several high-performance liquid chormatography-tandem mass spectrometric (LC-MS/MS) methods for the analysis of ENNs in food and feed have been described. Broadly, the most used extraction solvents were acetonitrile (Jestoi et al., 2009), ethyl acetate (Prosperini et al., 2013), methanol (Hu and Rychlikab, 2014; Oueslati et al., 2011) or mixes of these solvents (Juan et al., 2012; Streit et al., 2013). The extraction process could be repeated once or twice and the resultant extract, sometimes purified, is concentrated under a stream of nitrogen and the dry residue is resuspended, usually into the liquid chromatograph (LC) mobile phase. Extracts are filtered before being injected into LC, with volumes ranging from 5 to 20 µL.

Nevertheless, perhaps due to the few *in vivo* studies conducted so far, analytical methods for the simultaneous analysis of ENNs in biological matrices are scarce. The first studies about the acute toxicity of ENNs (B, B1,

A1) and death were performed in mice more than 15 years ago (Bosch at al., 1989; McKee et al., 1997), and from then only a few ENNs *in vivo* studies have been tested. Recently, new methods have been developed to facilitate the *in vivo* study of ENNs allowing their analysis from biological samples. However, any method for the simultaneous analysis of these four compounds in rat plasma, serum and feces has been reported. A method for ENN A quantitation in biological tissues and fluids including serum was developed in Wistar rats to perform a physiological test of ENN A toxicity in a 28-day repeated dose preliminary assay (Manyes et al., 2014). Another method for ENN A determination in feces, urine and serum was carried out on in Wistar rats to evaluate the immunotoxicity of ENN A after the 28-days ENN A supplementation (Juan et al., 2014). Afterwards, a method for the analysis of ENNs A, A1, B and B1, along with BEA in pig plasma was developed (Devreese et al., 2013) and a pilot toxicokinetic study and absolute oral bioavailability of ENN B1 was performed by analyzing pig plasma after oral and intravenously administration (Devreese et al., 2014).

The aim of this study is to develop a simultaneous extraction and determination method of four *Fusarium* mycotoxins (ENNs A, A1, B and B1) using liquid chromatography combined with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in three biological samples (serum, urine and feces) from Wistar rats. The method, once validated, was used for the analysis of Wistar rats samples that were administered a mixture of ENNs by a single oral dose, in a 8 hours duration experiment to simulate a situation of acute toxicity.

2. Material and methods

2.1 Chemical and reagents

Acetonitrile and methanol were purchased from Fisher Scientific (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). Standard solution stock (purity: 99%) of ENN A (mw: 681.92 g/mol), ENN B (mw: 639.82 g/mol), ENN A_1 (mw: 667.87 g/mol) and ENN B_1 (mw: 653.85 g/mol) were purchased from Sigma Aldrich (Madrid, Spain).

2.2 Preparation of standards

The standards of ENNs A, A_1, B and B_1 were obtained from Sigma-Aldrich (Madrid, Spain). All stock solutions were prepared by dissolving 1mg of mycotoxin in 1ml of pure methanol, obtaining a 1 mg/ml (1000mg/L) solution. These stock solutions were diluted with methanol in order to obtain the appropriate multi-compounds working standard solutions. All standards were kept at -20ºC.

2.3 LC/MS/MS Analysis

Detection and quantification was performed with 3200 QTrap® LC/MS/MS System (Applied Biosystems, Foster City, CA) equipped with a Turbo V® ionspray ESI source coupled to Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation of analyte was performed with a reversed-phase analytical column (Gemini ® C18 column, 3-µm particle size, 150×2 mm, I.D.), equipped with a C18 (4×2

mm, I.D.; 5 µm security guard cartridge) all from Phenomenex, Madrid, Spain. Elution was carried out in binary gradient mode. Both mobile phases contained 5 mM ammonium formiate and were composed of water/formic acid 99:1 (v/v; eluent A) and methanol/formic acid 99:1 (v/v; eluent B). The flow rate was 0.250 ml/min. The gradient program started with 90 % A and 10 % B and was kept until 3 min, afterwards a linear gradient was applied, reaching 70 % B after 1.5 min (holding time, 3 min). Other linear gradients to 80 % B (6 min) and 90 % B (14 min) were included. Finally, gradient switched back (5 min) to 90 % A. MS/MS was performed in the selected reaction monitoring (SRM) mode in the positive ion electrospray mode. For each compound, two characteristic product ions were monitored; the first and most abundant one was used for quantification, while the second one was used for qualification. The instrument was operated using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature, 350 °C; desolvation temperature 270ºC, curtain gas 20 psi; ion source gas 1 (sheath gas) 50 psi; ion source gas 2 (drying gas) 55 psi, ion spray voltage 5500 V and collision gas energy 5 eV. Nitrogen served as nebulizer and collision gas. The aqcuistation of two SRM transitions per analyte allowed to confirm the identity of the positive results. Analyst version 1.5.1 software (Applied Biosystem/AB sciex) was used fot data acquistation and processing.

2.4 ENNs extraction of biological fluids

Sample processing consisted of ACN extraction added directly in the samples in 2/1 (v/v or v/w) proportion. The samples were homogenized by vortex mixer VWR international (Barcelona, Spain) at maximum speed and room temperature for 30 sec. and then were centrifuged at 4000 rpm and 4 °C

for 10 min. (Centrifuge 5810R, Eppendorf, Germany). Urine samples were salt added (MgSO4/NaCl: 0.2g + 0.2/ ml urine) previously centrifugation to obtain two extraction phases. The supernatant corresponding to organic phase was collected into new tubes and the process was repeated once more. The resultant extract was dryness evaporated under nitrogen flow at 30ºC and the reduced pressure of 5 psi (Turbovap LV, Zymark, Runcorn, UK). Dried samples were resuspended in 1 ml of methanol and filtered with a 0.22 µm filter (Phenomenex, Madrid, Spain) prior to their LC-MS analysis.

2.5 In vivo study design

Thirteen female Wistar rats (two months old and average bw: 250 g) were acquired from Pharmacy animal facility (Universitat de València, Spain). As they were acquired from the same facility were the experiment was carried on, no adaptation period was needed. The Institutional Animal Care and Use Committee of the University of Valencia (IACUC) approved all animal procedures (protocol nº A1338818442265). Animals were divided in two groups: 6 rats in the control group, 7 rats in the treated group. Rats were housed in a windowless room with a 12h light-dark cycle. The study rooms were maintained under controlled conditions appropriate for the species (temperature 22ºC, relative humidity 45-65%). First of all, the animals, fasted for 12 hours before the experiment, received a single dose of a mixture of ENNs (A, A_1, B and B_1) or water if control. Mycotoxins were administered dissolved in 1 ml of water by oropharyngeal administration using a metal cannula. The trial had 8 hours duration to simulate a situation of acute toxicity. They were placed individually into metabolic cages to facilitate the collection of organic waste samples during this time. During the test period

animals were without feed but water *ad libitum*. From the administration time, blood, urine and feces samples were collected every 2 hours. ENNs were naturally produced by a microbial fermentation of the strain *Fusarium Tricincum* CECT 1036 in wheat. The extract administered was obtained by methanol extraction following analysis by LC-MS/MS. ENNs concentrations were: ENN B= 513 µg/ml, ENN B1= 706 µg/ml, ENN A1= 1081 µg/ml and ENN A= 593 µg/ml. The dose administered were prepared by drying 0.5 ml of the extract and resuspending in 1 ml of water. The dose administered relative to body weight are shown in Table 1.

Table 1. Doses of ENNs B, B1, A1 and A (mg/Kg bw) administered to rats.

Mycotoxin	Dose (mg/Kg bw)
EN B	1.03
EN B1	1.41
EN A1	2.16
EN A	1.19

2.6 Method Validation

Linearity was evaluated by preparing matrix-matched calibration curves by applying ENNs standard solutions directly onto the blank samples over a concentration range of 2 and 1000 ng/ml. Calibration curve samples were prepared analyzing in triplicate 9 concentration levels (2, 10, 25, 50, 100, 250, 500, 800 and 1000 ng/ml) by application of standard solution directly onto the blank samples, and the correlation coefficients (r), which had to be ≥0.99%, were calculated. *Matrix effect,* undersood as the influence of one or more undetectable components from the sample on the measurement of the analyte concentration or mass, was calculated comparing the slope of the

resulting linear regression curve of the standard calibration solutions, with the related slope of the matrix-matched curve prepared by spiking a blank sample before extraction. The percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A), was calculated in serum, urine and feces using standard solutions by analyzing in triplicate 9 concentration (2, 10, 25, 50, 100, 250, 500, 800 and 1000 ng/ml) in theses samples matrix. If the ratio (B/A x 100), defined as the absolute matrix effect (ME %), is 100% indicates that there is no absolute matrix effect whereas there is signal enhancement or suppression if the value is < or > 100%, respectively. *Limit of detection (LOD) and limit of quantification (LOQ)* were calculated as signal-to-noise ratio, S/N=3 and S/N=10, respectively, by analyzing decreasing concentrations of the spiked biological samples in calibration curves and using the analyst version 1.5.1 software (Applied Biosystem / AB Sciex). For the *recovery* test samples of 0.5 ml (serum and urine) and 0.5g (feces) were fortified in three replications and in three concentration levels (20, 100 and 500 ng/ml) and each sample was extracted with ACN 30 min after spiking. Accuracy and precision were calculated. Intraday and interday precision parameters (*repeatability* and *reproducibility*) were determined by analyzing by triplicates blank samples spiked at 3 different concentration levels (repeatability) and by repeating the analysis in 3 different non-consecutive days (reproducibility).

2.7 Calculations

Recoveries of fortified biological samples were calculated as the percentage of the ENNs (A, A1, B and B1) amount detected related to the total ENNs spiked in each of them. Recovery studies were performed in triplicate

and the spiking levels were 20, 100 and 500 ng/ml. For the biological samples of treated rats, the absolute amount of mycotoxins (ng) was calculated by multiplying the measured sample volume or weight by the ENNs concentration found.

3. Results and Discussion

3.1 Optimization of Mass Spectrometry Conditions

The optimization of MS/MS parameters was performed by flow injection analysis for each compound; entrance potential (EP) was set at 10V, for all analytes. The quantification and qualification ion transitions of the respective mycotoxins, the declustering potential, collision energy, and collision cell exit potential programmed are shown in Table 2, as well as the indicative retention times.

Table 2. Optimized MS\MS parameters for ENNs.

Mycotoxin	Retention time	Precursor ion (m/z)	Products ions (*m*/z)	DP (V)	CE (V)	CXP (V)
ENN A	12.02	699.4 [M+NH4]+	210,1^{Q} 228,2^{q}	66	35 59	14 16
ENN A1	12.23	685.4 [M+NH4]+	210,2^{Q} 214,2^{q}	66	37 59	8 10
ENN B	12.45	657.3 [M+NH4]+	196,1^{Q} 214,0^{q}	51	39 59	8 10
ENN B1	12.62	671.2 [M+NH4]+	214,1^{Q} 228,1^{q}	66	61 69	10 12

Product ion. Q: quantification transition; q: qualification transition, DP: declusteing potential, CE: collision energy, CXP: collision cell exit potential.

3.2 Method performance

The validation process was carried out using blank serum, urine and feces samples with no detectable mycotoxin. Linearity, evaluated by preparing matrix-matched calibration curves (2-1000 ng/ml) showed a linear trend with a correlation coefficient (r) >0.99. Little matrix effect was observed with values for ENN B, B1, A1 and A of 110, 104, 95 and 92%, respectively; and 107, 96 and 97% for serum, urine and feces respectively. These data, near 100%, indicate that there was no absolute matrix effect (Table 3). LODs and LOQs varied depending on the mycotoxin showing the lowest values for ENN B and B1 (LOD=0.2, LOQ= 2 ng/ml) followed by ENN A1 (LOD=0.5, LOQ= 5 ng/ml), and ENN A (LOD=1, LOQ= 10 ng/ml). The intraday and interday precision were evaluated on spiked samples at three concentration levels (20, 100 and 500 ng/ml) corresponding to 2, 10 and 50 times the maximum LOQ (10 ng/ml). The values <10% for the intraday test and <20% for the interday test demonstrated good repeatability and reproducibility for HPLC-MS/MS. Recoveries were satisfactory in the three matrices, with values for serum, urine and feces between 94-106%, 75-104% and 70-104%, respectively, and RSDs below 20%. Table 3 shows the results obtained with each matrix spiked at level of 100 ng/ml, corresponding to 10 times the maximum LOQ.

Table 3. LOD and LOQ, Recovery, intra- and inter-day precision, matrix effect and linearity in serum, urine and feces spiked at 100 ng/ml or ng/g.

Sample	Compound	LOD (ng/ml)	LOQ (ng/ml)	Recovery (R%±RSD)	Precision (%RSD)		Matrix effect (%)	Linearity (r^2)
					Intra-day	Inter-day		
Serum	EN B	0.2	2	94 ± 11	9	10	109	0.9917
	EN B1	0.2	2	106 ± 15	1	9	113	0.9908
	EN A1	0.5	5	94 ± 12	7	15	107	0.9979
	EN A	1	10	103 ± 15	7	18	99	0.9946
Urine	EN B	0.2	2	75 ± 10	3	7	116	0.9910
	EN B1	0.2	2	102 ± 13	7	14	67	0.9981
	EN A1	0.5	5	97 ± 14	6	11	108	0.9982
	EN A	1	10	104 ± 10	9	11	95	0.9973
Feces	EN B	0.2	2	95 ± 16	1	17	104	0.9914
	EN B1	0.2	2	70 ± 14	5	7	133	0.9987
	EN A1	0.5	5	96 ± 18	9	19	70	0.9927
	EN A	1	10	85 ± 15	5	11	82	0.9961

3.3 Analysis of Wistar rat biological samples.

The developed mycotoxin method was used to analyze biological samples from 13 Wistar rats that were administered a simple oral dose of ENNs mixture (A, A1, B and B1) by an oropharyngeal cannula. The ENNs dose administered were between 1.03 and 2.16 mg/Kg bw. All doses are shown in Table 1.

The administered concentrations did not cause observable adverse effects on the animals during the 8 hours of the experiment. Blood, urine and feces samples were collected before and at different time points after the administration (2, 4, 6 and 8h). Samples were kept at -20 ºC until their analysis. The concentrations of ENNs detected in serum for all time points were below LOQ. This could be due to the faster ENNs absorption, described by Devreese et al. (2013) in which the maximal concentration levels after oral bolus administration (0.05 mg/kg bw) of ENNs were between 20 and 30 min. ENNs in urine were also detected below LOQs indicating that the urine not seems to be the main elimination rout, at least for the unchanged compounds. May be the extensive ENNs metabolisation previously described by Faeste et al. (2011) and Ivanova (2011) could explain the low levels of intact compunds detected in urine.

ENNs B and B1 were detected in feces at all-time points in treated rat samples, reaching the maximum concentrations at 6 hours after administration. The concentrations levels detected at different time points for ENNs B and B1 are showed in Figure 2.

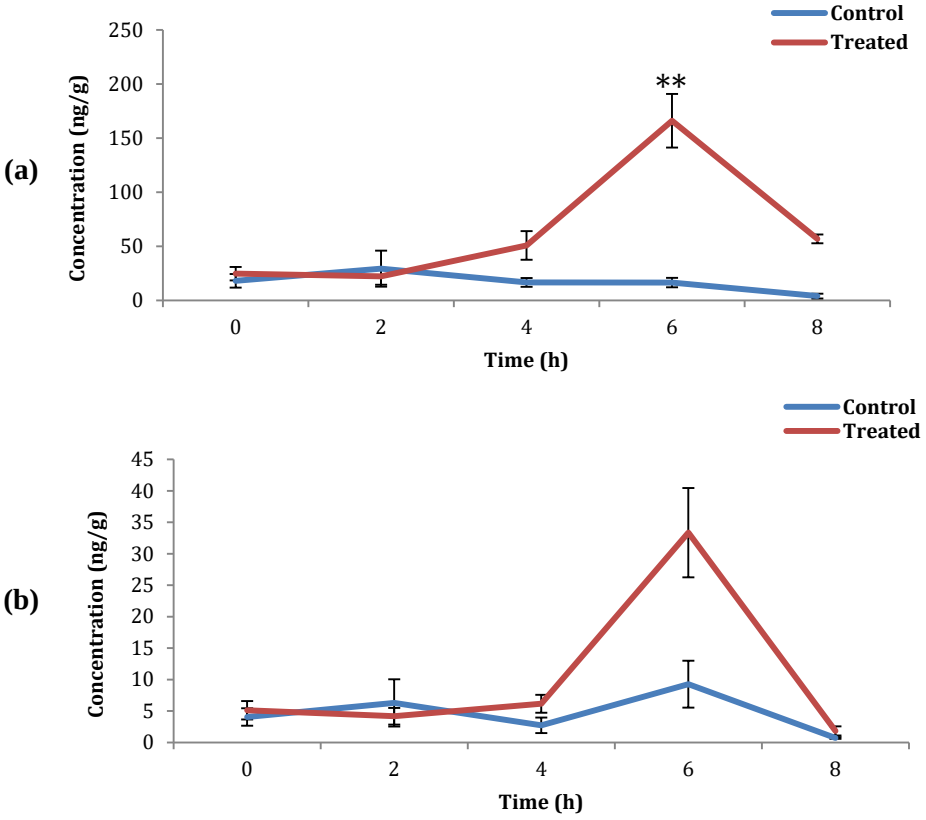

Figure 2. Concentrations detected in feces at 0, 2, 4, 6 and 8 hours after administration for ENN B (a) and ENN B1 (b) for control and treated groups. (** $P \leq 0.01$).

ENNs A and A1 were only detected at 6h after administration with concentrations close to LOQ. The concentrations detected in feces for all ENNs and for both control and treated groups at 6 hours after administration are shown in Table 4.

Table 4. Compound, administered concentration, concentration values of feces for control and treated animals obtained at 6 hours after administration. Nd: non detected.

	Concentrations in feces (6h)		
Compound	**Administered (µg/ml)**	**Detected (ng/g)**	
		Control	*Treated*
ENN B	128.25	16.46 ± 4.36	166.06 ± 20.80
ENN B1	173.5	9.26 ± 3.72	33.35 ± 7.10
ENN A1	270.25	n.d	8.08 ± 2.05
ENN A	148.25	n.d	10.52 ± 2.19

ENN B was also detected in control feces samples along with lesser concentrations of ENN B1, suggesting that the commercial feed usually employed to feed Wistar rats is probably contaminated with these compounds.

Figure 3 and Figure 4 show a LC-MS/MS chromatogram of feces samples from a control and treated rat, respectively.

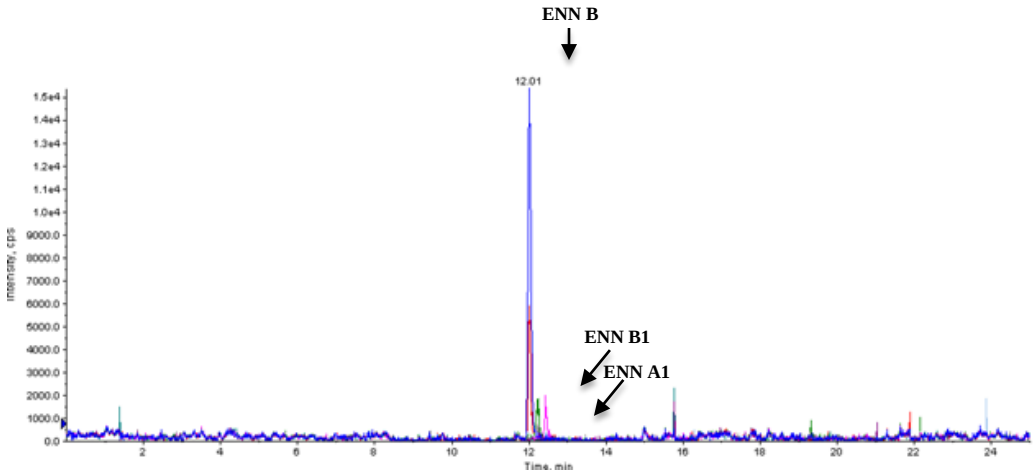

Figure 3. LC-MS/MS chromatogram showing the high concentration of ENNs B and the presence of ENNs B1 and A1 in a feces sample from one control rat.

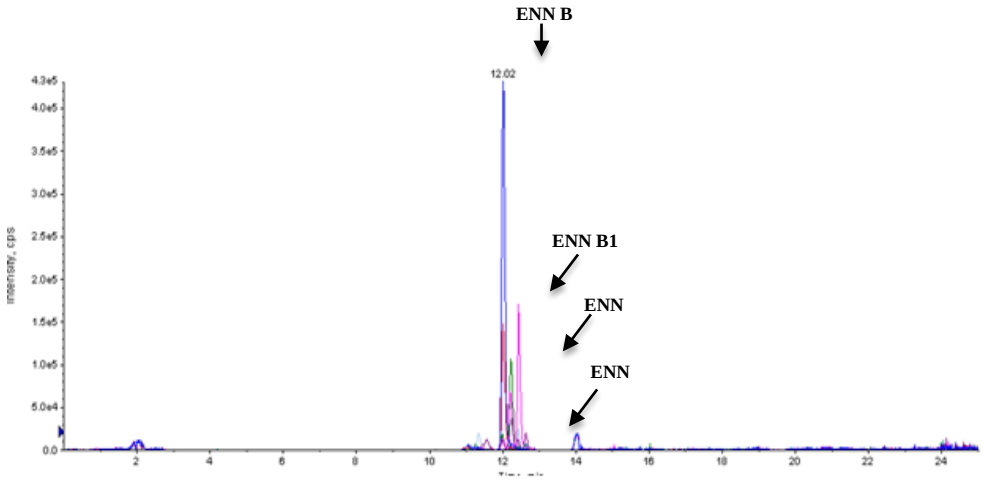

Figure 4. LC-MS/MS chromatogram showing the presence of ENNs B, B1, A1 and A in a feces sample from one treated rat at 6h after administration.

4. Conclusions

The validated method was accurate, precise and sensitive for the detection and quantification of ENNs A, A1, B, B1 in serum, urine and feces rat samples. The single oral administration of a mixture of ENNs to Wistar rats at concentrations ranging from 1.03 to 2.16 mg/kg bw did not cause observable adverse effects on the animals during the 8 hours of the experiment. ENNs in serum and urine samples collected at different time points were not quantifiable due to the low concentration levels detected. The fast absorption and extensive metabolization of these mycotoxins could explain these low levels. The four ENNs were detected in feces of treated group at all-time points collected samples. ENNs are in part excreted unchanged in feces following oral administration. ENNs reached the maximum concentration levels in feces at 6h after administration. The most detected mycotoxin was

the ENN B. ENN B with lesser concentrations of ENN B1 was also detected in control feces samples. The concentrations of ENNs B and B1 detected in feces along with their presence in control evidence that the feed probably contains these compounds. Further studies are needed.

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3.10 A Preliminary Study in Wistar Rats with Enniatin A Contaminated Feed

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A Preliminary Study in Wistar Rats with Enniatin A Contaminated feed

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Abstract

A 28-day repeated dose preliminary assay, using enniatin A naturally contaminated feed through microbial fermentation by a Fusarium tricinctum strain, was carried out employing 2-month-old female Wistar rats as in vivo experimental model. In order to simulate a physiological test of a toxic compound naturally produced by fungi, five treated animals were fed during 28 days with fermented feed. As control group, five rats were fed with standard feed. At the 28th day, blood samples were collected for biochemical analysis and the gastrointestinal tract, liver and kidneys were removed from each rat for enniatin A detection and quantitation. Digesta were collected from stomach, duodenum, jejunum, ileum and colon. Enniatin A present in organs and in biological fluids was analyzed by liquid chromatography-diode array detector (LC-DAD) and confirmed by LC-mass spectrometry linear ion trap (MS-LIT); also several serum biochemical parameters and a histological analysis of the duodenal tract were performed. No adverse effects were found in any treated rat at the enniatin A concentration (20.91 mg/kg bw/day) tested during the 28-day experiment. Enniatin A quantitation in biological fluids ranged from 1.50 to 9.00 mg/kg, whereas in the gastrointestinal organs the enniatin A concentration ranged from 2.50 to 23.00 mg/kg. The high enniatin A concentration found in jejunum liquid and tissue points to them as an absorption area. Finally, two enniatin A degradation products were identified in duodenum, jejunum and colon content, probably produced by gut microflora.

1. Introduction

Enniatins (ENs) are secondary fungal metabolites that have been known for several decades (Ivanova et al., 2006). Chemically there are six-membered cyclic depsipeptides, which are commonly composed of three D-a-hydroxyisovaleric acid (Hiv) residues linked alternatively to three L-configured N-methyl amino acid residues to give an 18-membered cyclic skeleton (Zhukhlistova et al., 1999). ENs are produced by strains of several species of fungal genera as Alternaria, Fusarium, Halosarpheia and Verticillium (Supothina et al., 2004). ENs produced by Fusarium subglutinans, Fusarium proliferatum and Fusarium tricinctum are cereal contaminants, especially maize and its derivatives. ENs have been found as worldwide natural contaminants of several food and feed products (Jestoi, 2008). A few years ago, Meca et al. (2010a) reported ENs contamination of cereals available in the Spanish market and their levels ranged from 0.51 to 11.78 mg/kg. Enniatins possess a wide range of biological activities: these substances are known as ionophores, phytotoxins, anthelmintic and antibiotics compounds (Jestoi, 2008). ENs antibiotic effects have been used in a pharmaceutical commodity with anti-inflammatory properties called fusafungine (Akbas et al., 2004). There are applications for ENs in respiratory tract infections treatment and it has been reported a positive effect on wound healing after tonsillectomy (Akbas et al., 2004). Several studies have indicated that ENs change the monovalent ion transport across membranes and disrupt the ionic selectivity of cell walls. This effect is particularly debilitating in mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation (Tonshin et al., 2010).

Several studies have evaluated the ENs cytotoxic activity in vitro using as experimental model rodent, monkey, porcine, insect and human cell lines (Behm et al., 2009; Dornetshuber et al., 2009; Fornelli et al., 2004; Hyun et al., 2009; Ivanova et al., 2006; Jestoi, 2008; Lee et al., 2008; Meca et al., 2010b, 2011; Vongvilai et al., 2004; Watjen et al., 2009). In the scientific literature, only few studies related to the ENs toxicity in vivo are available. In particular, Bosch et al. (1989) studied the toxicity of ENs, among other mycotoxins, in Fusarium contaminated feed on 20-day-old white female Sprague–Dawley rats, evidencing no toxic signs. To be sure about which mycotoxins where responsible of the effects, they administrated a mixture of ENs in a single oral dose [0.05 mg/g body weight (bw)]. McKee et al. (1997) studied the hypothetic ENs property to reduce the human immunodeficiency virus (HIV) growth using the hollow fiber assay and employing mice as biological model. They used an ENs A1, B and B1 purified mixture (from 1.25 to 40 mg/kg) injected intraperitoneally every 8 h during 6 days. Any anti- HIV properties were not found but 40, 20 and 10 mg/kg doses were lethal. Considering the lack of information in physiological conditions related to the ENs toxicity in vivo, the aims of this research were: (a) to study the EN A in vivo potential toxicity trough a repeated dose assay using standard rat feed contaminated by a microbial fermentation of Fusarium tricintum strain; (b) to evaluate the EN A presence in several rat organs after a 28-day continuous ingest and (c) to identify possible EN A degradation products from gut microflora.

2. Material and methods

2.1 Chemicals

Acetonitrile, methanol and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water (518MVcm_1 resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., Danbury, CT). Potato dextrose broth (PDB) was obtained from Insulab (Valencia, Spain). Phosphate-buffered saline (PBS), glycerol and EN A standard solution stock (purity: 99% molecular weight 682.92 g/mol) were purchased from Sigma Aldrich (Madrid, Spain).

2.2 Strain and culture conditions for the ENs production on rat feed

A solid medium represented by the rat feed (Autoclaved Harlan lab blocks, Castellar del Valle´s, Spain) was utilized in this study. The medium was prepared weighting 5 kg in two 2.5-l Erlenmeyer flasks and autoclaved at 121_C during 20 min. Each one was inoculated with 25 ml of a conidia suspension (106 conidia/ml sterile water) of Fusarium tricinctum CECT 20232 in PDB. Conidial concentration was measured by optical density at 600 nm in sterile water and adjusted to 106 conidia/ml PDB as reported Kelly et al. (2006). Fusarium tricinctum CECT 20232 strain was obtained from the Spanish Type Culture (CECT Valencia, Spain), in sterile 18% glycerol. Fermentations were carried out at 25 ºC on an orbital shaker (IKA Ks 260 basic, Stanfen, Germany) in batch culture for 30 days. At the end of fermentations, the solid culture was autoclaved at 121 ºC during 20 min to

promote fungi inactivation, and after drying and milling, ENs analysis was done.

2.3 EN A extraction from rat feed

A modified method based on Chelkowski et al. (2007) for mycotoxins extraction was performed. Briefly, ENs contained in 15 g of dried contaminated feed were extracted with 100 ml methanol:water mixture (75:25) using an Ika T18 basic Ultraturrax (Staufen, Germany) for 5 min. Samples were then filtered through Phenomenex No. 4 filter paper (Torrance, CA) and thereafter the solvent was removed under reduced pressure. Each extract was dissolved in 5 ml of methanol and filtered through a 0.22-mm filter Phenomenex before toxin identification and quantitation by liquid chromatography (LC)-DAD as reported by Meca et al. (2010a) (see ''EN A extraction from tissues'').

2.4 *In vivo* study design

Ten female Wistar rats (average body weight: 250 g) were acquired from Pharmacy animal facility (Universitat de València, Spain). The Institutional Animal Care and Use Committee of the University of Valencia approved all animal procedures (protocol no A1338818442265). Animals weredivided in two groups: five rats in the control group and five rats in the treated one. Each group was housed in one cage in a windowless room with a 12-h light–dark cycle. The study rooms were maintained under controlled conditions appropriate for the species (temperature 22 ºC, relative humidity 45–65%). After 7 days of adaptation, the control group was fed ad libitum with the Harlan autoclaved lab box feed, while the test group was fed ad

libitum with EN A contaminated feed (see ‘‘Strain and culture conditions for the ENs production on rat feed’’). Treatment was maintained for 28 days in order to simulate a preliminary subchronic study, a repeated dose assay, to be able to analyze EN A distribution.

Rats were observed and weighted weekly using a weighing scale. First weight measure was taken on day 0 and, the last measure was obtained, the sacrifice day. As none of them showed significant weight gain or loss, it was assumable that all of them ate a similar amount of feed during the assay. Considering that each animal consumed daily _11.82 g of contaminated feed, the EN A daily intake was of 5.50 mg per rat. Finally, considering that the mean weight of the treated animals was 263.48 g, the EN A daily intake was 20.91 mg/kg bw/day.

Rats were sacrificed by isoflurane gas asphyxiation and blood samples were collected via cardiac puncture. Blood samples were allowed to clot for 30 min and then the serum layer was separated by centrifugation at 1000 rpm for 30 min at 4 ºC. Serum was kept at -20 ºC until analysis. The gastrointestinal tract (from stomach to rectum) was removed from all rats and digesta were collected from stomach, duodenum, jejunum, ileum and colon. Digesta collection of the intestinal compartments was carried out flushing the tissues with 1ml PBS twice. Also, the liver, kidneys, heart, thymus and spleen of each animal were recollected after terminal sacrifice (Figure 1). Each organ collected was weighted for further comparison between the treated and the control animals.

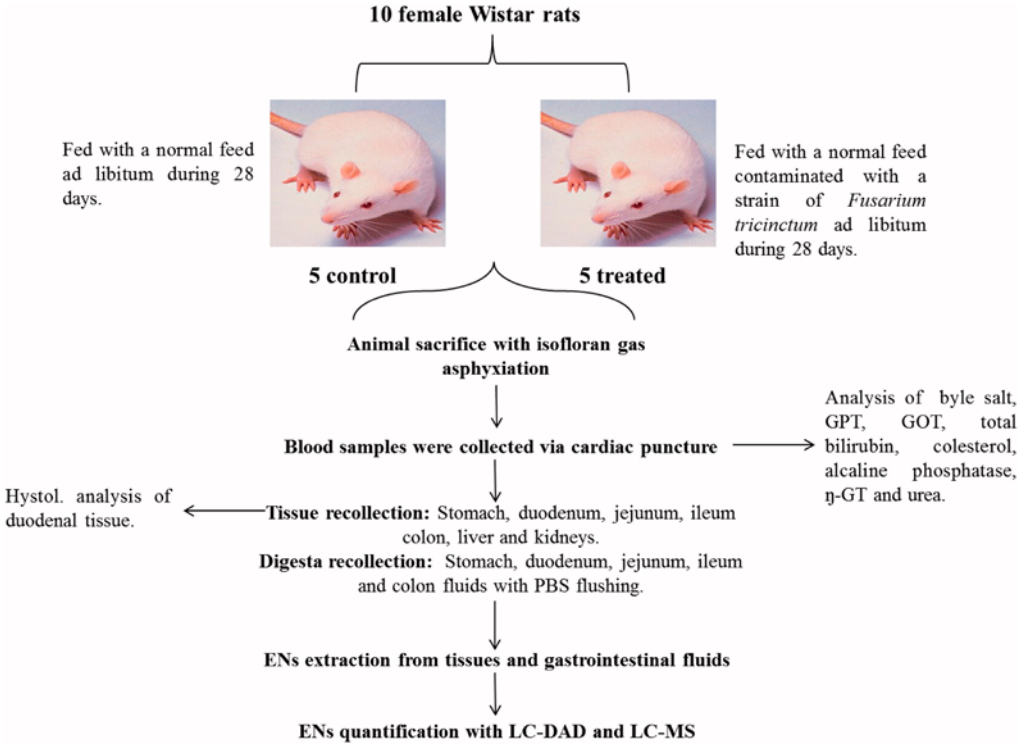


Figure 1. Schematic representation of the *in vivo* study carried out on EN A toxicity.

2.5 Histological and biochemical analysis

Histological analyses of duodenum tissue from treated and control rats, focused on enterocytes atrophy determination and on the presence of cells liquid in the gut tissue, were carried out by Echevarne laboratory (Barcelona, Spain). Duodenum tissue samples were fixed in formaldehyde (40% v/v in water), embedded in paraffin, sectioned at 4 mm and stained with haematoxylin and eosin before analysis. Biochemical parameters analyzed in serum were: bile salts, glutamic–pyruvic transaminase, glutamic–oxaloacetic transaminase, total bilirubin, cholesterol, alkaline phosphatase,

gamma-glutamyl transpeptidase and urea through ELISA kit analysis (Echevarne laboratory, Barcelona, Spain).

2.6 EN A surrogate recovery

Each tissue (0.5 g) and digest (0.5 ml) was placed in a 15-ml plastic test tube and fortified with 5 ml of EN A at 1000 ppm. About 30 min after spiking, each sample was extracted with 1ml of ethyl acetate using a vortex VWR international (Barcelona, Spain) for 3 min. Then, mixtures were centrifuged at 4000 rpm and at 4 _C during 15 min (Centrifuge 5810R, Eppendorf, Germany). Organic phases were collected into new tubes. Ethyl acetate addition, vortex, centrifugation and collection steps were repeated three times. The extracts were then evaporated for dryness under nitrogen flow at 30 ºC and reduced pressure (5 psi), in order to accelerate organic phase evaporation by decreasing the partial vapor pressure of the solvent just above the liquid surface (Turbovap LV, Zymark, Runcorn, UK). Dried samples were resuspended in 1ml of methanol and filtered with a 0.22-mm filter (Phenomenex, Madrid, Spain) prior to their LC analysis. EN A extraction from intestinal fluids To measure the amount of EN A contained in the stomach and intestinal fluids, they were extracted according to Meca et al. (2012). One milliliter of each intestinal fluid was placed in a 15-ml test tube, and extracted with 2ml of ethyl acetate using a vortex VWR international (Barcelona, Spain) for 3 min. Following steps were performed as described in the Section ‘‘EN A surrogate recovery’’.

2.7 EN A extraction from tissues

To measure the amount of EN A contained in the tissues collected from control and treated rats, it was extracted as follows: 0.5 g of each tissue was introduced in a 15-ml plastic tube and 2-ml of PBS (1_, pH 7.5) was added. Samples were completely grounded using an Ultraturrax T8 IKA (Staufen, Germany) during 3 min. EN A was extracted from the PBS solution using 4ml of ethyl acetate employing a vortex (VWR international, Barcelona, Spain) during 3 min. Following steps were performed as described in the section titled ''EN A surrogate recovery''.

2.8 LC-DAD analysis

Liquid chromatography analyses of EN A (Meca et al., 2010a) were performed using LC-10AD pumps and a diode array detector (DAD) (Shimadzu, Japan). A Gemini (150mm_4.6 mm, 5 mm) Phenomenex column was used. LC conditions were set up using a constant flow at 1.0 ml/min of acetonitrile:water (70:30 v/v) as a starting eluent system. The starting ratio was kept constant for 5 min and then it was linearly modified to 90% acetonitrile in 10 min. After 1 min, the mobile phase was set to the initial conditions in 4 min. All samples were filtered through a 0.22-mm syringe filter Phenomenex prior to injection (20 ml) into the column. EN A was detected at 205 nm. Mycotoxin identification was performed by comparing retention times and UV spectra of samples with those of pure standards. A further confirmation action was performed by co-injecting pure standards together with each sample. Mycotoxin quantitation was determined by comparing the

tested samples peak areas with a calibration curve performed with standards (n.4).

2.9 LC-MS-linear ion trap confirmation

An Applied Biosystem/AB SCIEX QTRAP_ linear ion traps (LIT) mass spectrometer (Concord, ON, Canada), coupled to a Turbo Ion Spray source was used. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole can also be operated as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration the Q TRAP can also operate in enhanced resolution scan (ER) and in enhanced product ion scan (EPI) modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing. A Gemini (150mm_2.0 mm, 5 mm) Phenomenex column was used. LC was set using a constant flow of 0.2 ml/min of acetonitrile:water (70:30 v/v) with 0.1% of HCOOH isocratically. The instrument was operated in the positive ion electrospray mode using the following parameters: cone voltage 40V, capillary voltage 3.80 kV, source temperature 350 ºC, desolvation temperature 270 ºC and collision gas energy 5 eV. EN A identification and quantitation was performed using the modality of ER, utilizing the mass range spectrometry associated to a linear ion trap permitted to obtain an enhanced characterization of the isolated compounds.

2.10 Mass spectrometry characterization of the EN A degradation products

Characterization of the newly formed compounds wasperformed as explained in the Section ‘‘LC-MS-linear ion trap confirmation’’ using the LC coupled to LIT in ER mode.

2.11 Calculations

Recoveries of fortified tissues and biological fluid samples were calculated as the percentage of the EN A detected amount related to the total EN A spiked in each of them. Recovery studies were performed in triplicate (n.3) and the mean and the SD were calculated. The spiking levels were 1.0, 5.0 and 10 mg/g. For the treated rats liquid contents and tissues, the absolute amount of mycotoxins (mg) was calculated by multiplying the measured sample volume or weight by the EN A concentration found. The mean and the SD were calculated for each organ and tissue.

3. Results and discussion

3.1 Method performance

Mean recovery of fortified tissues and biological fluid samples (n.3) at 3 levels of EN A (1.0, 5.0 and 10 mg/g) was of 97.8% (range.70–156%) with a relative SDs of 3.5% (range.1.5–5.5%). Intra-day (n.5) and inter-day (five different days) precisions were 2.4% and 9.0%, respectively. These values were below ±10%, which is the maximum variation for certification exercises for several mycotoxins (2002/657/EC). The limit of detection (LOD) and the limit of quantitation (LOQ) calculated as signal-to-noise ratio, S/N.3 and S/N.10, were 0.2 and 0.6 mg/g, respectively (Table 1). The high recovery data obtained for the mycotoxin in gastric content (156%) maybe due to the composition of this sample. In particular, the matrix present in this organ is very complex considering that it contains the majority of the macronutrients present in the feed and is not completely digested. These factors may increase the matrix effect on the recovery of the EN A.

Table 1. EN A mean recoveries, inter-day and intra-day variations, limit of detection (LOD) and limit of quantitation (LOQ) of the analytical method applied to the different matrices analyzed in this study in which EN A has been detected.

Sample	Mean recovery (%)	Inter-day variation (%)	Intra-day variation (%)	LOD (mg/kg)	LOQ (mg/kg)
Gastric content	156.3 ± 1.5	2.0	8.7	0.2	0.6
Duodenum content	97.2 ± 3.4	2.2	9.6	0.2	0.6
Duodenum	97.1 ± 2.6	2.5	9.2	0.2	0.6
Jejunum content	98.2 ± 3.4	2.5	7.5	0.2	0.6
Jejunum	97.5 ± 2.0	3.1	10.2	0.2	0.6
Colon content	70.2 ± 2.2	2.4	8.8	0.2	0.6
Colon	71.4 ± 3.1	2.0	9.6	0.2	0.6
Liver	93.1 ± 2.0	2.6	9.1	0.2	0.6
Serum	100.0 ± 2.8	2.4	8.6	0.2	0.6

3.2 EN A quantitation of contaminated feed

Feed contamination by fungi strain was carried out in order to reproduce experimentally the natural mycotoxin present in a food matrix. Fusarium tricinctum strain CECT 2032, through microbial fermentation, produced the mycotoxin in rat feed. Figure 2(a) shows the LC-MS-LIT chromatogram of the EN A detected at 465 mg/kg in the contaminated feed. Moreover, Figure 2(b) shows the MS-LIT spectrum of the bioactive compound EN A, with three characteristic signals that identify the structure of this bioactive compound as the molecular weight (MW.682.92 g/mol), the sodium and the potassium adduct. The EN A identification was also confirmed by the

comparison of the retention time (RT.27.61 min) of the EN A standard solution with the peak of the EN A present in the sample.

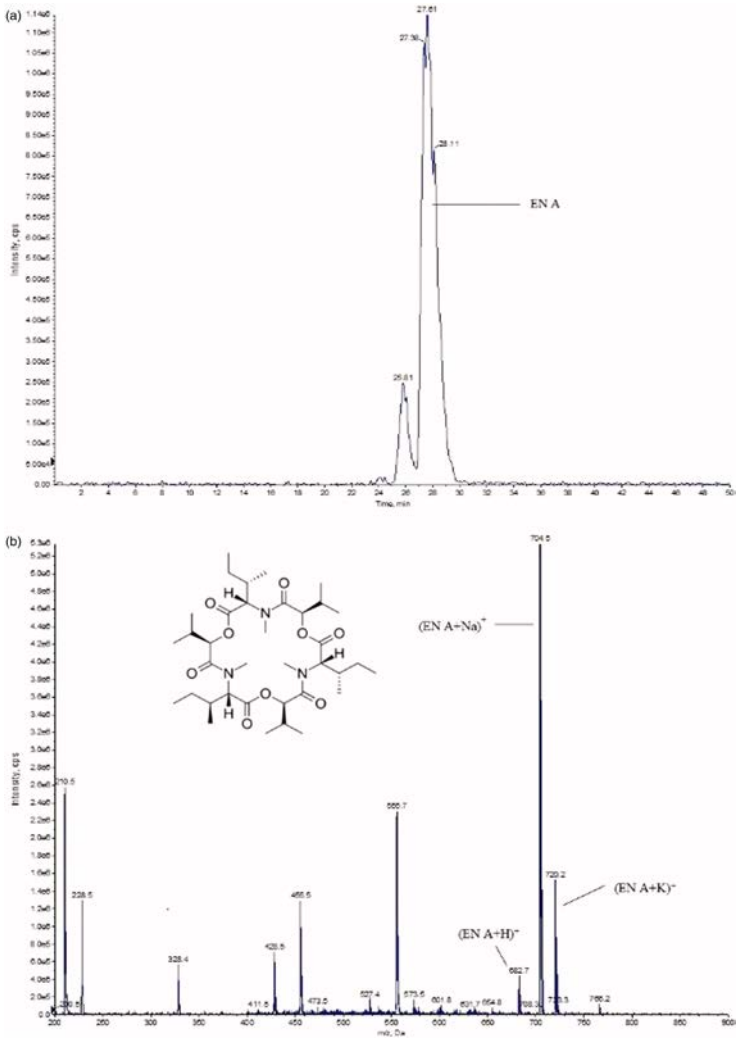


Figure 2. (a) LC-MS-LIT chromatogram of the EN A present in the feed contaminated with the strain of Fusarium tricinctum CECT 20232 and (b) mass spectrum in linear ion trap (MS-LIT) of the EN A.

3.3 EN A distribution in rat tissues and biological fluids

After the animals were sacrificed, they were examined and neither visible weight nor morphological tissue or organ changes were observed (Table 2). The histological analysis of the duodenum tissue was focused on enterocytes atrophy and cellular infiltration determination in the analyzed tissue. No differences were found between the treated and control animals. Biochemical blood parameters analyzed in the treated and control animals serum did not show any significant differences between them (Table 3). Bile salts, GTP and GOT showed lower values in treated than in control rats, but there were no statistically significant differences between both the animal groups. All biochemical parameters analyzed were within the standard healthy range. No adverse effects were observed in treated rats at the mycotoxin concentration used during the 28-day treatment. The lack of toxic effects produced by ENs on the animal model studied is in agreement with the data published by Bosch et al. (1989). The authors tested the toxic effect of deoxynivalenol (DON), zearalanenone (ZEN), moniliformin (MON), fusaraneone-X (FX), 3–15 Acetyl-DON and ENs A, A1, B, B1 naturally present in contaminated corn on 20-day-old white virgin female Sprague–Dawley rats. During 5 days, treated animals were fed with a 1:1 mixture of fermented Fusarium rice culture and complete rat diet, whereas control rats received only complete rat diet.

Table 2. Weekly body weight measurements during the study of the rats used. At week 0, first day of the 28 day-study. Comparison of organs weight of these rats measured at terminal sacrifice.

	Control rats				
	1	**2**	**3**	**4**	**5**
Organ weight (g)					
Week 0	242.0	269.8	289.2	229.0	236.4
Week 1	244.6	273.2	295.2	228.4	244.6
Week 2	252.0	274.0	296.0	229.0	250.0
Week 3	253.4	290.0	270.8	227.6	252.2
Week 4	255.4	285.3	262.6	230.3	257.1
Organ weight (g)					
Liver	8.40	9.31	9.82	8.07	7.61
Kidneys	1.61	1.85	1.59	1.41	1.36
Heart	0.84	0.97	1.03	0.72	0.92
Thymus	0.53	0.53	1.02	0.43	0.53
Spleen	0.42	0.51	0.50	0.38	0.46
	Treated rats				
	1	**2**	**3**	**4**	**5**
Organ weight (g)					
Week 0	268.8	261.4	252.6	236.6	255.4
Week 1	262.4	255.8	256.2	233.0	248.2
Week 2	272.2	269.8	253.8	234.8	259.2
Week 3	279.6	274.6	265.2	230.2	267.8
Week 4	284.4	270.2	268.1	222.3	274.3
Organ weight (g)					
Liver	7.54	8.12	7.43	7.15	7.58
Kidneys	1.53	1.66	1.46	1.59	1.63
Heart	0.92	0.89	0.97	0.93	0.78
Thymus	0.62	0.55	0.56	0.51	0.79
Spleen	0.57	0.54	0.55	0.50	0.63

Table 3. Biochemical parameters analyzed in control ($n = 5$) and treated ($n = 5$) rat serum.

	Controls rats	Treated rats
Bile salts (µmol/l)	47.6 ± 3.1	28.5 ± 3.2
GPT (U/l)	41.2 ± 3.9	28.4 ± 3.9
GOT (U/l)	98.2 ± 9.7	84.8 ± 9.8
Total bilirubin (mg/dl)	0.1 ± 0.05	0.1 ± 0.03
Cholesterol (mg/dl)	92.6 ± 9.6	85.6 ± 8.7
Alkaline phosphatase (U/dl)	71.5 ± 7.1	68.8 ± 8.6
γ-GT (U/l)	<5	<5
Urea (mg/dl)	42.6 ± 3.5	47.2 ± 5.4

To be certain of the ENs effects, 2mg of ENs mixture was administered orally to rats weighting 40 g approximately each. The observation lasted for 5 days and no toxic signs were found. This result is comparable with the data observed in our study. McKee et al. (1997) administrated intraperitoneally to mice ENs in a concentration range from 1.25 to 40 mg/ kg bw/8 h during 6 days. The top three doses of the EN mixture (40, 20, 10 mg/kg bw) tested in the hollow-fiber assay were toxic to all mice in the tested groups. With the highest dose, most deaths occurred between days 2 and 3, while for the 20 and 10 mg/kg bw dose groups, deaths occurred between days 4 and 5. For all surviving groups, there was a dose-dependent weight loss. These toxic effects indicated that a maximum-tolerated dose for the ENs was achieved within the tested dose range. Unfortunately, a comparison between the results reported by McKee et al. (1997) and those reported in this study was not possible due to the different species assayed as well as the different route of the toxin administration chosen. The use of solvents to dissolve compounds to test is not the best approach to study any molecule toxicity in vivo due to the response that the animals can have to the solvent. EN A oral administration of

a naturally contaminated rat feed was chosen for our approach in order to simulate a natural intake of the compound studied. Usually, the bioactive compound administration in animal experiments through alternative methodologies to the oral intake as intraperitoneal injection, promotes the reaching of observed adverse effect levels due to the bypass of the gastrointestinal digestion reaction that can influence the structure of the studied compound (Jestoi, 2008). The last important point is the interaction between the compound studied and the matrix effects generated by the other feed components. This phenomenon is absent in the experiments carried out with standard solutions of toxic compounds intraperitoneally injected as proven by McKee et al. (1997). The EN A concentration was determined in several organs and biological fluids. Digesta from stomach, duodenum, jejunum, ileum and colon were evaluated. The gastrointestinal tract and the kidneys were also analyzed. The EN A chromatogram present in the liver sample of a treated animal with the contaminated feed compared with the control rat is shown in Figure 3. No trace of EN A was found in any control rat organ or tissue. As shown in Figure 4(a), the lowest EN A concentration was detected in colon and duodenum with 2.2±0.7 mg/kg and 2.9± 0.6 mg/kg, respectively, probably because of a weak absorption of the bioactive compound in those gastrointestinal tract parts. The highest EN A concentration was observed in liver with 22.7±1.0 mg/kg and it may be related to its detoxification function of bioactive compounds transported from the intestine through the portal vein and others present in the rat. The molecules transported are normally accumulated into the hepatocytes where they are metabolized by the enzymes present in the bile that can modify their

chemical structure. Liver and kidneys are particularly susceptible to organ toxicity as they are the sites of toxin filtration and toxin metabolic breakdown. The secondary products produced by toxic compound metabolism can also be accumulated in the liver and may be potentially toxic for the animal body (Kerns & Di, 2008). However, no EN A was detected neither in kidneys, stomach nor ileum. Regarding the intraintestinal liquids, the highest EN A data were observed in the jejunum content with 9.6±1.1 mg/kg, whereas the lowest was detected in the duodenal liquid with 1.3±0.2 mg/kg. Significant EN A concentrations were measured in the colon content with 7.3±0.7 mg/kg, whereas the EN A data found in the gastric content and in serum were of 4.6±0.2 and 5.0±0.5 mg/kg, respectively (Figure 4b). No EN A was observed in ileum content.

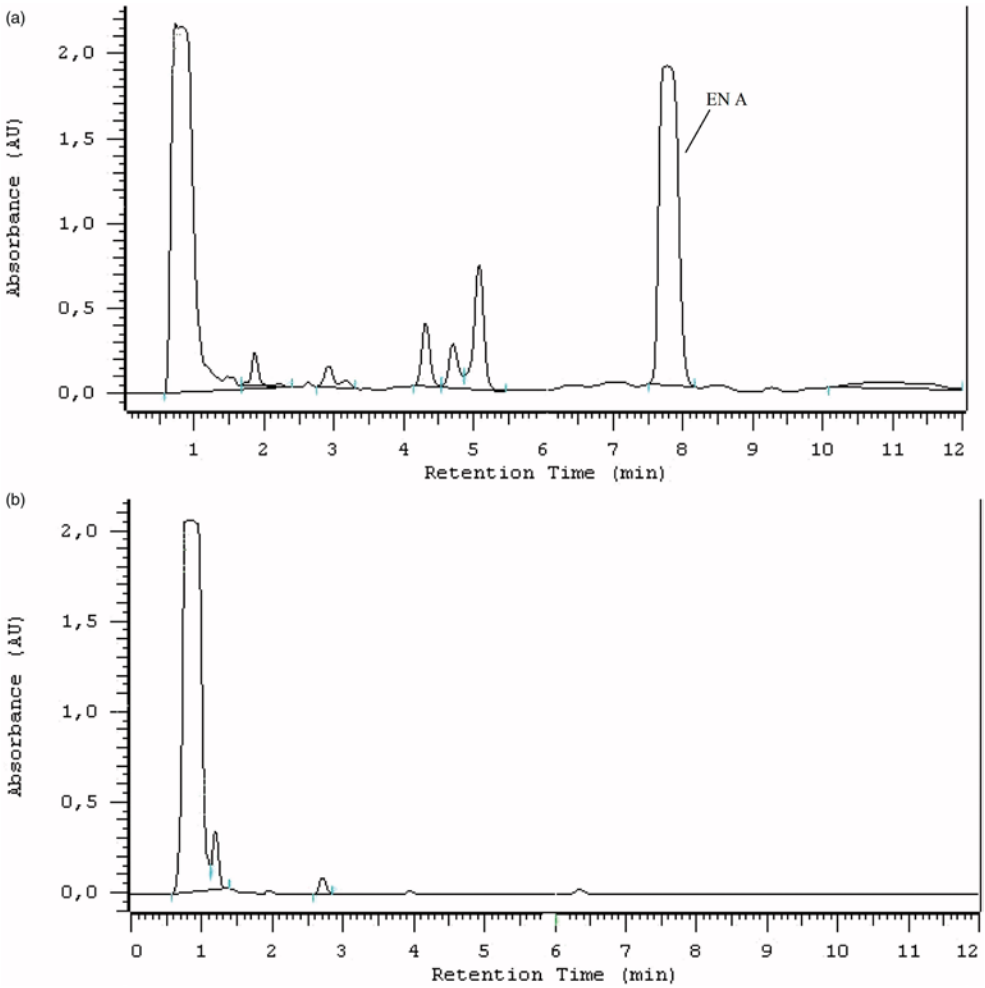


Figure 3. (a) LC-DAD chromatogram of the ENA present in the liver of the rat treated with the feed contaminated with the EN A, compared with (b) the liver of the control animals.

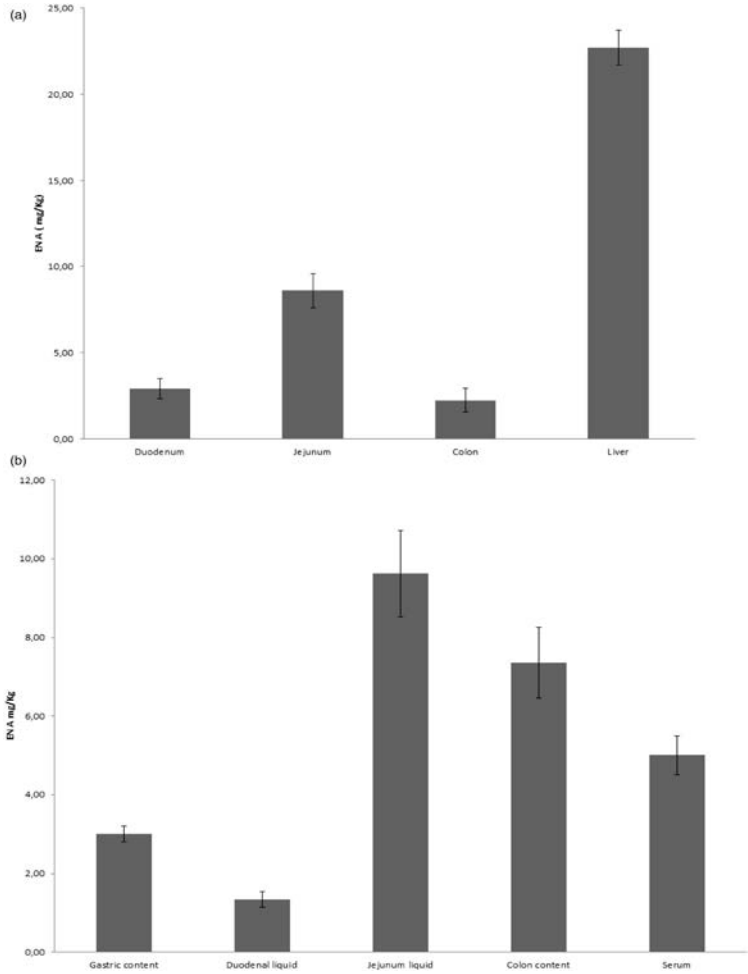


Figure 4. EN A concentration detected by LC-DAD in organs, gastrointestinal liquids and serum of treated female rats ($n = 5$). Their diet consisted in EN A contaminated feed (465 mg/kg) *ad libitum* during 28 days. Control rats ($n = 5$) ate standard feed and no trace of EN A was detected during the whole analysis in any sample. (a) Different organs from treated rats. (b) Mycotoxin concentration present in the gastrointestinal liquids and serum of treated animals.

3.4 LC-MS-LIT determination of ENs degradation products and adducts

The gastrointestinal content extracts were also injected in the LC-MS-LIT to identify possible degradation products produced through the gastrointestinal fermentation by gut microflora. Two degradation products were detected in the duodenal compartment represented by the EN A with the loss of an isoleucine (Ile) group, an amino acid characteristic of the ENs structure and by the EN A with the loss of a hydroxivaleric acid unit (HyLv) (Figure 5). The concentration in duodenum digesta of these two degradation products was of 89.7±3.2 and of 123.55± 4.1 mg/l, respectively. The presence of these newly formed compounds was confirmed employing the technique of the LC-MS coupled to the LIT.

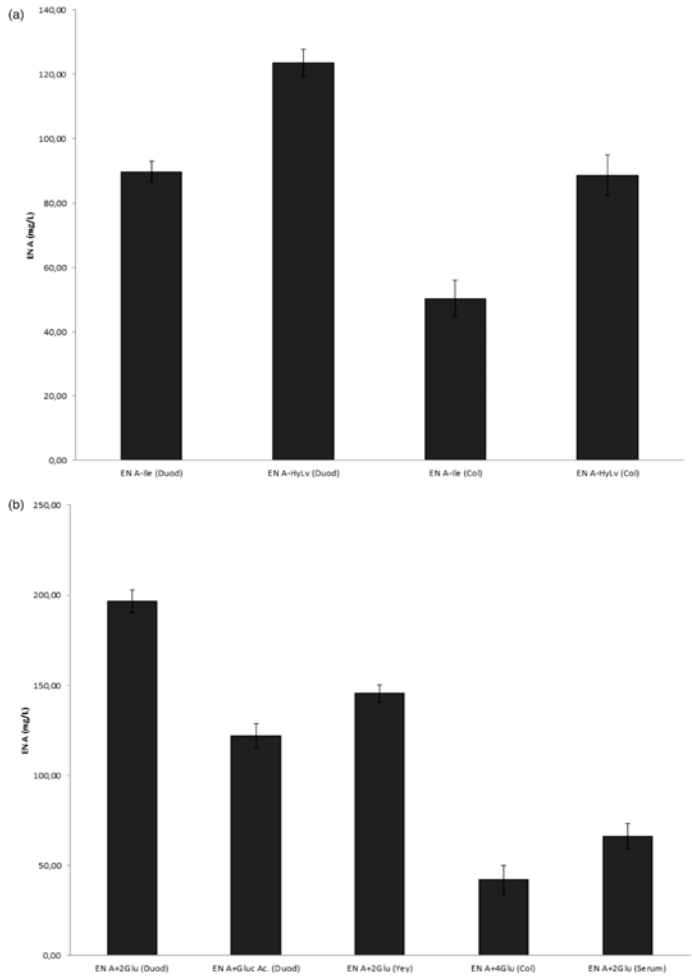


Figure 5. Quantification of the (a) EN A degradation products originated by the microbial fermentation of the ENA present in the rat feed by the intestinal microflora and (b) adducts of formation of the EN A with the glucose in the liquid of several intestinal compartments. Ile, isoleucine; HyLv, hydroxivaleric acid; Duod, duodenum; Gluc. Ac., glucuronic acid; Glu, glucose; Col, colon.

As explained in Table 4, the structure of the degradation compound ENA-Ile was confirmed by the fragment with m/z.577.1 that represents the molecular weight (MW) of the compound formed. By fragmentation of this signal, two diagnostic signals were obtained in MS2 with m/z of 547.3 represented by the EN A-Ile with the loss of a carbonyl group and m/z of 292.4, the EN A with the loss of two Ile group. The last confirmation of the structure of this degradation product was obtained by the MS3 spectra, which evidenced the characteristic fragments of the two principal ENs components as the Ile and HyLv. The MS1 fragment of the degradation product composed by the EN A with the HyLv group loss presents an m/z of 637.4. The structure of this product formed was confirmed by the fragments in MS2 with an m/z of 537.1 and MS3 with an m/z of 533.4. They represent the EN A with the loss of four molecules of water and two HyLv units. Definitive confirmation fragments in MS^3 were the signals with m/z of 84 represented by one HyLv unit and 168.0 by two HyLv units. The presence of this important EN A structural component in MS^3 confirmed the structure of the degradation product formed. The adducts formed between the EN A and the macronutrients present in rat feed detected and characterized are described in Table 5. In the duodenum and jejunum compartments the adduct formed with the EN A and two molecules of glucose were detected. As shown in Table 5, this newly formed compound presents a m/z of 1022.0. The structure of the bioactive compound formed was confirmed by the fragments obtained in MS2 and represented by the signals with m/z 937.3 and 916.3 that confirmed the loss by the structure of the adduct of an EN A component as the HyLv. In MS^3 spectra an important diagnostic signal was observed with an m/z of

181.16 represented by the MW of a glucose unit. The concentrations calculated for this newly formed compound in the duodenum and jejunum compartments were 196.74±6.3 and 149.39±4.9 mg/l, respectively. Another important adduct detected in the duodenal compartment was the reaction product originated by the reaction between the EN A and the glucuronic acid. Uridine diphosphate glucuronosyltransferase (UDP-GT or UGT) is a family of inducible microsomal isoenzymes associated with the liver, intestine, lung and olfactory epithelium. These isozymes catalyze glucuronidation, the transfer of glucuronic acid from the high-energy nucleotide UDP-glucuronic acid (UDP-GA) to an electronegative group on a wide variety of endogenous and xenobiotic substrates (Hayes, 2007). The concentration found for this adduct in the duodenal compartment was 121.98±6.8 mg/l. The confirmation of the adduct formed was carried out using the LC-MS coupled with the LIT operating in MS1, MS2 and MS3. The ER spectra in MS1 evidenced a diagnostic fragment with an m/z of 112.3 that represents the EN A coupled with two units of glucuronic acid. The presence of that compound was confirmed in the MS2 spectra with a fragment with an m/z of 914.4. The final confirmation of the coupling adduct obtained with the reaction between the EN A and the glucuronic acid was evidenced in the MS3 spectra with the fragment corresponding to the MW of the glucuronic acid with an m/z of 195.1.

Related to the adducts formed with EN A and macronutrients, in the colonic compartment the product of the reaction was detected between the EN A and four glucose units. This product was detected at the concentration of 42.02±8.2 mg/l and presents an MW of 1517.8 g/mol in MS1 spectra. The presence of the glucose in the adduct structure was confirmed by the

localization of several fragments in the MS^2 spectra and, in particular, one fragment present in the MS^3 spectra with an m/z of 724.6, represented by four glucose units. Among the adducts detected in several intestinal compartments, only the reaction product between the EN A and two glucose units was found in serum (Figure 6). This compound can be considered the only adduct detected in this study that was absorbed by the intestinal epithelium and was detected in the rat blood (66.11±7.1 mg/l). The reasons for the presence of this compound in the systemic circulation are possibly because of the high concentrations detected in the duodenum and jejunum compartments that favored the absorption of the adduct formed. The structure of this adduct observed in serum was confirmed by several diagnostic fragments: the ion detected in MS1 spectra with an m/z of 1065.1 represents the MW of the adduct, the signal detected in the MS2 spectrum with an m/z of 1013 represents the adduct with the loss of an EN A structural component such as Ile and, definitely, the presence of the ion with an m/z of 181.6 in the MS3 spectrum confirmed the presence of glucose in the structure of the newly formed compound. To sum up, EN A intestinal degradation products and adducts are described for the first time. Further investigation is needed to fill the gap of the metabolic routes affecting EN A.

Table 4. EN A intestinal degradation products. Two degradation fron EN A found in the intestinal compartment of the treated rats, compartment where they were discovered and their MS1, MS2 and MS3 fragments.

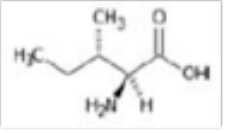
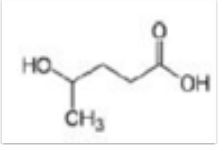
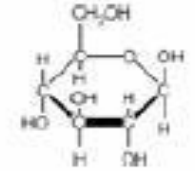
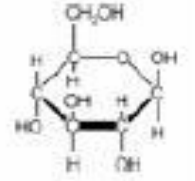
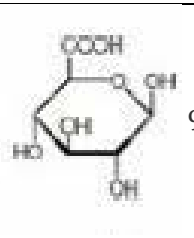
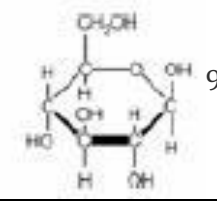
					MS2 fragments		MS3 fragments	
Degradation product	**Biological liquid**	**[M + H]+ m/z**	**Fragment**	**Structure**	**m/z**	**Fragment**	**m/z**	**Fragment**
(EN A + K-Ile)+	Duodenum	577.1	Ile		547.3	(EN A + K-Ile-C=O)+	85.0	(HyLv)+
	Colon				292.4	(EN A + K-2Ile)+	144.2	(Ile)+
(EN A + K-HyLv)+	Duodenum	637.4	HyLv		537.1	(EN A + K-4H2O)+	84.0	(HyLv)+
	Colon				533.4	(EN A + K-2HyLv)+	168.0	2(HyLv)+

Table 5. ENN A intestinal adducts. Four adducts formed with ENN A and macronutrients present in the rats feed, in which compartment where they were discovered and their MS1, MS2 and MS3 fragments.

					MS^2 fragments		MS^3 fragments	
Adducts	**Biological liquid**	**[M + H] + m/z**	**Adduct Fragment**	**Structure**	**m/z**	**Fragment**	**m/z**	**Fragment**
(EN A + 2Glu-H2O)−	Duodenum Jejunum	1022.0	Glu		937.3	(EN A + 2Glu-H2O-HyLv)+	181.16	(Glu)−
					916.3	(EN A + 2Glu-2H2O-HyLv)+	84.0	(HyLv)−
(EN A + K + 4Glu)−	Colon	1517.8	Glu		1442.4	(EN A + K + 4Glu-HyLv)+	84.0	(HyLv)−
					1373.8	(EN A + K + 4Glu-HyLv-Ile)+	144.2	(Ile)−
					1042.4	(EN A + K + 4Glu-HyLv- 3Ile-2H2O)+	724.6	4(Glu)−

(EN A + 2Gluc Ac.)−	Duodenum	1112.3	Gluc Ac.		914.4	(EN A + Gluc Ac.)+	195.1	(Gluc Ac.)−
							390.2	2(Gluc Ac.)−
(EN A + Na + 2Glu)−	Serum	1065.1	Glu		903.9	(EN A + Na + 2Glu-Ile-H2O)+	181.16	(Glu)−

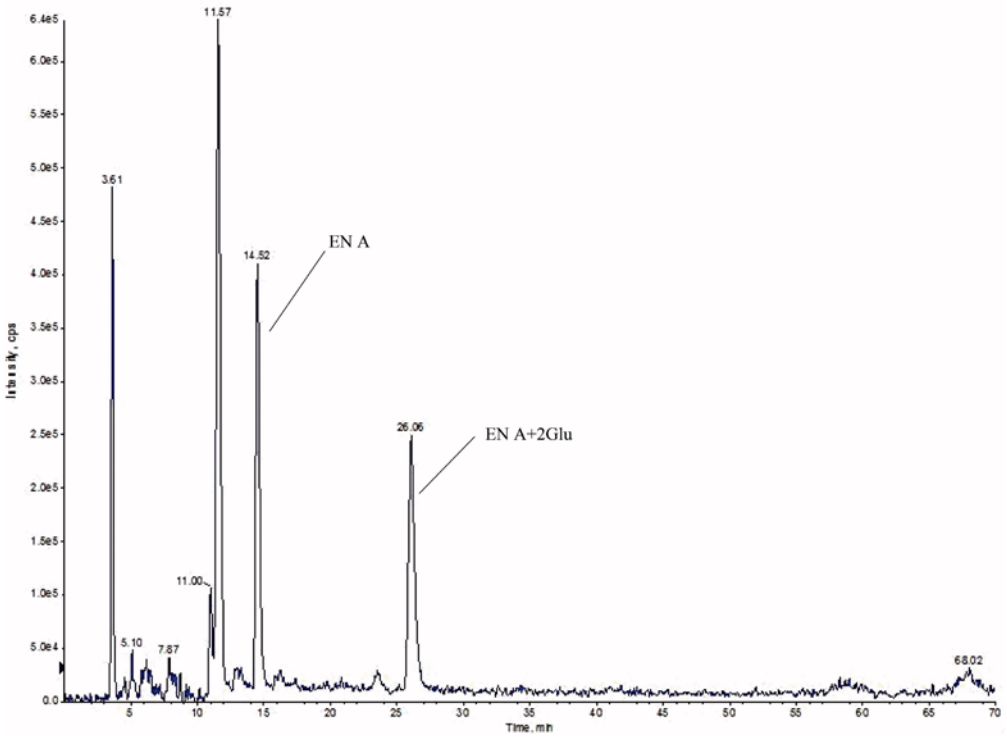


Figure 6. LC-MS-LIT chromatogram of the EN A and of the adduct of formation between the minor *Fusarium* mycotoxin and the glucose evidenced in the serum of treated animals. Glu, glucose.

4. Conclusion

The results obtained in this study confirmed that the EN A concentration of 20.91 mg/kg bw/day present in rat feed through a microbial fermentation by a strain of Fusarium tricinctum, used during 28 days on Wistar rats simulating a preliminar subchronic toxicity study, does not provoke any observed adverse effect on the animals. No statistical differences on the biochemical blood parameters or on the histological analysis carried

out on the duodenum tissue were found when comparing controls with treated animals. Thus, we can confirm that 20.91 mg/kg bw/day of EN A is a potential non-toxic level for young adult rats in medium term ingestion. One of the most important aspects of the mycotoxins risk assessment is the determination of the human exposure degree. Serrano et al. (2012) calculated the estimation of the daily intake (EDI) for EN A in Spanish infants: 35.2 mg/kg bw/day; for the sum of ENs (A, A1, B and B1) in Spanish infants: 234.6 mg/kg bw/day; and for the sum of ENs in adults: 2.35 mg/kg bw/day. The concentration tested in this work is 594-fold, 89-fold and 8897-fold higher than each of them, respectively. Regarding the results obtained, it is possible to affirm that the EN A EDI calculated in Spanish infants is a potential non-toxic concentration in the medium term. For the sum of ENs, both in infants and in adults, it is more complicated to extrapolate the results because of the possible interactions between molecules as synergies. EN A was detected not only in several organs and contents of the gastrointestinal tract, but also in serum confirming its intestinal absorption. EN A degradation products and adducts, probably produced by gut microbial fermentation, were identified and characterized. For the first time experimental data of interest are presented that give information about toxicokinetic processes and potential effects after in vivo oral administration of emerging mycotoxins that may be of interest to international institutions when conducting risk evaluation assessment. Further investigation should be focused on the calculation of the lowest-observed-adverse-effect-level (LOAEL) for the ENs in order to establish a dose–response relationship, a fundamental step to assess the risk related to the intake of these mycotoxins.

Acknowledgement

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3.11 Transcriptomic Study of the Toxic Mechanism Triggered by Beauvericin in Jurkat Cells

Toxicology Letters (2017)

Transcriptomic Study of the Toxic Mechanism Triggered by Beauvericin in Jurkat Cells

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Abstract

Beauvericin (BEA), an ionophoric cyclic hexadepsipeptide mycotoxin, is able to increase oxidative stress by altering membrane ion permeability and uncoupling oxidative phosphorylation. A toxicogenomic study was performed to investigate gene expression changes triggered by BEA exposure (1.5, 3 and 5 μM; 24h) in Jurkat cells through RNA-sequencing and differential gene expression analysis. Perturbed gene expression was observed in a concentration dependent manner, with 43 differentially expressed genes (DEGs) overlapped in the three studied concentrations. Gene ontology (GO) analysis showed several biological processes related to electron transport chain, oxidative phosphorylation, and cellular respiration significantly altered. Molecular functions linked to mitochondrial respiratory chain and oxidoreductase activity were over-represented (q-value<0.01). Pathway analysis revealed oxidative phosphorylation and electron transport chain as the most significantly altered pathways in all studied doses (z-score>1.96; adj p-value<0.05). 77 genes involved in the respiratory chain were significantly down-regulated at least at one dose. Moreover, 21 genes related to apoptosis and programmed cell death, and 12 genes related to caspase activity were significantly altered, mainly affecting initiator caspases 8, 9 and 10. The results demonstrated BEA-induced mitochondrial damage affecting the respiratory chain, and pointing to apoptosis through the caspase cascade in human lymphoblastic T cells.

Keywords: beauvericin, Jurkat, immunotoxicology, RNA-seq, toxicogenomics, transcriptomics.

1. Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi species with low-molecular weight and high variety of structural compounds. They represent one of the most important categories of biologically produced natural toxins relative to human health and economic impact worldwide, being found in numerous commodities of plant origin, especially cereals grains (Ruiz et al., 2011). Mycotoxins are practically unavoidable contaminants in food and feed representing among the natural food contaminants a major issue in food safety, and actually posing critical challenges in food toxicology (Dellafiora and Dall'Asta, 2017). Mycotoxin ingestion may induce various chronic and acute effects on humans and animals, such as cytotoxic, hepatotoxic, neurotoxic, genotoxic, immunosuppressive, estrogenic, nephrotoxic, mutagenic, teratogenic, and/or carcinogenic effects (Smith et al., 2016). Biochemically, the modes of action of mycotoxins can be divided into four categories: interactions with deoxyribonucleic acid (DNA), inhibition of different steps in protein synthesis, effects on cell membranes, and interfering on energy metabolism (Celik et al., 2010). From a molecular perspective, the primal mechanisms of toxic action commonly affect the integrity, functionality, and turn over of biological macromolecules -such as DNA, RNA, and proteins- or the biochemistry of the multitude of low-molecular-weight molecules (e.g. the production of reactive chemicals in cells) (Dellafiora and Dall'Asta 2017).

Among the thousands of fungal secondary metabolites currently known, only a few groups of mycotoxins are important from the safety and economic points of view; namely aflatoxins, ochratoxin A, zearalenone, fumonisins and trichothecenes. However, several Fusarium species can

produce a group of lesser-studied toxins called emerging mycotoxins, which includes beauvericin (BEA), enniatins, moniliformin, and fusaproliferin (Escrivá et al., 2015).

BEA is a cyclic hexadepsipeptide mycotoxin, which contains three D-hidroxyisovaleryl and three N-methylphenylalanil residues in an alternating sequence (Mallebrera et al., 2014). It shows insecticidal and antiviral properties, strong antibacterial activity against human, animal and plant pathogenic bacteria, and immunosuppressive effects (Wang and Xu, 2012). BEA, as lipophilic and ionophoric compound, was reported to increase ion permeability in biological membranes by forming a complex with essential cations (Ca2+, Na+, K+) and cation-selective channels in lipid membranes, affecting the ionic homeostasis and uncoupling the oxidative phosphorylation (Tonshin et al., 2010). Consequently, BEA particulary disturbs the ion balance and the cytoplasmic pH in the mitochondrial membrane by the accumulation of Ca2+, leading to increased levels of intracellular reactive oxygen species (ROS) and reduced intracellular glutathione (GSH) as signs of oxidative stress. The loss of mitochondrial membrane integrity may finally be conducive to degeneration and cellular necrosis and/or apoptosis (Schoevers et al., 2016).

The molecular cytotoxic mechanisms of many mycotoxins in animals still remain unclear and require further investigation, especially the depiction of the intact pathway and the alterations of key enzymes in the pathway driven by mycotoxins. The mitochondrial alterations and the relationship between oxidative stress and mitochondrial physiology induced by mycotoxins offer important research potential for elucidating the key steps of mycotoxicosis initiation (Wen et al., 2016).

Toxicogenomics aims to elucidate the molecular mechanisms of toxicity and modes of action by analyzing chemical induced changes in gene expression patterns (Li et al., 2016). Toxicogenomic approach could be used to understand the complex effects of chemicals and to assist in health risk assessment, and it has already generated interesting results for predicting compound genotoxicity and carcinogenicity (Wilson et al., 2013). Next generation sequencing (NGS) techniques will certainly become the gold standard for understanding molecular mechanisms of toxicity and they are revolutionizing genomic/proteomic studies providing high-throughput datasets with unprecedented precision and accuracy (Fang et al., 2012; Caiment et al., 2015). RNA-seq allows profiling gene expression and composition, and provides a combination of transcriptome-wide coverage, sensitivity, and accuracy for a comprehensive view of gene expression changes, contributing to a more detailed look at the transcriptome than the microarrays (Conesa et al., 2016).

A transcriptome analysis experiment was designed to characterize all transcriptional activity (coding and non-coding) in human Jurkat lymphoblastic T cells. The Jurkat cell line was selected as in vitro model system since it has frequently been used successfully in traditional in vitro studies of the immune system maybe because of its well established reliability (Shao et al 2014). On the other hand, previous in vivo studies demonstrated that the emerging mycotoxins may reach bloodstream in detectable concentrations, allowing them to enter into direct contact with cells and other blood components (Manyes et al., 2014). The responses of Jurkat cells to immunotoxic mycotoxin deoxynivalenol were comparable with the responses of human peripheral blood mononuclear cells (Katika et al., 2012). Moreover,

the Jurkat cell line makes experiments less labor-intensive and more reproducible as compared to primary cells, and they are of human origin facilitating extrapolation to the human situation.

The aim of this study was to investigate the changes in gene expression profile triggered by BEA in human Jurkat lymphoblastic T cells after 24 h of exposure (1.5, 3 and 5 μM).

2. Materials and Methods

2.1 Reagents

The reagent grade chemicals and cell culture components used, RPMI-glutamax medium, penicillin/streptomycin, phosphate buffer saline (PBS), and BEA (783.95 g/mol, 97% purity) were purchased by Sigma chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and methanol were obtained from Fisher Scientific (Madrid, Spain). Deionised water (resistivity <18 MV cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Stock solution of BEA was prepared in methanol and maintained at -20 ºC. Final concentrations of BEA in the assay were achieved by their dilution in the culture medium. The final methanol concentration in the medium was 1% (v/v).

2.2 Cell Culture and Beauvericin Exposure

Jurkat cells (ATCC TIB-152) derived from human T lymphocyte peripheral blood were maintained in RPMI-glutamax medium supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FBS inactivated. Incubation conditions were pH 7.4, 37 ºC under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two

days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma Aldrich, St Louis Mo. USA). Before contamination, cells were plated in 12-well tissue culture plates at a density of $5x10^5$ cells/well. Jurkat cells were exposed for 24 h in standard conditions to 1.5 µM, 3 µM, and 5 µM BEA in 1% methanol and this solvent concentration as control (each condition n=3) in maintenance medium.

2.3 RNA Extraction and Next Generation Sequencing (NGS)

Total RNA of the control and exposed human T lymphocytes cells was isolated using Direct-zol™ RNA MicroPrep kit and treated with RNase free DNaseI (Zymo Research) to remove genomic DNA contamination. The extracted RNA of each sample was firstly checked for quantity and quality using Agilent 2100 Bioanalyzer (Agilent Technologies). All sequenced samples were generated from high quality RNA samples having a RIN number above 8. The standard Illumina protocol was followed to develop RNA-seq libraries. The Illumina NextSeq 500 platform was used for sequencing. Then, one archive for each sample was obtained, 12 in total. RNA quality control and sequencing were carried out by the Genomics section of the Central Service for Experimental Research (SCIE, University of Valencia).

2.4 Data Processing

The latest versions of different analytical tools were used in order to achieve a RNA-seq differential gene expression analysis. Raw sequencing reads were pre-processed and quality control was first performed by FastQC software. Sequencing reads were aligned by Bowtie2 (v2.2.6) and mapped to the Ensembl Human genome sequence (GRCh38) using default parameters.

Read counts of each gene were quantifyed using RSEM (v.1.2.28) and Normalization fitting a negative binomial distruction and pair comparison analysis (treated vs. control) were performed and DEGs were obtained using DESeq2 package. All the analyses were performed in R.

2.5 Gene Expression Analysis

All of the DEGs were subjected to over-representation and gene ontology (GO) analysis by ConsensusPathDB. GO annotations and functional classifications of DEGs were obtained. Pathway assignments were carried out using PathVisio software with *Hs_Derby_Ensembl_85 bridge* gene dataset. Adjusted $p \leq 0.05$ and z-score > 1.96 were used as the threshold to identify the significantly enriched GO terms and pathways.

2.6 Primer Design and Quantitative Real-Time PCR Assays

Gene-specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) using default criterion of the software with amplified products ranging from 75 to 150 bp and Tm at 59°C. Primer sequences used in the qRT-PCR analyses are presented in Table 1.

Gene Symbol	Primer sequences (5' to 3') FP/RP	E (%)	Regression coefficient (R^2)
MATN4	TGCAGGGCCATTGACTACTG / TCGCTCACACACTGGAACTC	117	0,966
MT-ND3	GACTACCACAACTCAACGGC / GGGCTCATGGTAGGGGTAAA	82	0,991
18S rRNA	CGGCTACCACATCCAAGGAA / GCTGGAATTACCGCGGCT	76	0,994

Table 1. Primers Used for Real-Time Quantitative RT-PCR Analysis.

Standard RT-PCR was performed for all the primer pairs and a single amplification product of the expected size for each gene was obtained by the melting curve assay. Primer amplification efficiency was determined from standard curve generated by serial dilution of cDNA (5 fold each) for each gene in triplicate. Correlation coefficients (R^2 values) and amplification efficiencies (E) for each primer pairs were calculated from slope of regression line by plotting mean Cq values against the log cDNA dilution factor in Stepone software. Real-time amplification reactions were performed in 96 well plates using SYBR Green detection chemistry and run in triplicate on 96-wells plates with the StepOne Plus Real-time PCR machine (Applied Biosystems). Reactions were prepared in a total volume of 10 µl containing: 3 µl of 1:2 diluted template, 1 µl of each amplification primer (5µM) and 5 µl of 2X Fast SYBR Green (Applied Biosystems). Non-template controls (NTC) were also included for each primer pair, replacing the template by water DNAse and RNAse free from the RNA extraction kit (Zymo Research). The cycling conditions were set as default: initial denaturation step of 95°C for 5 min to activate the Taq DNA polymerase, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 30s. The melting curve was generated by heating the amplicon from 60 to 90°C. Baseline, threshold cycles (Ct) and statistical analysis were automatically determined using the StepOne Plus Software version 2.3 (Applied Biosystems). All the experiments were done according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009).

3. Results and Discussion

3.1 RNA Extraction, Sequencing and Quantification

The highly abundant ribosomal RNA (rRNA) constitutes over 90% of total RNA in the cell and should be removed by an appropriate RNA-extraction protocol to acquire the messenger RNA (mRNA) (Conesa et al., 2016). RNA-seq data acquisition, which consists of several steps from obtaining raw reads to read alignment and quantification, was specifically checked at each step to monitor the quality of the data. Percentage of mapped reads, considered an important mapping quality parameter, was 43458513 indicating high overall sequencing accuracy and low presence of contaminating DNA and it was calculated by FastQC, version 0.11.3 (Andrews, 2010). The RNA Integrity Number (RIN) was above 8 and rRNA ratio (28S/18S) was above >1.7, both obtained by the Eukaryote Total RNA Nano assay (Bioanalyzer, Applied biosystems).

3.2 Gene Expression Profile

The overall gene expression of lymphocytes T treated cells with three BEA doses (1.5, 3 and 5 μM) for 24 h significantly differed from the expression of untreated cells. Perturbed gene expression was shown in all studied concentrations when compared with control samples, with an increased number of differential expressed genes (DEGs) with increased dose, pointing to a concentration dependent damage. The number of DEGs in comparison of control samples raised from 44 to 2403 and 5511 when BEA exposure doses to Jurkat cells increased from 1.5 to 3 and 5 μM, respectively. The total number of identified DEGs considering all three BEA concentrations was 5719, with 60.4% down-regulation and 39.6% up-regulation (3457 and 2262

genes, respectively). This indicated a slight trend towards down-regulation against up-regulation after BEA exposure to Jurkat cells, also evidenced when analyzing each independent dose. In this way, the down-regulated genes ranged 61-80% of the total DEGs for each individual concentration, while the up-regulated ones represented between 21 to 44%.

On the other hand, considering that the total human genome contains ~21000 protein coding genes (HGNC database), the results showed that BEA may compromised the normal expression levels up to 27.3% of human genome perturbation (16.5% down-regulation and 10.8 % up-regulation). As it was expected, the highest dose (5 μM) compromised most the gene expression profile of the human genome, with similar values that those obtained in the general overview (10% and 16% up- and down- regulation, respectively). Among these 5719 DEGs identified for at least one BEA dose, 62% (3523 genes) were significantly perturbed in one single dose, mainly 5 μM (58%) and in lesser extent 3 μM (4%), with no one identified in lowest concentration (1.5 μM). On the other hand, around 38% (2154 genes) overlapped in two doses, almost all (99.9%) in the highest ones, 3 and 5 μM, indicating evidence of dose-dependent expression. Finally, 43 differentially expressed genes were found for each of the three studied BEA concentrations, considering them the most relevant geneset (Figure 1).

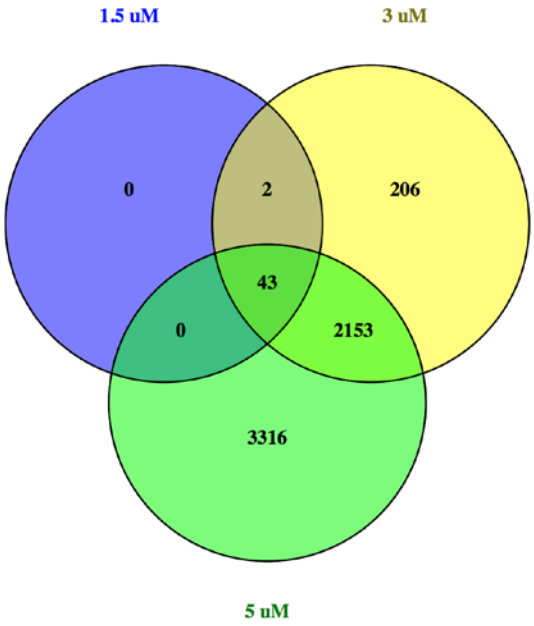

Figure 1. Venn diagram for the differentially expressed genes in Jurkat cells treated with BEA at 1.5, 3 and 5 μM.

3.3 Differentially Expressed Genes (DEGs)

Generating an accurate list of differentially expressed genes is the basis for pathway or gene set enrichment analysis (Fang et al., 2012). From the 43 DEGs found in the present study overlapping in the three studied BEA concentrations, 36 were down-regulated and 7 up-regulated. Moreover, 11 genes were located in the mitochondrial DNA, representing the 26% of the geneset. Interestingly, these 11 mitochondial DNA genes corresponded to the 10 most strongly down-regulated genes plus the most strongly up-regulated one, reaching Log2 fold change (Log2FC) values up to 3.6 in over-expression and -1.30 for repression. These results bring greater relevance to

mitochondria as a target site for BEA induced cytotoxicity in cellular models. Moreover, the Log2FC values show a dose dependent expression pattern for all three BEA concentrations (Figure 2).

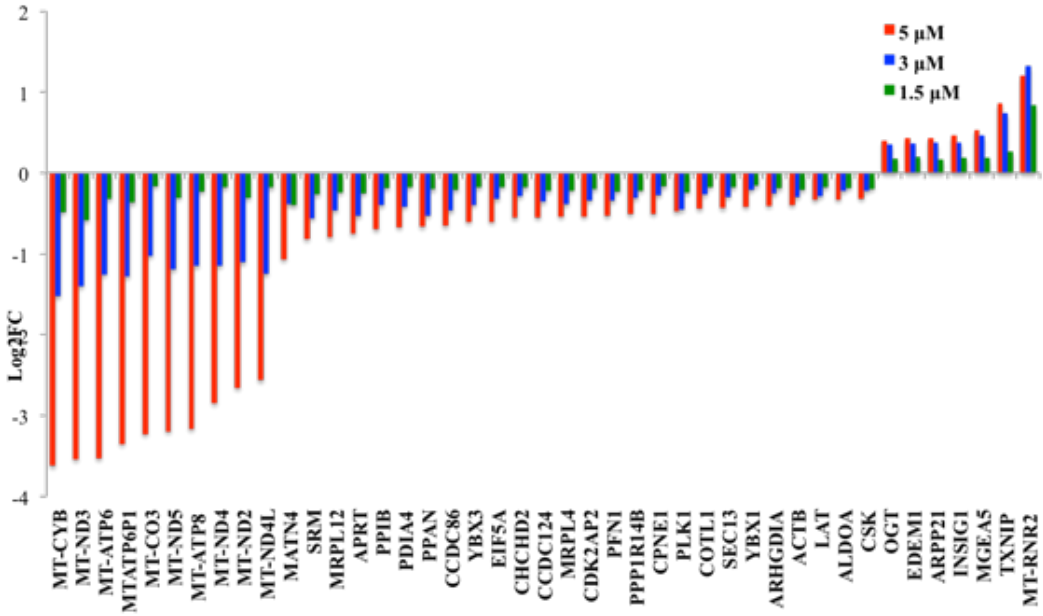

Figure 2. Log2FC values for the differentially expressed genes overlapped in the three BEA concentrations; 1.5, 3 and 5 µM.

Mitochondria are the center of cellular energy homeostasis and redox regulation, and integrate numerous metabolic pathways, which may play a crucial role of initiative apoptosis. Though the majority (98%) of mitochondrial proteins is nuclear-encoded, the mitochondrial genome retains some genes encoding proteins involved in the respiratory chain. The mitochondrial respiratory chain complex gene family includes genes found in both nuclear DNA as well as mitochondrial DNA, and mutations in either nuclear or mitochondrial genes may cause diseases (Qi et al., 2017). In terms

of apoptosis signaling, the mitochondria are a central sensor and integration point for diverse apoptosis signals (Lee et al., 2008). Mitochondrial integrity is central to both caspase-dependent and -independent cell death pathway, since its membrane perforation serves to induce both. The release of pro-apoptotic factors from the mitochondrial intermembrane space is a key event in a cell's commitment to die and is under the tight regulation of the Bcl-2 family. However, the underlying mechanisms governing the efflux of these pro-death molecules are largely unknown (Donovan et al., 2004). BEA-treated cells have the ability to complete the entire apoptotic process and result in cell fragmentation (Jow et al., 2004). The results obtained in the present study increase the scientific evidence through the transcriptomic approach that mitochondria are the main cell organelle responsible of BEA induced toxicity.

The rest of the 43 DEGs were distributed into different chromosomes highlighting chromosomes 16 (12%), 17 and 7 (9% each), followed by 1, 3, 11 and 19 (7% each). Table 2 shows the geneset list including the HGNC symbol, gene description and chromosome location.

HGNC Symbol	Gene Desription	Location
MT-CYB	mitochondrially encoded cytochrome b	Chr. MT
MT-ND3	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 3	Chr. MT
MT-ATP6	mitochondrially encoded ATP synthase 6	Chr. MT
MTATP6P1	mitochondrially encoded ATP synthase 6 pseudogene 1	Chr. 1
MT-CO3	mitochondrially encoded cytochrome c oxidase III	Chr. MT
MT-ND5	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 5	Chr. MT
MT-ATP8	mitochondrially encoded ATP synthase 8	Chr. MT
MT-ND4	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4	Chr. MT
MT-ND2	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 2	Chr. MT
MT-ND4L	mitochondrially encoded NADH:ubiquinone oxidoreductase core subunit 4L	Chr. MT
MATN4	matrilin 4	Chr. 20
SRM	spermidine synthase	Chr. 1
MRPL12	mitochondrial ribosomal protein L12	Chr. 17
APRT	adenine phosphoribosyltransferase	Chr. 16
PPIB	peptidylprolyl isomerase B	Chr. 15
PDIA4	protein disulfide isomerase family A member 4	Chr. 7
PPAN	peter pan homolog (Drosophila)	Chr. 19
CCDC86	coiled-coil domain containing 86	Chr. 11
YBX3	Y-box binding protein 3	Chr. 12
EIF5A	eukaryotic translation initiation factor 5A	Chr. 17
CHCHD2	coiled-coil-helix-coiled-coil-helix domain containing 2	Chr. 7
CCDC124	coiled-coil domain containing 124	Chr. 19

MRPL4	mitochondrial ribosomal protein L4	Chr. 19
CDK2AP2	cyclin dependent kinase 2 associated protein 2	Chr. 11
PFN1	profilin 1	Chr. 17
PPP1R14B	protein phosphatase 1 regulatory inhibitor subunit 14B	Chr. 11
CPNE1	copine 1	Chr. 20
PLK1	polo like kinase 1	Chr. 16
COTL1	coactosin like F-actin binding protein 1	Chr. 16
SEC13	SEC13 homolog, nuclear pore and COPII coat complex component	Chr. 3
YBX1	Y-box binding protein 1	Chr. 1
ARHGDIA	Rho GDP dissociation inhibitor alpha	Chr. 17
ACTB	actin beta	Chr. 7
LAT	linker for activation of T-cells	Chr. 16
ALDOA	aldolase, fructose-bisphosphate A	Chr. 16
CSK	c-src tyrosine kinase	Chr. 15
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase	Chr. X
EDEM1	ER degradation enhancing alpha-mannosidase like protein 1	Chr. 3
ARPP21	cAMP regulated phosphoprotein 21	Chr. 3
INSIG1	insulin induced gene 1	Chr. 7
MGEA5	meningioma expressed antigen 5 (hyaluronidase)	Chr. 10
TXNIP	thioredoxin interacting protein	Chr. 1
MT-RNR2	mitochondrially encoded 16S RNA	Chr. MT

Table 2. 43 DEGs (for all BEA concentrations): HGNC Symbol, gene description, and chromosome location.

3.3 Gene Ontology and Pathway Analysis

One of the important steps in a standard transcriptomics study is the characterization of the molecular functions or pathways in which DEGs are involved by comparing a list of DEGs against the rest of the genome for over-represented functions, and gene set enrichment analysis, which is based on ranking the transcriptome according to the measurement of differential expression (Conesa et al., 2016). In this sense, the over-representation analysis using ConsensusPathDB of the selected 43 DEGs geneset provided a list of gene ontology (GO) terms in which several biological processes related to respiratory and electron transport chain, oxidative phosphorylation, cellular respiration, generation of precursor metabolites and energy, mitochondrial respiratory chain complex I biogenesis and assembly, etc. were most over-represented. Molecular functions linked to oxidoreductase activity, NADH dehydrogenase and NADH dehydrogenase (quinone) activity were statistically more significative in the DEGs set, while cellular components such as mitochondrial membrane, mitochondrial respiratory chain, mitochondrial envelope, NADH dehydrogenase and oxidoreductase complexes, mitochondrial protein complex and respiratory chain were significantly represented in BEA treated Jurkat cells compared to control samples.

Pathway analysis using PathVisio showed a total of 7688 data points (N) and an increased number of data points meeting criterion (R) with increasing doses, i.e. 26, 1096 and 2398 for 1.5, 3 and 5 μM, respectively. When all concentrations data where integrated 25 data points meeting criterion (R) were overlapped. Three pathways were statistically significant in the three studied doses (z-score>1.96; adj p-value<0.05); oxidative phosphorylation, electron transport chain and nucleotide metabolism.

Moreover, the number of genes involved in the pathways, as well as, the percentage of positive/measured genes showed a dose-dependent relationship. The analysis revealed 6, 23 and 50 DEGs included in the oxydative phosphorylation pathway for 1.5, 3 and 5 µM, respectively, with the highest z-score (12.99) among all the pathways. Furthermore, electron transport chain pathway (z-score: 11.61) included 7, 36 and 82 DEGs, while nucleotide metabolism pathway (z-score: 3.79) showed 1, 7 and 16 DEGs for 1.5, 3 and 5 µM, respectively (Figure 3). Other statistically significant pathways were found overlapped in the two highest BEA concentrations (3 and 5 µM), such as DNA damage response, G1 to S cell cycle control, and integrated cancer pathway.

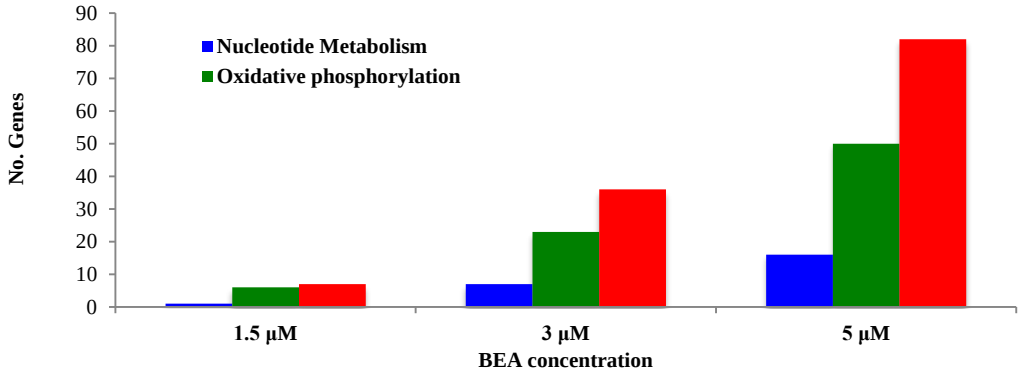

Figure 3. Number of DEGs involved in the oxydative phosphorylation, electron transport chain, and nuecleotide metabolism pathways for the three BEA concentrations (1.5, 3 and 5 µM).

Figure 4 shows the genes invoved in the electron transport chain pathway for Homo sapiens, indicating in red and blue the down-regulated and up-regulated genes, respectively, for the three BEA concentrations (1.5, 3 and 5 µM).

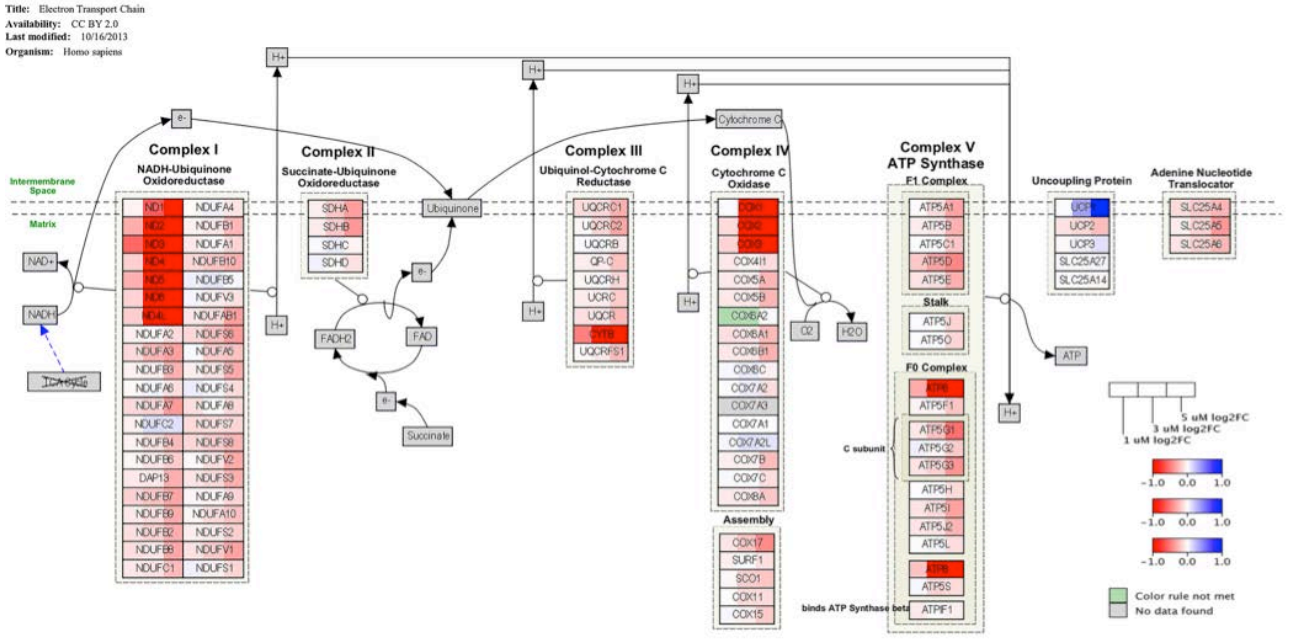

Figure 4. Electron transport chain pathway (*Homo sapiens*) obtained with the DEGs by PathVisio analysis. Highlighted in red the down-regulated and in blue the up-regulated genes for the three BEA concentrations (1.5, 3 and 5 μM).

3.4 Functional Profiling of DEGs

The whole list of 5719 DEGs included several genes belonging to ATPase H+ transporting subunits, cytochrome c oxidase subunits, NADH: ubiquinone oxidoreductase subunits, ubiquinol-cytochrome c reductases, ribosomal proteins, and mitochondrial ribosomal proteins, among others. All DEGs were individually characterized and studied to find relevant genesets related to some specific biological routs and pathways. In this way, functional related gene groups mainly belonging to respiratory chain, apoptosis, necrosis process, and caspases cascade activation were deeply studied.

3.4.1 Respiratory Chain

Respiration is the core of mitochondrial metabolism for free energy release and ATP production, and depends on the function of the electron transport chain, composed of five respiratory complexes (Qi et al., 2017). During respiration, electrons from NADPH and FADH2 are transferred to O2 generating ATP and oxidized NADP1 and FAD1. Depending on the substrate, electrons are transported from complex I (NADH dehydrogenase [ubiquinone]) and complex II (succinate dehydrogenase) through ubiquinone and complex III (ubiquinol-cytochrome c reductase) to cytochrome-c and to complex IV (cytochrome-c oxidase), which produces water, while ATP is generated by complex V (ATP synthase) (Dudkina et al., 2006).

In the present study several genes related to respiratory chain were significantly altered. From the 96 genes involved in the respiratory chain (HGNC database), 77 genes were differentially expressed at least at one dose (all down-regulated), indicating that BEA induced perturbation of 80% of the respiratory chain genes. Although the 5 complexes were affected, complex I,

complex III, and complex V showed the highest number of perturbed genes (84-90%). Among these 77 genes, 9 were differentially expressed for all three BEA concentrations, mainly belonging to complex I (5 genes), followed by complex V (2 genes), and complexes III and IV (1 gene each). As shown in Figure 5, all 9 genes were down-regulated in a clear dose-dependent manner.

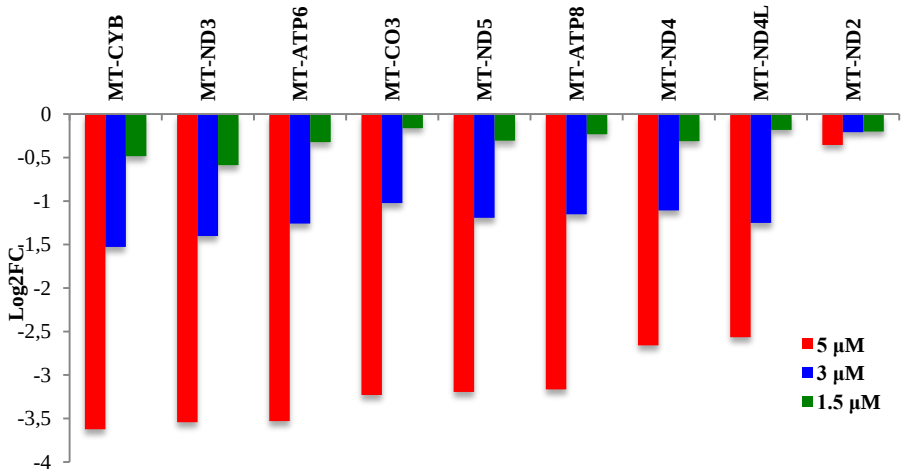

Figure 5. Respiratory chain genes altered in the three BEA concentrations; 1.5, 3 and 5 µM.

Similar gene expression results were recently observed by microarray technique after 4 h expossure of the emerging mycotoxin EN B (1, 10 and 20 µM) on rat primary hepatocytes. The number of down-regulated genes was five times higher than up-regulated ones, with over-represented genes associated with apoptotic processes, apoptotic mitochondrial changes, and cell signal transduction. Moreover, it was revealed the alteration of energy metabolism due to effects on mitochondrial organization and function, and the assembly of complex I of the electron transport chain (Jonsson et al., 2016).

3.4.2 Necrosis, Apoptosis and Programmed Cell Death

Cell death generally proceeds through two molecular mechanisms: necrosis and apoptosis. Apoptosis is highly regulated cell death program triggered through the extrinsic (or death receptors) pathway or the intrinsic (or mitochondrial) pathway (Boussabbeh et al., 2015). It is considered an important physiological process involved in cell deletion during organogenesis and in the control of cell proliferation and differentiation in adult tissues, which characterized by distinct biochemical features. Apoptosis can be triggered by numerous mediators including receptor-mediated signals, withdrawal of growth factors, and environmental agents (Elmore, 2007). In the present study 21 genes (14 down-regulated and 7 up-regulated) related to apoptosis and programmed cell death were differentially expressed at least at one dose (5 µM and/or 3 µM). On the other hand, 4 necrosis related genes were perturbed (3 down-regulated and 1 up-regulated) but only in treated Jurkar cells with the highest BEA concentration (5 µM), while low (1.5 µM) and medium (3 µM) BEA doses did not show significant alteration in the expression of the necrosis involved genes. Figure 6 shows the differentially expressed genes related to apoptosis and programmed cell death (3 and 5 µM), and genes linked to necrosis process (5 µM).

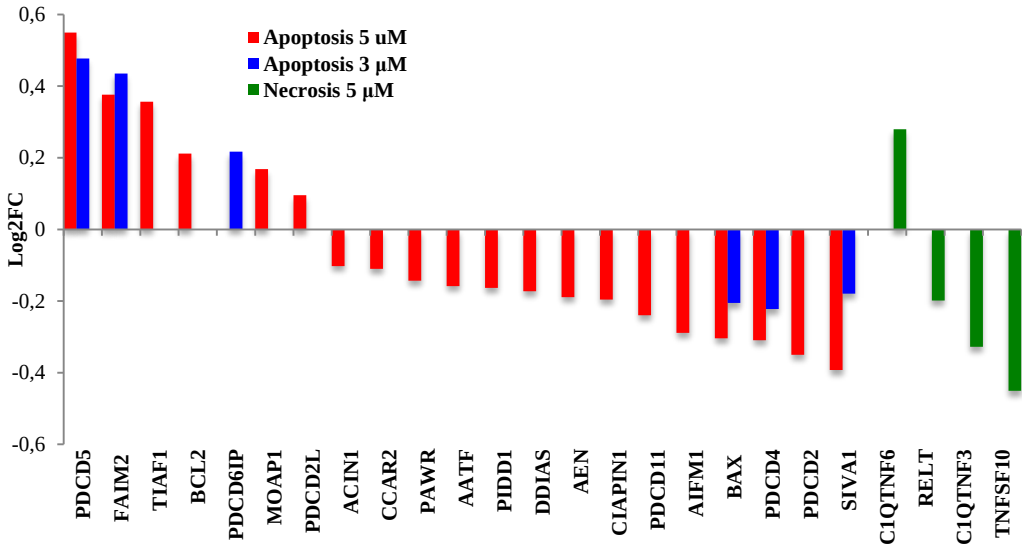

Figure 6. Apoptosis and necrosis related genes altered in Jurkat cells treated with 3 and/or 5 µM BEA (apoptosis), and with 5 µM BEA (necrosis), respectively.

The cytotoxicity of BEA has been previously described based on apoptosis induction via the mitochondrial pathway for several human cancer cell lines; such as non small cell lung cancer [NSCLC] A549 (Lu et al., 2016; Lin et al., 2005), acute lymphoblastic leukemia [CCRF-CEM] (Jow et al., 2004), cervix carcinoma [KB, KBv200 and KB-3-1] (Tao et al., 2015; Dornetshuber et al., 2009), and colon adenocarcinoma [Caco-2] (Prosperini et al. 2013). Moreover, BEA induced apoptosis in activated T cells (Wu et al., 2013), Chinese hamster ovary [CHO-K1] (Mallebrera et al., 2016), rat liver hepatoma [H4IIE] (Watjen et al., 2014), porcine kidney PK15 (Klaric et al., 2008), turkey peripheral blood lymphocytes (Dombrink-Kurtzman, 2003), and rodent cholangiocytes (Que et al., 1997). However, the molecular mechanism

underlying the BEA-induced apoptotic process is not yet clearly understood (Lin et al., 2005). The available literature demonstrated that the induction of apoptosis by BEA involves multiple cellular/molecular pathways and the molecular mechanisms are still controversially discussed. BEA ionophoric action could be responsible for the alteration of the lipid membrane structures and production of ROS, which decrease intracellular GSH content, contributing to apoptosis and necrosis (Klaric et al., 2008). Some studies showed that BEA induced apoptosis by decreasing the mitochondrial membrane potential, which leads to the release of cytochrome c into cytosol and caspase-3 activation. Loss of mitochondrial membrane potential may be an early event in the apoptotic process and required for BEA-induced cytochrome c release into cytosol, that later triggered the cleavage and activation of mitochondrial downstream caspases (Lin et al., 2005). However, it may not be an early requirement for apoptosis and may be a consequence of the apoptotic-signaling pathway (Ly et al., 2003). It was also suggested that intracellular Ca2+ plays an important role as a mediator in cell death signalling induced by BEA, since it increases cytosolic Ca2+ concentration in a dose-dependent manner leading to irreversible cell damage if it is not immediately compensated (Chen et al., 2006). Moreover, the rise in Ca2+ is a point of convergence among many downstream mechanisms, i.e., decreases the mitochondial membrane potential, releases of cytochrome c, increases caspase activation and apoptosis (Mallebrera et al., 2016). Other results showed that mitochondrial integrity and apoptosis were mainly regulated by the expression of Bcl-2 family proteins of cell death regulatory molecules (Lee et al., 2008).

An increase in early apoptotic cells was observed in Caco-2 and CHO-

K1 cells with inhibition of cell proliferation arresting cells in G0/G1 (Prosperini et al., 2013; Mallebrera et al., 2016). In human NSCLC A549 cells BEA-induced apoptosis in concentration- and time-dependent manner by mitochondrial transmembrane depolarization and significant reduction of mitochondrial membrane potential, increase in the release of mitochondrial cytochrome c, and activation of caspase 3 (Lin et al., 2005). Significant increase of apoptosis was later reported in the same cell line by the activation of MEK1/2 (mitogen-activated protein kinase kinase)-ERK42/44 (extracellular signal-regulated kinases)-90RSK (ribosomal s6 kinase) signaling pathway, with the up-regulation of the pro-apoptotic proteins Bax, Bak, Bad, and down-regulation of anti-apoptotic protein Bcl-2 (Lin et al., 2005). BEA inhibited activated T cells via PI3K/Akt inhibition, thereby suppressing cell activation/proliferation, reducing cytokines production and resulting in cell apoptosis, by collapse of mitochondrial membrane potential, release of cytochrome c to the cytosol, down-regulation of Bcl-2, phosphorylation of Bad, activation of caspase-12, 9, 3 and PARP cleavage (Wu et al., 2013). Apoptosis by mitochondrial pathway, including decrease of ROS generation, loss of mitochondrial membrane potential, release of cytochrome c, activation of Caspase-9 and -3, and cleavage of PARP was also described in KB and KBv200 cells, without signs of Bcl-2 or Bax alteration (Tao et al., 2015). In human promyelocytic leukaemia cells (HL60) and human cervix carcinoma cells (KB-3-1) cells generation of ROS was not involved in BEA-induced apoptosis (Dornetshuber et al., 2009).

On the other hand, cell death by necrosis as the ultimate endpoint of lethal cell injury has been also reported, nevertheless apoptosis remains as the main cell death process induced by BEA exposure. Increased necrotic

CHO-K1 cells was observed after 48 h of exposure, although BEA 24 h exposure produced apoptosis but not necrosis (Mallebrea et al., 2016). Necrosis was evidenced in C6 glioma BEA exposed cells, but apoptosis prevails in H4IIE hepatoma cells with significant increase in caspase 3/7 activity and nuclear fragmentation, as a molecular mechanism of cell death (Watjen et al., 2014). Apoptosis and necrosis were observed in porcine kidney PK15 cells depending on the concentration used and time of exposure, with an increase in caspase-3 activity after 48 h of exposure leading to apoptosis (Klaric et al., 2008).

3.4.3 Caspase Cascade

Caspases have been shown to be activated during apoptosis in many cell systems and play critical roles in both the initiation and execution of apoptosis (Jow et al., 2004). Caspases are expressed as inactive proenzymes in living cells and become activated when cells receive an apoptosis- inducing signal. Once activated, they cleave a number of key substrates, resulting in their activation or inactivation. Activation of caspases leads to the morphological and biochemical features of apoptosis. Two caspase activating cascades that regulate apoptosis have been described: one is initiated from the cell surface death receptor and the other is triggered by changes in mitochondria integrity (Yuan et al., 2003). A family of at least 13 related cysteine proteases has been identified in mammals, existing in the cell as inactive precursors that undergo proteolytic processing and activation. Once activated, initiator caspases can proteolyse additional effector caspases, generating a proteolytic cascade that cleaves key structural components as well as proteins critical for cell survival in a highly sequence-specific fashion,

ultimately resulting in the systemic and controlled destruction of the cell (Donovan et al., 2004).

In the present study, 12 genes involved in caspase cascade activation were differentially expressed (10 up-regulated and 2 down-regulated) at least at one BEA dose (3 and/or 5 µM), pointing to mainly affect initiator caspases 8, 9 and 10 (Figure 7).

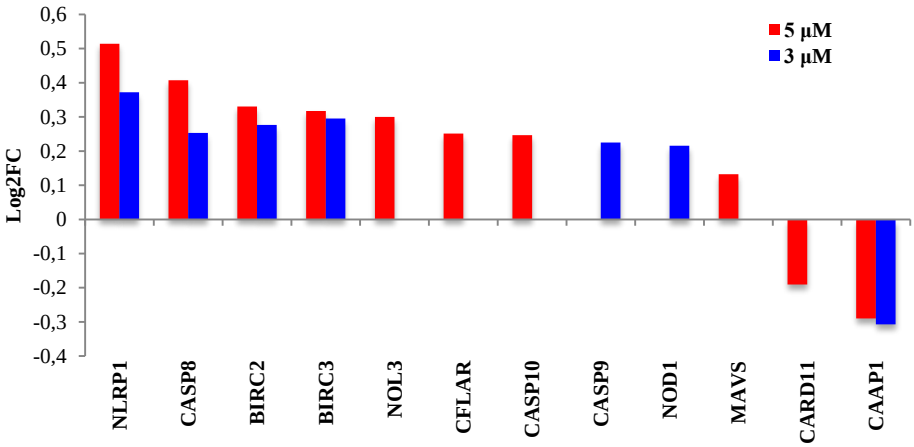

Figure 7. Caspases cascade related genes altered in Jurkat cells treated with 3 and 5 µM BEA concentrations for 24 h.

Activation of caspase 3, which can occur as a consequence of caspase 9 activation, is a central mechanism of apoptosis in the death receptor pathway, leading to intracellular events in the apoptosis cascade, and essential for DNA fragmentation (Jow et al., 2004; Lin et al., 2005). Since the apoptosome cascade or intrinsic pathway involves activation of procaspase 9 by cytochrome c released from the mitochondria (Lee et al., 2008), gene expression alteration of initiator caspases shown in the present study may indicate an apoptosis early step, which may lead to the activation of the

executioner procaspases (caspase 3, 6 and 7) that cleave PARP and other apoptotic protein substrates. Recent results obtained in our laboratory showed that BEA induced apoptosis in Jurkat cells after 24 and 48 h exposure in a concentration and time dependent manner. Moreover, caspase-3 activation was already observed after 24 h of BEA exposure and reached 60% of activated cells after 48 h (data not shown).

Summarizing, the perturbed genes involved in apoptosis and programmed cell death identified in the present study showed overall trend towards apoptosis after BEA exposure in Jurkat cells. In this way PDCD5, which encodes a protein over-expressed during apoptosis, and MOAP1, which mediates caspase-dependent apoptosis, were up-regulated. On the other hand, the up-regulation of caspases genes CASP8, CASP9, CASP10 and NLRP1 gene, known to be key mediator of programmed cell death whose over-expression induce apoptosis by strongly interaction with caspase 2 and weakly with caspase 9, supports the apoptosis induction by BEA. Moreover, the down-regulation of CAAP1, a caspase activity and apoptosis inhibitor that encodes and anti-apoptotic protein modulator of caspase-10 dependent mitochondrial and caspase-3/9 feedback amplification loop is in agreement with the apoptosis path. Nevertheless, some specific genes perturbation, such as the up-regulation of NOL3 -anti-apoptotic gene- could not be explained. With regard to the bcl-2 family genes, the present study showed down-regulation of Bax and up-regulation of Bcl-2, contrary to other studies where the up-regulation of the pro-apoptotic protein Bax and the down-regulation of anti-apoptotic protein Bcl-2 were reported, among with caspases genes up-regulation (Lin et al., 2005; Wu et al., 2013). Either Bcl-2 is up-regulated and Bax down-regulated to attenuate BEA induced apoptosis or Bcl-2 may act as a

downstream death substrate of caspases, i.e. Bcl-2 is cleaved by caspase 3, thereby not only inactivating its anti-apoptotic function but also enhancing cell death (Cheng et al., 1997). Eitherway, the results shown in the present work still agree with the other studies with regard to the caspases activation as main possible path for the apoptosis event.

3.5. Confirmation of NGS Results by PCR

The sequencing-based results were validated using qPCR. The expression of MT-ND3, a mitochondrial DNA gene, and MATN4, a nuclear DNA gene, was measured in Jurkat cells after exposure to BEA in the same conditions than in the NGS assay. The results confirm the down-regulation of both genes compared to the control (Figure 8).

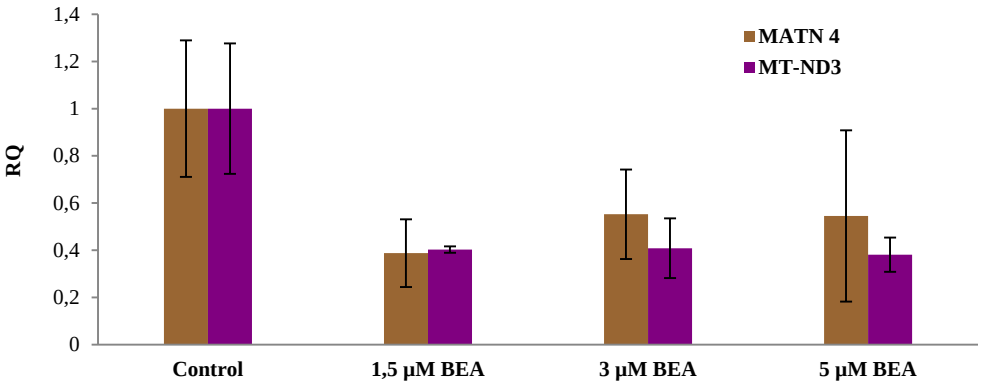

Figure 8. Bar plot showing Jurkat cells relative expression of selected genes when compared to control (RQ=1) after 24h-exposure to different concentrations of BEA by qPCR. RQ, relative quantification.

House-keeping gene 18S rRNA was used as endogenous gene control (Fig. 8). S18 has not been differently expressed at any concentration exposed

and it has already been valited as a good house-keeping gene (Banda et al., 2008).

4. Conclusion

This is -to our knowledge- the first transcriptomic study based on RNA-seq addressing BEA cytotoxicity in human Jurkat lymphoblastic T cells. The transcriptomic analysis revealed that BEA altered gene expression profile in the human genome in a dose-dependent manner. RNA-seq showed a large number of differentially expressed genes mainly related to respitatory chain, apoptosis, and caspase cascade activation. Biological processes related to respiratory and electron transport chain, oxidative phosphorylation, and cellular respiration were most over-represented. Molecular functions linked to oxidoreductase activity, NADH dehydrogenase and NADH dehydrogenase (quinone) activity were statistically more significative in the DEGs set, while cellular components such as mitochondrial membrane, mitochondrial respiratory chain, mitochondrial envelope, NADH dehydrogenase and oxidoreductase complexes, mitochondrial protein complex, and respiratory chain were significantly represented in BEA treated Jurkat cells compared to control samples. Finally, oxidative phosphorylation, electron transport chain, and nucleotide metabolism pathways were dose-dependent significantly altered in the three studied concentrations (z-score>1.96; adj p-value<0.05). These results assist in understanding the mechanisms of BEA induced cytotoxicity and strongly suggest the implication of mitochondrial damage pointing to apoptosis through the caspase cascade activation in human lymphoblastic T cells.

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3.12 Enniatin B Induces Expression Changes in the Electron Transport Chain Pathway Related Genes in Lymphoblastic Cell Line

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Enniatin B induces expression changes in the electron transport chain pathway related genes in lymphoblastic cell line

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Abstract

EN B is an ionophoric and lipophilic mycotoxin which reaches the bloodstream and has the ability to easily penetrate the cellular membranes. The purpose of this study was to reveal changes in the gene expression profile caused by ENB in human Jurkat lymphoblastic T-cells after 24 h of exposure at 1.5, 3 and 5 μM by next generation sequencing. It was found that up to 27% of human genome expression levels were significantly altered (5750 genes for both down-regulation and up-regulation). In the three EN B concentrations 245 DEGs were found to be overlapping, 83 were down-regulated and 162 up-regulated. ConsensusPathDB analysis of over-representation of the selected 245 DEGs, provided a list of gene ontology (GO) terms in which several biological processes related to nucleoside monophosphate metabolic process, respiratory chain complex, electron transport chain, oxidative phosphorylation, cellular respiration were most overrepresented. Electron transport chain pathway and oxidative phosphorylation pathway showed 23, 42 and 43 DEGs for 1.5, 3 and 5 μM both down-regulated. In summary, the transcriptomic analysis based on RNA-seq addressing EN B cytotoxicity in human Jurkat lymphoblastic T cells revealed that mitochondria are the organelles showing more related DEGs. Consequently, DEGs involved in biological processes, molecular functions and pathways which take place in them were significantly changed and it has been evidenced in the results obtained.

Keywords: Enniatins; transcriptomics; electron transport chain; Jurkat; RNA-seq.

1. Introduction

In the last years, due to globalization and long-term grain storage, mycotoxins have become a great issue in food safety as they are principal contaminants in food and feed, which has turned into a critical challenge for researchers and institutions working on food toxicology (Ruiz et al., 2011). The toxicity caused by mycotoxins ingestion can vary from chronic to acute effects on human and animals and it can be manifested in many different ways: cytotoxic, hepatotoxic, neurotoxic, genotoxic, immunosuppressive, estrogenic, nephrotoxic, mutagenic, teratogenic, and/or carcinogenic effects (Fernández-Blanco et al., 2015). There are also other classifications which focus on their biochemical action: interactions with deoxyribonucleic acid (DNA), inhibition of different steps in protein synthesis, effects on cell membranes, and interfering on energy metabolism (Celik et al., 2010).

Enniatins (ENs) are mycotoxins structurally related with a large group of cyclic hexadepsipeptides in common. Their chemical structure also contents D- hydroxy-isovaleryl-(2-hydroxy-3-methylbutanoic acid) and N-methylamino acid residues. Up to date, there have been found in nature 29 analogues, finding Enniatin B and Enniatin B1 (ENB and ENB1) as the most frequent in food, feed and unprocessed grain. ENB potential toxicity has become an issue of concern for human and animal health in the last decade (EFSA, 2014). ENB isoforms have been described as phytotoxins, with a wide range of toxicological effects, such as cytotoxic, insecticidal, antibacterial, antifungal and phytotoxic properties (Prosperini et al., 2014). ENB cytotoxicity has been proved to produce, via the mitochondrial pathway, early reactive oxygen species (ROS) inducing lipid peroxidation (LPO), apoptosis and necrosis (Prosperini et al., 2013).

In vitro cell culture models are commonly used as a suitable model for agricultural grains toxicity studies, even if they have different target sites in biological systems. Cells answer rapidly to toxic stress by altering their metabolic rates and cell growth changes. Therefore, cytotoxic studies are useful to estimate through different parameters the effects of mycotoxins. Indeed, it has previously reported cytotoxic effects of ENs against mammalian and cancer cell lines: Caco-2, Hep-G2 and HT-29 (Meca et al., 2011, 2012), IPEC1 ((Kolf-Clauw et al., 2013), Min-6 (Tonshin et al., 2010), and CHO-K1 (Lu et al., 2012).

A recent discovery showed dietary intake of ENs reaching the bloodstream in detectable concentrations and it determined the need for a next step by selecting a cell line from the immunological system to investigate its effects (Manyes et al., 2014). Thus, human lymphoblastic T-cells were elected as a validated model in immunotoxicity experiments. It is also a reliable, stable cell line easy to manipulate and reproduce (Shao et al., 2014). Moreover, it has been used in the past to evaluate toxic effects induced by exposure to mycotoxins: aflatoxins AFB1 and AFM1 (Luongo et al., 2014), satratoxin H (Nielsen et al., 2009), deoxynivalenol (DON) (Katika et al., 2012, Pestka et al., 2005) and nivalenol (NIV) (Taranu et al., 2010, Severino et al., 2006), fumonisin B1 (FB1), alpha-zearalenol (alpha-ZEA) (Luongo et al., 2008), 4 beta-acetoxyscirpendiol (4-MAS), 15-acetoxyscirpenol (15-MAS), 4,15-diacetoxyscirpenol (4,15-DAS), and 30-acetyldiacetoxyscirpenol (TAS) (Lee et al., 2006).

The embracement of big data by geneticians and bioinformaticians has opened an exciting path for toxicology researchers as it opens a much more precise and real information that other approaches as microarrays.

Toxicogenomics is one of the most innovative –omics to date and it is certain that its importance will gain weight as technology advances (Afshari et al., 2010). It could be defined as the field which tries to understand by analyzing chemical alterations in gene expression patterns molecular mechanisms of toxicity. Until now, the most utilized technique in toxicogenomics was microarrays which can measure the changes in transcription through hybridization of the target with a very large set of short, labeled probes (Li et al., 2015). However, Next Generation Sequencing (NGS) is becoming a common technique because its cost has lowered in the last years. It is based in solid-phase bridge amplification (© Illumina) and its accuracy, efficiency and the amount of information that provides through data treatment, makes NGS the key technique in genomic or proteomic studies in the following years (Jagadish et al., 2014). The RNA-seq gives detailed information of the gene composition and expression. It also furnishes information about the transcriptome to understand the expression changes (Conesa et al., 2016).

The purpose of this study was to reveal changes in the gene expression profile caused by ENB in human Jurkat lymphoblastic T-cells after 24 h of exposure at the following concentrations: 1.5, 3 and 5 μM.

2. Materials and Methods

2.1 Reagents

The reagent grade chemicals and cell culture components used, RPMI-glutamax medium, penicillin/streptomycin, phosphate buffer saline (PBS), and EN B (783.95 g/mol, 97% purity) were purchased by Sigma chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) and methanol were obtained from Fisher Scientific (Madrid, Spain). Deionised water (resistivity <18 MV

cm) was obtained using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Stock solution of EN B was prepared in methanol and maintained at -20 °C. Final concentrations of EN B in the assay were achieved by their dilution in the culture medium. The final methanol concentration in the medium was 1% (v/v).

2.2 Cell Culture and EN B exposure

Jurkat cells (ATCC TIB-152) derived from human T lymphocyte peripheral blood were maintained in RPMI-glutamax medium supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin and 10% (v/v) FBS inactivated. Incubation conditions were pH 7.4, 37 °C under 5% CO_2 and 95% air atmosphere at constant humidity. Culture medium was changed every two days. Absence of mycoplasma was checked routinely using the Mycoplasma Stain Kit (Sigma Aldrich, St Louis Mo. USA). Before contamination, cells were plated in 12-well tissue culture plates at a density of $5x10^5$ cells/well. Jurkat cells were exposed for 24 h in standard conditions to 1.5 µM, 3 µM, and 5 µM ENB in 1% methanol and this solvent concentration as control in maintenance medium (each condition triplicated).

2.3 RNA Extraction and Next Generation Sequencing (NGS)

Total RNA of the control and exposed human T lymphocytes cells was isolated using Directzol™ RNA MicroPrep kit and treated with RNase free DNaseI (Zymo Research) to remove genomic DNA contamination. The extracted RNA of each sample was firstly checked for quantity and quality using Agilent 2100 Bioanalyzer (Agilent Technologies). All sequenced samples were generated from high quality RNA samples having a RIN number above 8.

The standard Illumina protocol was followed to develop RNA-seq libraries. The Illumina NextSeq 500 platform was used for sequencing. Then, one archive for each sample was obtained, 12 in total. RNA quality control and sequencing were carried out by the Genomics section of the Central Service for Experimental Research (SCIE, University of València).

2.4 Data Processing

The latest versions of different analytical tools were used in order to achieve a RNA-seq differential gene expression analysis. Raw sequencing reads were pre-processed and quality control was first performed by FastQC software. Sequencing reads were aligned by Bowtie2 (v2.2.6) and mapped to the Ensembl Human genome sequence (GRCh38) using default parameters. Read counts of each gene were quantified using RSEM (v.1.2.28) and Normalization fitting a negative binomial distribution and pair comparison analysis (treated vs. control) were performed and DEGs were obtained using DESeq2 package. All the analyses were performed in R.

2.5 Gene Expression Analysis

All of the DEGs were subjected to over-representation and gene ontology (GO) analysis by ConsensusPathDB. GO annotations and functional classifications of DEGs were obtained. Pathway assignments were carried out using PathVisio software with Hs_Derby_Ensembl_85 bridge gene dataset. Adjusted $p \leq 0.05$ and z-score > 1.96 were used as the threshold to identify the significantly enriched GO terms and pathways.

2.6 Primer Design and Quantitative Real-Time PCR Assays

Gene-specific primers were designed using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/) using default criterion of the software with amplified products ranging from 75 to 150 bp and Tm at 59°C. Primer sequences used in the qRT-PCR analyses are presented in Table 1. Standard RT-PCR was performed for all the primer pairs and a single amplification product of the expected size for each gene was obtained by the melting curve assay. Primer amplification efficiency was determined from standard curve generated by serial dilution of cDNA (5 fold each) for each gene in triplicate. Correlation coefficients (R^2 values) and amplification efficiencies (E) for each primer pairs were calculated from slope of regression line by plotting mean Cq values against the log cDNA dilution factor in StepOne software. Real-time amplification reactions were performed in 96 well plates using SYBR Green detection chemistry and run in triplicate on 96-wells plates with the StepOne Plus Real-time PCR machine (Applied Biosystems). Reactions were prepared in a total volume of 10 µl containing: 3 µl of 1:2 diluted template, 1 µl of each amplification primer (5µM) and 5 µl of 2x Fast SYBR Green (Applied Biosystems). Non-template controls (NTC) were also included for each primer pair, replacing the template by water DNAse and RNAse free from the RNA extraction kit (Zymo Research). The cycling conditions were set as default: initial denaturation step of 95°C for 5 min to activate the Taq DNA polymerase, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 59°C for 30s. The melting curve was generated by heating the amplicon from 60 to 90°C. Baseline, threshold cycles (Ct) and statistical analysis were automatically determined using the StepOne Plus Software version 2.3 (Applied Biosystems). All the experiments were done

according to MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) guidelines (Bustin et al., 2009).

3. Results

3.1 RNA Extraction, Sequencing and Quantification

RNA-extraction protocol to acquire the messenger RNA (mRNA) consists on several steps. Ribosomal RNA constitutes over 90% of total RNA in the cell and needs to be removed (Conesa et al., 2016). To control the data quality, RNA-seq data acquisition, every step from the obtention of raw reads to alignment and quantification was monitored. The Eukaryote Total RNA Nano assay (Bioanalyzer, Applied biosystems) showed that rRNA ratio (28S/18S) was >1.7 and RNA Integrity Number (RIN) was above 8. Total number of reads per FASTQ ranged from 22M to 46M, being the median 40188286, which indicates high overall sequencing accuracy and low presence of mapped reads considered an important mapping quality parameter.

3.2 Gene Expression Profile

The untreated cells overall gene expression in 24 h differs significantly from the EN B treated ones (doses 1.5, 3 and 5 μM). Those treatments to Jurkat cells increased in comparison to control samples from 367 DEGs at 1.5 μM to 5022 DEGs at 3 μM and 4644 DEGs at 5 μM. Considering all three EN B concentrations the total number of DEGs was 5750, with 54% down-regulation and 46% up-regulation. The down-regulated genes ranged from 44 to 73% of the total DEGs while the up-regulated ones represented between 27 to 66%, if taken into account the overall percentages for the three conditions.

EN B could also compromised the usual expression levels up to 27% of human genome (5750 genes for both down-regulation and up-regulation) considering that the total human genome contains ~21000 protein coding genes (HGNC database).

The number of differential expressed genes (DEGs) found for the three conditions showed variability and significant differences between the two highest concentrations (3 and 5 μM) of study compared to first concentration (1.5 μM) and control sample. Around 65% (3746 genes) overlapped in the highest doses 3 and 5 μM. For each condition the DEGs were as follow: 367 genes at 1.5 μM, 5022 genes at 3 μM and 4644 genes at 5 μM. There were 1712 altered in one single dose: 2% at 1.5 μM, 62% at 3 μM and 36% at 5 μM. 245 differentially expressed genes were found to be affected for each one of the three studied EN B concentrations, which conformed the most relevant geneset (Figure 1).

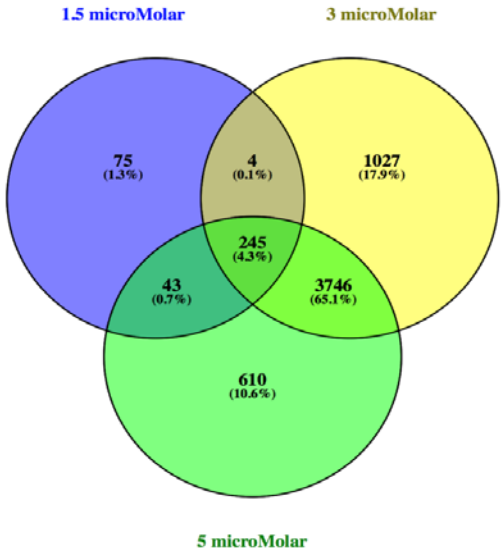

Figure 1. Venn diagram for the differentially expressed genes in Jurkat cells treated with BEA.

3.3 Differentially Expressed Genes (DEGs)

The basis for pathway or gene set enrichment analysis is to generate a precise list of differentially expressed genes (Fang et al., 2012). In the three EN B concentrations 245 DEGs were found to be overlapping, 83 were down-regulated and 162 up-regulated. Discrepancy between differential expression and dose affected only 3 genes, being one up-regulated at 1.5 μM while down-regulated at 3 and 5 μM and two down-regulated at 1,5 μM while up-regulated at 3 and 5 μM.

Statistical analysis of the DEGs showed that the most up-regulated genes were the same 3 for each condition and the most down-regulated were similar also for the 3 samples. The most up-regulated gene was the ChaC1 with a Log2FC between 1.29 and 2.44. The most down-regulated gene was COX2 with a Log2FC between -1.28 and -2.79.

The number of genes involved in the poly(A) RNA binding molecular process was remarkably affected with 86 at 1.5 μM, 408 at 3 μM and 401 at 5 μM. The mitochondria was another target of EN B toxicity with almost 57% of the genes involved in the mitochondrial structure affected in all 3 conditions. The respiratory electron transport chain was down-regulated in a 34% while the oxidative phosphorylation process suffered almost a 16% down-regulation. Moreover, there were 19 genes altered in these two biological processes from which 47% were also altered in the respiratory complex I function and 84% in the respiratory chain complex (Supplementary data).

3.4 Gene Ontology and Enriched protein complex

In a standard transcriptomics study, ranking the transcriptome by comparing a list of DEGs against the rest of the genome for over-represented

functions according to measurement of differential expression is an important step in the characterization of the molecular functions or pathways in which DEGs are involved (Conesa et al., 2016). ConsensusPathDB analysis of over-representation of the selected 245 DEGs, provided a list of gene ontology (GO) terms in which several biological processes related to nucleoside monophosphate metabolic process, respiratory chain complex, electron transport chain, oxidative phosphorylation, cellular respiration were most over-represented. 238 of 245 Ensembl accession numbers (97.1%) from the input list were mapped to 238 distinct genes in ConsensusPathDB and 36195 of 60677 accession numbers (59.7%) from the background list were mapped to 36211 distinct genes.

Table 2. Relevant GOs obtained from the over-representation analysis of the 245 overlapped DEGs in ConsensusPathDB

Gene Ontology term		Category, level	Candidates contained	p-value	q-value
GO:0044429	mitochondrial part	CC 3	55 (5.7%)	4.2e-22	3.53e-20
GO:0098800	inner mitochondrial membrane protein complex	CC 4	21 (18.6%)	3.72e-19	3.13e-17
GO:0098798	mitochondrial protein complex	CC 3	22 (16.7%)	6.12e-19	2.57e-17
GO:0005740	mitochondrial envelope	CC 4	41 (5.8%)	1.01e-16	4.24e-15
GO:0098803	respiratory chain complex	CC 4	16 (21.9%)	4.37e-16	1.22e-14
GO:0009123	nucleoside monophosphate metabolic process	BP 5	29 (9.8%)	4.61e-18	1.56e-15
GO:0022904	respiratory electron transport chain	BP 4	18 (17.0%)	7.44e-16	3.5e-13
GO:0022900	electron transport chain	BP 3	18 (16.7%)	1.05e-15	3.09e-13
GO:0006119	oxidative phosphorylation	BP 4	18 (16.4%)	1.47e-15	3.5e-13
GO:0045333	cellular respiration	BP 4	21 (12.1%)	3.07e-15	4.86e-13
GO:0003723	RNA binding	MF 4	59 (3.7%)	1.03e-14	8.83e-13
GO:0044822	poly(A) RNA binding	MF 5	49 (4.2%)	2.85e-14	1.57e-12
GO:0003954	NADH dehydrogenase activity	MF 4	9 (20.0%)	3.23e-09	1.39e-07
GO:0050136	NADH dehydrogenase (quinone) activity	MF 5	9 (20.0%)	3.23e-09	8.88e-08
GO:0016491	oxidoreductase activity	MF 2	29 (4.0%)	2.84e-08	9.38e-07

Molecular functions linked to RNA binding, NADH dehydrogenase and NADH dehydrogenase (quinone) activity were statistically more significant in the DEGs set, while cellular components such as mitochondrial respiratory chain, mitochondrial membrane, mitochondrial envelope, mitochondrial protein complex and respiratory chain were significantly affected but in a lower degree.

Regarding the protein complexes, 148 genes (62.2%) from the input list are present in at least one protein complex. The total number of genes from the background list present in at least one protein complex is 9086.

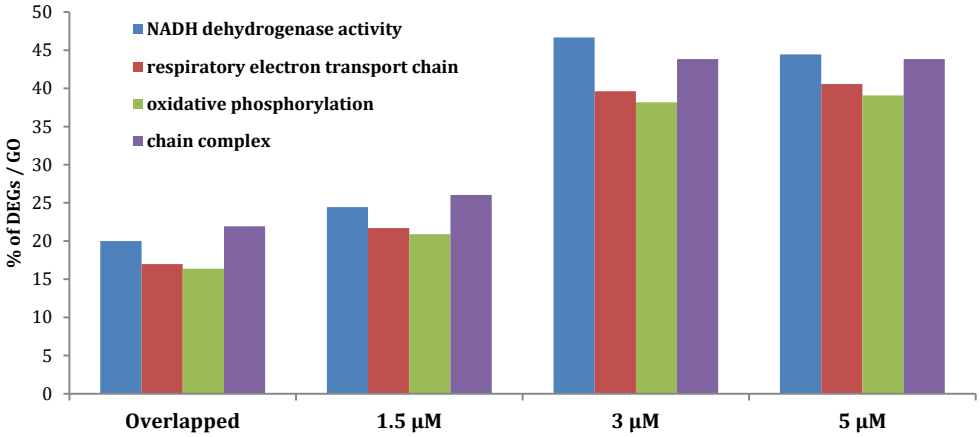

Figure 2. % of DEGs involved per GO regarding the 245 DEGs overlapped in the three concentrations of EN B tested and the DEGs obtained individually. MF, molecular function GO:0003954 NADH dehydrogenase activity; BP, biological process GO:0022904 respiratory electron transport chain and GO:0006119 oxidative phosphorylation; CC, cellular component GO:0098803 respiratory chain complex.

3.5. Pathway Analysis

PathVisio showed a total of 7688 data points (N) and a higher number of data points meeting criterion (R) was found in the higher doses: 367, 5022

and 4644 for 1.5, 3 and 5 μM, respectively. 131 data points meeting criterion (R) were overlapped when all concentrations data where integrated. 21 pathways were statistically significant for the three studied doses (z-score>1.96; adj p-value<0.05). The most relevant pathways affected by the number of genes were the electron transport chain and the oxidative phosphorylation with 22 and 19% affected genes, respectively (Table 3). The analysis also showed a significant alteration on the TCA Cycle and Deficiency of Pyruvate Dehydrogenase complex with 22% and 23% of genes altered across all the studied doses.

Table 3. Pathways overlapped in all EN B conditions by PathVisio.

Pathway	positive (r)	measured (n)	total	%	Z Score
Electron Transport Chain	23	103	118	22,3%	16,3
Oxidative phosphorylation	12	62	68	19,4%	10,8
TCA Cycle	4	17	48	23,5%	7,0
TCA Cycle and Deficiency of Pyruvate Dehydrogenase complex (PDHc)	3	16	35	18,8%	5,3

Electron transport chain pathway (z-score: 16.3) and oxidative phosphorylation pathway (z-score: 10.8) showed 23, 42 and 43 DEGs for 1.5, 3 and 5 μM both, all down-regulated. The strongest down-regulated genes in the electron transport chain pathway belonged to the complex I with 7, complex III with 1, complex IV with 3 and complex V with 2. Another consistent data with EN B toxicity reported were the most up-regulated genes which belonged to the uncoupling protein (Figure 2).

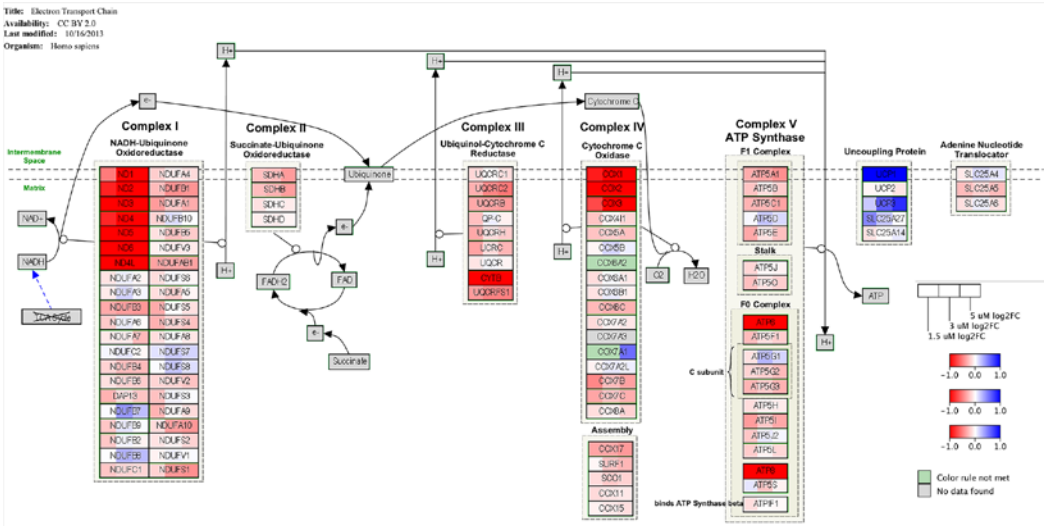

Figure 3. Genes involved in the electron transport chain pathway for Homo sapiens are shown: in red the down-regulated and in blue the up-regulated genes for the three EN B conditions (1.5, 3 and 5 µM).

The results ground the scientific evidence, using the transcriptomic approach, that mitochondria are the main cell organelle responsible of EN B induced toxicity.

3.6. Confirmation of NGS results by PCR

The sequencing-based results were validated using qPCR. The expression of COX2, a mitochondrial electron transport chain gene, SLC7A11, an amino acid transporter gene, and STC2, a calcium ion balance gene, was measured in Jurkat cells after exposure to EN B in the same conditions than in the NGS assay. The results confirmed the strong downregulation of COX2 and the upregulation of SLC7A11 and STC2 genes compared to the control (Figure 3). House-keeping gene 18S Rrna was used as endogenous gene control (Fig. 3). S18 was not differently expressed at any concentration when exposed and

it has already been validated as a consistent house-keeping gene (Banda et al., 2008).

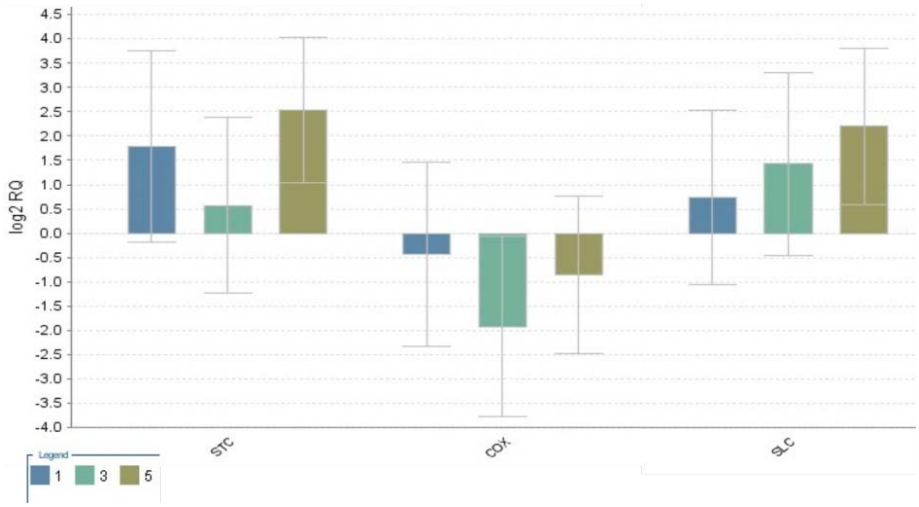

Figure 4. Bar plot showing Jurkat cells relative expression of selected genes when compared to control (Log2RQ=0) after 24h-exposure to different concentrations of EN B by qPCR. RQ, relative quantification.

4. Discussion

EN B is a ionophoric and lipophilic mycotoxin which has the ability to easily penetrate the cellular membranes (Kamyar et al. 2004). It forms dimers which work as ionophore transporting ions into the cell changing the pH and the ionic balance (Prosperini et al., 2017). Cell membranes have different electrochemical potentials and mitochondria has the highest membrane potential in the cell, so EN B toxicity seems to focus on mitochondria l pathways. Indeed, the cytotoxic activity of EN B could be based on the induction of apoptosis via the mitochondria l pathway (Dornetshuber et al. 2007; Wätjen et al. 2009). Moreover, Tonshin et al., 2010 has studied the

ionophoric properties of EN B in rat liver mitochondria showing that it functioned as an uncoupling inhibitor of the respiration. As the epicenter for cell regulation and homeostasis, mitochondria plays a crucial role in most of the main cellular activities, so any variability could induce cell death pathways such as the caspase-dependent and –independent. However, the mechanisms responsible for the spread of these pro-death molecules are still largely unknown (Donovan et al. 2004). The respiratory chain involves five different complexes with the ability to produce Adenosin Triphosphate (ATP) and oxygen.

Mitochondrial metabolism is responsible for respiration in order to produce ATP and free energy release and it is dependent on the electron transport chain function composed of five respiratory complexes (Qi et al., 2017). Several genes related to respiratory chain were significantly altered in the present study. There are 96 genes implicated in the respiratory chain (HGNC database): 35 genes were down-regulated in at least one dose, indicating that EN B induced perturbation in 36% of the respiratory chain genes. Complex II and complex V showed the highest number of perturbed genes (47-50%) although four complexes were affected. Among these 35 genes, 9 were differentially expressed for all three EN B concentrations: complex I and II (1 gene each), and complexes III and V (3 genes each). As shown in Figure 3, all 9 genes were down-regulated in a clarifying dose-dependent manner. From NADPH and FADH2, the electrons are transferred to oxygen generating ATP and oxidized NADP1 and FAD1. Reliant on the substrate, electrons are transported from complex I (NADH dehydrogenase (quinone)) and complex II (succinate dehydrogenase) through ubiquinone and complex III (ubiquinolcytochrome c reductase) to cytochrome-c and to

complex IV (cytochrome-c oxidase), which produces water, whereas ATP is produced by complex V (ATP synthase) (Dudkina et al., 2006).

The results show a slight trend towards down-regulation against up-regulation after EN B exposure to Jurkat cells. Analogous gene expression profile was recently observed by microarray technique after 4 h exposure of the emerging mycotoxin EN B at 10 μM on rat primary hepatocytes. Down-regulated genes were five times more numerous than up-regulated ones. Moreover, it was discovered the variation of energy metabolism due to alterations on mitochondrial organization and function, and the assembly of complex I of the electron transport chain (Jonsson et al., 2016). Interestingly, even if numbers are not comparable because of different cell line used and concentration of exposure, it is worthy to mention that in Jurkat cells, 23 DEGs belong to NDUF family and also 23 were from TRIMM family genes, in at least one of the three concentrations. Besides, 13 of the 34 rat primary hepatocytes DEGs involved in mitochondrion organization were also found among Jurkat DEGs and, related to proliferation process, two kinases and five regulators are identical in both studies.

Furthermore, these data suggest a tendency towards a maximum of EN B genotoxic effects as a similar number of genes were altered for 3 μM and 5 μM doses after 24h. Transcriptomic changes were dose dependent between 1.5 and 3-5 μM, but between 3 and 5 μM it was similar, suggesting two possibilities, or time dependent transcriptomic damage as early as an exposure of 3 μM or a maximum of damage independent of time because no more EN B penetrates into the Jurkat cell and they die before accumulating more DEGs. Future research will clarify these hypotheses.

5. Conclusion

The transcriptomic analysis based on RNA-seq addressing EN B cytotoxicity in human Jurkat lymphoblastic T cells revealed that mitochondria are the organelles showing more related DEGs, as it has been reported for rat primary hepatocytes at a higher dose and a shorter exposure. Consequently, biological processes, molecular functions, protein complexes and pathways, which are placed in these mitochondria are affected and it has been evidenced in the results obtained.

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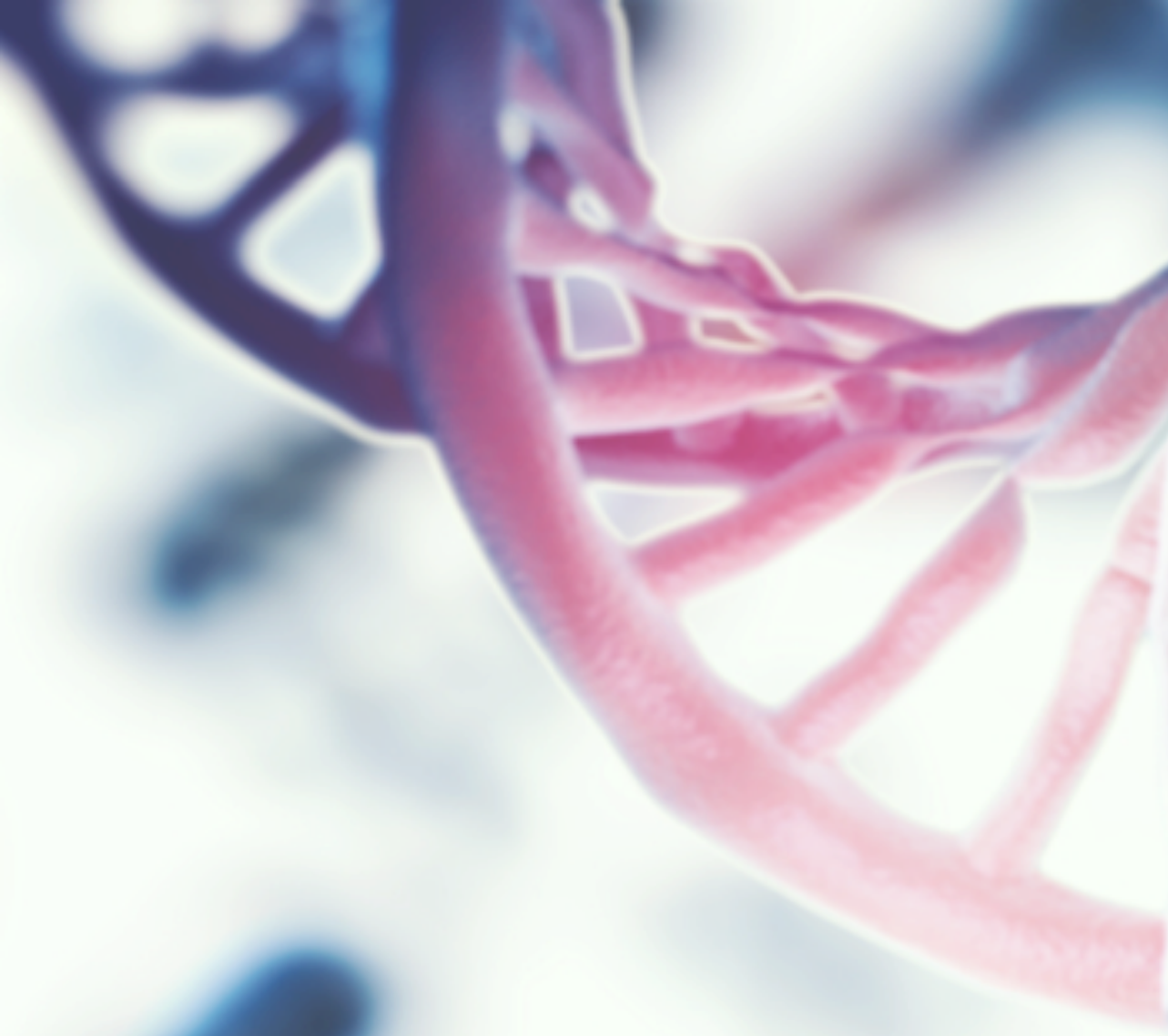

4. GENERAL DISCUSSION

4. DISCUSIÓN GENERAL

Acorde con los objetivos propuestos, en la presente tesis doctoral se evaluó la toxicidad de las micotoxinas emergentes BEA, EN A y EN B a través de (1) estudios *in vitro* en la línea celular Jurkat linfocitos T humanos; (2) estudios *in vivo* en rata Wistar, y (3) estudios transcriptómicos mediante la técnica de secuenciación masiva RNA-seq. Asimismo, se analizó el contenido micotoxigénico en piensos destinados a ratas de laboratorio y en orina humana mediante técnicas de separación cromatográfica y detección por espectrometría de masas. Para ello, fue necesario desarrollar, optimizar y validar multi-métodos analíticos eficaces, exactos y sensibles capaces de garantizar una buena calidad desde el punto de vista analítico.

4.1 Desarrollo y Validación de Métodos Analíticos

Durante la presente investigación se han desarrollado una serie de métodos analíticos con diversas finalidades, desde la determinación de micotoxinas emergentes de *Fusarium* en muestras biológicas de rata Wistar formando parte de experimentos *in vivo*, hasta el análisis multi-micotoxinas de piensos animales y orina humana. Todas las metodologías desarrolladas han sido validadas previamente a su aplicación obteniendo resultados satisfactorios.

Como se observa en la Tabla 1, el análisis de ENs en muestras biológicas de rata como suero, orina, heces, hígado, riñón e intestino se realizó mediante la metodología de extracción LLE con los disolventes EtOAc y ACN, adicionando NaCl a este último en el caso de las muestras de orina, favoreciendo así la formación de dos fases.

Para el análisis de 11 micotoxinas en orina humana se realizó un

estudio comparativo de las metodologías de extracción SALLE, miniQuEChERS y DLLME, en el que se optimizaron las condiciones de extracción para cada técnica (tipo y volumen de disolvente, adición de una etapa de purificación), se validaron los métodos de manera individual y se evaluó la eficiencia de los mismos para finalmente seleccionar la metodología más apropiada. Las condiciones óptimas se obtuvieron con el uso de ACN como disolvente de extracción, combinado con EtOAc en el caso de DLLME, técnica que requiere dos disolventes; dispersante y extractante. Se consiguió así validaciones satisfactorias para las tres metodologías, seleccionando DLLME como la técnica más adecuada mostrando mayor sensibilidad y precisión.

La extracción de 19 de micotoxinas en piensos se realizó mediante UAE, es decir LLE asistida con ultrasonidos, con la mezcla acidificada ACN/H_2O (80:20). Los extractos obtenidos fueron por una parte inyectados en LC-MS/MS para el análisis de 11 compuestros entre los que se encuentran las ENs, y por otro lado evaporados y derivatizados para el posterior análisis de 7 tricotecenos mediante GC-MS/MS. La Tabla 1 recoge las metodologías validadas especificando las micotoxinas de análisis, la matriz y método de extracción, el método de separación-detección, así como los valores de sensibilidad, exactitud y precisión.

Tabla 1. Métodos de análisis de micotoxinas validados en la presente tesis doctoral.

Método de Análisis				Validación del Método		
Micotoxinas	**Matriz**	**Extracción**	**Detección**	**Sensibilidad LODs/LOQs** *(µg/kg o µg/L)*	**Exactitud (R%)**	**Precisión (RSD%) Intra-/Inter-**
EN A	Muestras biológicas rata	LLE-EtOAc	HPLC-MS/MS	0.2/10	70-156	<3/<10
ENs A, A1, B, B1	Muestras biológicas rata	LLE-ACN (*NaCl)	HPLC-MS/MS	0.2-1/2-10	70-106	<9/<19
BEA, EN A, EN B, EN A1, EN B1, AFB1, AFB2, AFG1, AFG2, OTA, ZEN	Orina humana	SALLE-ACN-NaCl/C18	HPLC-MS/MS	0.1-10/0.5-40	70-108	<14/<24
		miniQuEChERS-ACN-MgSO4/C18	HPLC-MS/MS	0.1-15/0.5-35	71-109	<14/<24
		DLLME-ACN/EtOAc (10:100, v/v)	HPLC-MS/MS	0.05-2/0.1-4	79-113	<12 <15
EN A, EN A1, EN B, EN B1, AFB1, AFB2, AFG1, AFG2, FB1, FB2, T-2, HT-2	Piensos rata	UAE-ACN/H2O/HCOOH (79:20:1, v/v)	HPLC-MS/MS	1-5/5-15	73-112	<9/<13
DON, 3-ADON, 15-ADON, NIV, NEO, DAS, FUS-X	Piensos rata	UAE-ACN/H2O/HCOOH (79:20:1, v/v) Derivatización BSA+TMCS+TMSI	GC-MS/MS	0.5-5/1-10	62-97	<24/<29

4.2 Presencia de Micotoxinas en Alimentos y Piensos

En los últimos años, numerosos productos alimentarios han sido analizados para investigar la presencia de micotoxinas de *Alternaria,* principalmente Alternariol (AOH), Alternariol Metil Éter (AME), Tentoxina (TEN), Ácido Tenuazónico (TeA), Altenuene (ALN), Alterotoxinas (ATX) y Macrosporina. Los alimentos objeto de análisis son en su gran mayoría cereales, frutas y vegetales, así como derivados de los mismos, con especial énfasis en materias primas como cereales, tomates y productos elaborados a base de tomate, manzanas, y lentejas, donde además se detectan las mayores concentraciones (Escrivá et al., 2017d). Aunque no existen normativas regulatorias internacionales para ninguna de las micotoxinas de *Alternaria*, la EFSA emitió en 2011 una opinión científica sobre los riesgos para la salud humana y animal asociados a la exposición a estas micotoxinas a través de alimentos y piensos, indicando un posible riesgo potencial a pesar de no haber finalizado la evaluación del riesgo debido a la limitada información científica disponible sobre la presencia y toxicidad de estos compuestos (EFSA, 2011).

En el caso de las micotoxinas del género *Fusarium*, algunas de las cuales (DON, ZEN, FBs, T-2 y HT-2) se encuentran actualmente legisladas y bajo un estricto control a nivel europeo, su presencia en numerosos productos para el consumo humano y animal sigue siendo frecuente y en ocasiones preocupante (Lee and Ryu, 2017). Asimismo, las micotoxinas emergentes de *Fusarium,* sobre las que la limitada información dificulta el establecimiento de normativas regulatorias, se encuentran frecuentemente y en concentraciones elevadas en alimentos, principalmente cereales y derivados, frutos secos y especias (EFSA, 2014).

Respecto a la contaminación de piensos, la presencia de micotoxinas

se ha informado durante décadas en todo el mundo (Streit et al., 2012; Kim et al., 2014). No es de extrañar si se tiene en cuenta la composición de los piensos, principalmente elaborados a base de mezclas de materias primas cereales y proteínas de semillas, lo que los hace altamente susceptibles a la contaminación micotoxigénica. Se ha demostrado que durante el procesamiento de cereales las micotoxinas se concentran en las fracciones utilizadas comúnmente como alimento para animales (Pinotti et al., 2016)

La Unión Europea establece valores máximos (AFB1) y valores orientativos para algunas micotoxinas (DON, ZEN, FBs, AFs, OTA, T-2 y HT-2) en distintos tipos de piensos destinados a animales de granja o mascotas de compañía (European Commission 2002, 2006b, 2013). Sin embargo, la normativa vigente no especifica valores para piensos destinados a animales de laboratorio, donde la contaminación por micotoxinas podría no solo afectar a la salud animal, si no también repercutir negativamente en los resultados obtenidos en ensayos *in vivo*.

Para el estudio de piensos se recolectaron 35 muestras de pienso comercial para ratas de laboratorio obtenidos de diferentes animalarios y centros de investigación de España. El análisis de los mismos mediante las metodologías previamente descritas demuestra una muy elevada incidencia de contaminación por multi-micotoxinas con combinaciones entre 3 y 6 compuestos, siendo las más prevalentes EN B, EN B1, ZEN y DON, alcanzando esta última concentraciones superiores a 2 mg/kg. El alto porcentaje de muestras positivas concuerda con la literatura disponible y reitera la necesidad de una monitorización periódica de los piensos, en concreto los destinados a animales de experimentación, destacando la importancia de someterlos a análisis previamente a la realización de experimentos *in vivo*

*(*Escrivá et al., 2016a; 2016b).

Los niveles máximos para muchas micotoxinas deben todavía ser deliberados por la EFSA y regulados por la Unión Europea en un futuro próximo, con el fin de mejorar la calidad, tanto de alimentos como piensos, y preservar así la salud de consumidores y animales.

4.3 Presencia de Micotoxinas en Muestras Biológicas

Algunas micotoxinas son térmicamente estables y han demostrado capacidad de bioacumulación y transferencia a órganos, tejidos, leche materna o huevos tras su ingesta a través de alimentos contaminados. Como consecuencia de la transferencia y la bioacumulación, la contaminación por micotoxinas se ha evaluado no solo en productos agrícolas, alimentos y piensos, sino también en productos derivados de animales, así como en tejidos y fluidos biológicos de humanos y animales. Los niveles generalmente detectados en muestras biológicas son extremadamente bajos por lo que son numerosas las metodologías analíticas que han sido desarrolladas y aplicadas al análisis de micotoxinas con diversas finalidades como la biomonitorización y evaluación de la exposición humana y animal; el estudio de la distribución, bioacumulación, transferencia y persistencia de micotoxinas en tejidos; el estudio toxicocinético y absorción, distribución, metabolismo y excreción (ADME), y el estudio del papel de las micotoxinas en ciertas patologías. Dada su recolección no invasiva y fácil manejo, la orina humana ha sido, en la última década, la muestra biológica más analizada, seguida de órganos como hígado y riñón de animales que incluyen cerdo, rata y pollo, y finalmente gran variedad de muestras biológicas como suero, heces y leche materna (Escrivá et al., 2017a).

Gracias al análisis de micotoxinas en muestras biológicas se dispone de datos de persistencia y bioacumulación en órganos y tejidos de muchos de estos compuestos tóxicos naturales. Se ha demostrado la distribución y bioacumulación de OTA principalmente en el riñón, considerado el principal órgano diana (Soto et al., 2015), así como la transferencia transplacentaria en cerdos y humanos (Minervini et al., 2013); la bioacumulación de ZEN en organismos vivos llegando a seres humanos a través de todos los niveles de la cadena alimentaria (Kwasniewska et al., 2015); así como de DON y sus metabolitos que han sido detectados en órganos y tejidos destacando hígado, riñón y músculo de cerdos (Schneweis et al., 2005) y ratones (Amuzie et al., 2008) tras su administración oral. La FB1 se bioacumula principalmente en hígado y el riñón tras su administración oral e intravenosa en pavos (Tardieu et al., 2008), mientras que la toxina T-2 es rápidamente absorbida y metabolizada, principalmente a HT-2, siendo ambas rápidamente distribuidas en tejidos que incluyen hígado, riñón, intestino, pulmones, bazo y tejido graso en pollos, mostrando además considerable persistencia (Osselaere et al., 2013; Sun et al., 2014; Yang et al., 2015). Sin embargo, T-2 no se ha sido detectada en ratas tras la administración oral de concentraciones superiores (Chandratre et al., 2014). Las tasas de transferencia observadas de AFB1 en forma de su metabolito AFM1 en leche de vacas y burras son prácticamente insignificantes (Britzi et al., 2013; Winkler, et al., 2015; Tozzi et al., 2016). Finalmente, debido a su naturaleza lipofílica, varios estudios han indicado la distribución y bioacumiulación de BEA y ENs en órganos y tejidos de rata, ratón y pollo (Jestoi et al., 2007; Manyes et al., 2014; Rodríguez-Carrasco et al., 2016).

La información obtenida del análisis de micotoxinas en órganos,

tejidos y fluidos evidencia, por tanto, la importancia del análisis de muestras biológicas como herramienta útil para la monitorización de la exposición humana y animal a micotoxinas, así como la necesidad de considerar los aspectos de transferencia y bioacumulación de estos compuestos a la hora de realizar la evaluación de riesgo de micotoxinas.

4.4 Evaluación de la Exposición a Micotoxinas: Análsis de Orina y Técnicas de Reducción.

Debido a la considerable establilidad de las micotoxinas durante el procesado de alimentos resulta prácticamente imposible eliminar estos compuestos tóxicos de los alimentos una vez contaminados. Es por ello la necesidad de implementar estrategias para minimizar la exposición humana, ya sea estableciendo niveles máximos en productos comúnmente contaminados, como monitorizando los niveles de micotoxinas en seres humanos y animales expuestos (Lee and Ryu, 2017). Muchas autoridades nacionales e internacionales de salud pública y gubernamentales, como Food and Drug Administration (FDA), Organización Mundial de la Salud (OMS), Food Agriculture Organization (FAO) y EFSA están prestando seria atención a la contaminación de alimentos y piensos por micotoxinas, abordando el problema global mediante la adopción de normativas y directrices para las principales clases de micotoxinas, contando actualmente con alrededor de 100 países que disponen de límites establecidos sobre la presencia de estos contaminantes en alimentos y piensos (Alshannaq and Yu, 2017).

Los estudios de exposición a micotoxinas se basan generalmente en la combinación de datos sobre la presencia de micotoxinas en alimentos junto con datos poblacionales de consumo de los mismos. Sin embargo, este

enfoque indirecto presenta limitaciones originadas por la distribución heterogénea de las micotoxinas en los alimentos, la subestimación y sobreestimación de los datos de consumo y las diferencias individuales en cuanto a la absorción, distribución, metabolismo y excreción (Huybrechts et al., 2015). Los estudios de monitorización de micotoxinas en muestras biológicas como sangre y orina se consideran un enfoque útil y fiable para evaluar la exposición real a micotoxinas a corto y largo plazo, ya que permiten una evaluación de la exposición más precisa y objetiva a nivel individual cubriendo todas las fuentes de exposición, y reduciendo así las incertidumbres relacionadas con las tasas de incidencia y de consumo (Song et al., 2013). En esta línea, se han realizado estudios de biomonitorización de micotoxinas a través del análisis de orina en diferentes países incluyendo España, Bélgica, Portugal, Alemania, Italia y Austria, demostrando una elevada frecuencia de micotoxinas en muestras biológicas a pesar de que, en la mayoría de los casos, el cálculo de la ingesta diaria probable (PDI) no supera los valores de ingesta diaria tolerable (TDI) (Escrivá et al., 2017a). Por otra parte, además de la monitorización humana y evaluación de la exposición a micotoxinas, el análisis de muestras de orina permite el establecimiento de rangos de población de referencia e identificación de grupos de población vulnerables (Mally et al., 2016), así como relacionar los niveles urinarios de micotoxinas con algunas enfermedades como la nefropatía (Hmaissia Khlifaa et al., 2012).

Motivado por la ubicua, extensa y prácticamente inevitable contaminación de alimentos y piensos por micotoxinas, el desarrollo de medidas efectivas para contrarrestar y/o reducir la toxicidad de las mismas se considera un aspecto importante a considerar en la inocuidad de los

alimentos. Como consecuencia, se observa creciente interés en las investigaciones encaminadas a las técnicas de reducción y/o descontaminación de micotoxinas mediante el uso de agentes adsorbentes como aglutinantes (carbón activo, aluminosilicatos, arcillas, polímeros químicos, productos de glucano, levaduras, etc.) que agregados a la dieta interactúan *in vivo* con las micotoxinas restringiendo su absorción en el tracto gastrointestinal de seres humanos y animales (JRC European Commission, 2010). De forma similar, los productos alimentarios que contienen polifenoles podrían contribuir a disminuir el riesgo toxicológico asociado a la exposición a micotoxinas. La ingesta de antioxidantes en la dieta es una forma plausible y efectiva de aumentar y fortalecer los sistemas de defensa endógenos, ya que muchos de ellos pueden actuar como eliminadores de radicales libres induciendo así a la citoprotección. En esta línea, la quercetina (QUER) es, por sus propiedades, un compuesto adecuado para estudiar la citoprotección contra la exposición a micotoxinas, tanto *in vitro* como *in vivo*. Debido a su estructura química, QUER contrarresta el estrés oxidativo generado como resultado de ROS, el cual puede contribuir a la génesis de aterosclerosis, hipertensión, diabetes, cardiopatía isquémica e insuficiencia cardíaca (Salem et al., 2014).

Varios estudios *in vitro* han descrito la actividad protectora de QUER frente al efecto citotóxico de micotoxinas como PAT, AFB1, DON, T-2, ENs y BEA, sugiriendo que el consumo de alimentos ricos en QUER podría considerarse una buena estrategia para prevenir la toxicidad inducida por micotoxinas (Boussabbeh et al., 2015). Sin embargo, para evidenciar efectos beneficiosos para la salud por la ingesta de antioxidantes a través de los alimentos, es necesario que estos compuestos alcancen concentraciones

plasmáticas suficientes para desencadenar el efecto biológico, por lo que son necesarios más estudios sobre el mecanismo de acción de QUER y sus metabolitos bioactivos, así como de su biodisponibilidad y bioaccesibilidad (Escrivá et al., 2017b)

A pesar de la importancia de las investigaciones sobre la descontaminación y reducción de la toxicidad de las micotoxinas, la prevención de las infecciones fúngicas es, sin duda, la manera más racional y eficiente de evitar la contaminación de micotoxinas en productos agrícolas, y por extensión en alimentos y piensos destinados al consumo humano y animal.

4.5 Estudio *in vitro* de Micotoxinas Emergentes

Son numerosos los estudios *in vitro* que informan de los efectos citotóxicos de BEA y ENs en varios tipos celulares, sin embargo, a pesar de que algunas micotoxinas como DON, AFB1 y OTA han sido clasificadas como inmunotóxicas, la respuesta tóxica de las micotoxinas emergentes en el sistema inmunológico está todavía por elucidar (Shao et al., 2014). Las células Jurkat han sido ampliamente utilizadas en estudios del sistema inmune obteniendo respuestas de toxicidad similares a las de células mononucleares de sangre periférica frente a micotoxinas como DON (Katika et al., 2012). Sin embargo, a pesar de que otras micotoxinas como AFB1 (Luongo et al., 2014), NIV (Taranu et al., 2010), y FB1 (Luongo et al., 2006) han sido ya testadas en este tipo celular, los estudios de micotoxinas emergentes en celulas Jurkat son a día de hoy escasos.

En el presente trabajo se investigaron los mecanismos de citotoxicidad *in vitro* de las micotoxinas emergentes BEA y EN B en células Jurkat mediante

ensayos de viabilidad celular, producción de ROS, análisis del ciclo celular, muerte celular por apoptosis y/o necrosis, activación de las caspasa-3 y daño del ADN (Manyes et al., 2018 under review).

Tras la exposición de células Jurkat a BEA (1 y 15 μM; 24, 48 y 72 h) se observó reducción de la viabilidad celular con valores de IC50 entre 3 y 7.5 μM, mientras que la reducción de la viabilidad celular tras la exposición a las mismas concentraciones de EN B fue en todos los casos inferior al 30%, indicando un IC50 >15 μM. Para ambas micotoxinas la reducción de la viabilidad se mostró dependiente de la dosis y el tiempo. BEA además aumentó el porcentaje de células apoptóticas/necróticas de manera muy superior a EN B. Tanto BEA como EN B mostraron incremento de la actividad caspasa-3, sin observarse diferencias en la producción de ROS respecto al control, y revelando arresto en fase S para ambas micotoxinas tras el estudio del ciclo celular. En cuanto a la genotoxicidad, BEA aumentó el porcentaje de ADN en la cola entre 18-20% con respecto al control a las concentraciones de 3 y 5 μM, mientras que EN B no mostró alteraciones significativas en el daño del ADN a ninguna de las concentraciones testadas.

Los resultados obtenidos en células Jurkat concuerdan con los efectos tóxicos previamente descritos para BEA y ENs en líneas celulares como CHO-K1 (Mallebrera et al., 2016), Caco-2 (Properini et al., 2013a; 2013b) y Hep-G2 (Juan-García et al., 2015) en los que se indica apoptosis, necrosis, daño en el ADN y alteración del potencial de membrana mitocondrial, con efectos más evidentes y marcados en el caso de BEA en comparación con las ENs. Por ejemplo, valores de IC50 entre 2-9 μM para BEA frente a >15 μM para las ENs fueron descritos en células Caco-2 bajo las mismas condiciones de exposición (Prosperini et al., 2013a; 2013b). Acorde con los resultados obtenidos, la

reducción de la viabilidad celular tras la exposición a EN B en células HT-29, Caco-2 y Hep-G2 durante 24 h no alcanzó el 50% a las mayores dosis de ensayo (30 μM) indicando un IC50 superior (Meca et al., 2011). Tras evaluar los efectos tóxicos de BEA y ENs en células Hep-G2 se propone un orden de potencialidad tóxica decreciente BEA > EN B1 > EN A1 considerando el número e intensidad de los efectos tóxicos observados (Juan-García et al., 2015). La inhibición del flujo mediado por las proteínas de transporte ABCG2 y ABCB1 mostró ser más eficaz en el caso de BEA en comparación con las ENs en las líneas celulares humanas A549, GLC-4, KB-3-1 y HL-60 (Dornetshuber et al., 2009a). Los efectos inhibidores de BEA y EN B sobre la proliferación de células progenitoras sanguíneas como glóbulos blancos (CFU-GM), plaquetas (CFU-MK) y glóbulos rojos (BFU-E) varió en función de la micotoxina de estudio, con valores de IC50 similares para ambas micotoxinas en células BFU-E, pero menores para BEA en células CFU-GM y CFU-MK, indicando en este caso mayor citotoxicidad potencial de BEA frente a EN B (Dornetshuber et al., 2009b).

Con respecto a la genotoxicidad, estudios previos han indicado el potencial genotóxico de BEA en linfocitos humanos mediante ensayos de aberraciones cromosómicas, intercambios entre cromátidas hermanas, micronúcleos, e índices de división mitótica, proliferativa y nuclear (Celik et al., 2010). La fragmentación del ADN internucleosómico tras la exposición a BEA ha sido también observada en células CCRF-CEM (Jow et al., 2004), PK15 (Klaric et al., 2010), células sanguíneas de ave (Dombrink-Kurtzman, 2003), y leucocitos humanos (Klaric et al., 2010), apuntando además a la afección de las funciones inmunes al suprimir la proliferación e inducir la apoptosis en linfocitos (Dombrink-Kurtzman, 2003).

Son menos los estudios de genotoxicidad realizados con EN B, sin embargo, y acorde con los resultados obtenidos en células Jurkat, todos ellos coinciden en que el mecanismo de toxicidad de la micotoxina no parece estar relacionado con el potencial genotóxico (Prosperini et al., 2017). Así bien, no se observaron alteraciones significativas a nivel de ADN tras la exposición a EN B en células Caco-2 (Prosperini et al., 2013b), A549, GLC-4, KB-3-1, y HL-60 (Dornetshuber et al., 2009b).

Por otro lado, el exceso y generación temprana de ROS puede conducir al estado de estrés oxidativo celular originando estructuras macromoleculares oxidadas como lípidos de membrana, proteínas y ADN, parada del ciclo celular y apoptosis (Ferrer et al., 2009). A pesar de que existen datos previos que sustentan el incremento de ROS y LPO desencadenado por BEA y ENs en Caco-2 (Prosperini et al., 2013a; 2013b), en la presente investigación no se muestran indicios de incremento de ROS en células Jurkat tratadas con las micotoxinas.

Finalmente, los datos obtenidos indican incremento de la actividad caspasa-3 como respuesta a la exposición de BEA y EN B en células Jurkat, proponiendo así la vía apoptótica dependiente de las caspasas como uno de los posibles mecanismos de acción para la muerte celular inducida por estas micotoxinas emergentes. La diferencia entre la respuesta a una u otra micotoxina reside en la relación con el incremento del tiempo de exposición tras el tratamiento de BEA y, en el caso de EN B, con el incremento de la dosis. Acorde con el mecanismo propuesto, algunos estudios indican la inducción de apoptosis por BEA por disminución del potencial de membrana mitocondrial y liberación de citocromo c en el citosol tras la escisión y activación de las

caspasas mitocondriales, como la caspasa-3, en células NSCLC A549 (Lin et al., 2005).

La investigación *in vitro* realizada parece indicar que la exposición de células Jurkat a BEA y EN B disminuye la viabilidad celular de modo tiempo y dosis dependiente, aumenta el porcentaje de células apoptóticas con incremento de la actividad de caspasa-3, sin afección en la producción de ROS. Los efectos observados fueron más marcados en todos los casos tras la exposisción a BEA, la cual mostró tasas significativas de genotoxicidad en células Jurkat. Los resultados sugieren que, a pesar de su similitud estructural, BEA y EN B podrían desencadenar la respuesta tóxica por vías similares aunque con distinta potencialidad tóxica en células Jurkat. Sin embargo, a pesar de las investigaciones realizadas hasta la fecha, elaborar una clasificación de respuestas tóxicas desde el punto de vista mecanístico es todavía complicado y sugiere la aplicación de técnicas genómicas y moleculares.

4.6 Estudio *in vivo* de Micotoxinas Emergentes

La limitada información sobre las micotoxinas emergentes de *Fusarium* dificulta el establecimiento de normativas y regulaciones, así como el desarrollo de un plan de monitorización integral capaz de evaluar el riesgo y definir los límites de tolerancia a estos compuestos (Escrivá et al., 2015a). Todavía a día de hoy los datos de toxicidad y toxicocinéticos *in vivo* son escasos a pesar de que las fusarotoxinas emergentes han ganado mayor interés en las últimas décadas debido a la frecuente contaminación de alimentos y piensos por estos compuestos tóxicos (Fraeyman et al., 2017). Tal y como concluyó la EFSA, la exposición aguda a BEA y ENs no es una

preocupación para la salud humana, sin embargo no se han podido establecer conclusiones con respecto a la exposición crónica debido a la falta de datos de toxicidad *in vivo* relevantes. Se necesita por tanto, estudiar los efectos individuales y combinados de las micotoxinas emergentes *in vivo* para poder actualizar la evaluación de riesgo que dictaminó la EFSA en 2014.

En la presente investigación se evaluó la toxicidad subaguda de la EN A en rata Wistar tras la administración de la micotoxina vía oral durante 28 días en forma de pienso contaminado (20.91 mg/kg pc/día). Durante este periodo no se detectaron efectos adversos observables en los animales tratados respecto al grupo control. Tanto el peso corporal de los animales, como el peso de los principales órganos una vez transcurrido el periodo de ensayo se mantuvo sin diferencias estadísticamente significativas entre los grupos tratado y control. Tras evaluar los parámetros bioquímicos sanguíneos y realizar un examen histológico del tracto duodenal y las microvellosidades intestinales no se observaron diferencias significativas entre ambos grupos (Manyes et al., 2014). Tampoco se observaron efectos adversos tras la administración única por vía oral de una mezcla de ENs A, A1 B y B1 (1-2.2 mg/kg pc) en rata Wistar durante las 8 horas posteriores a la administración, ni tras el examen morfo- e histológico de órganos y tejidos realizado post-mortem (Escrivá et al., 2015b). La ausencia de efectos toxicológicos *in vivo* tras la administración oral de micotoxinas emergentes concuerda con estudios previos en rata (Juan et al., 2014) y ratón (Bosch et al., 1989; Rodríguez-Carrasco et al., 2016). Sin embargo, la administración de dosis elevadas de ENs (10-40 mg/kg pc) vía intraperitoneal en ratones inmunodeprimidos resultó ser letal (McKee et al., 1997). Se ha demostrado en cerdos que la formación de metabólitos de ENs es mayor tras la absorción

intestinal en comparación con la administración intravenosa (Ivanova et al., 2017), por lo que la ausencia del paso de las ENs a través del tracto gastrointestinal en la administración intravenosa e intraperitoneal podría favorecer el alcance de los niveles tóxicos en sangre al influir en mayor o menor grado en la estructura química de las micotoxinas (Jestoi, 2008), suponiendo, en cualquier caso, una aproximación menos representativa de la situación real de ingesta de alimentos contaminados.

Datos previos coinciden en la ausencia de efectos adversos observables en aves de corral tras la ingesta de dietas multi-contaminadas con micotoxinas emergentes, entre otros compuestos como DON, T-2, HT-2, y ZEN, alcanzando concentraciones de hasta 4 y 8.9 mg/kg para ENs y BEA (Callebaut et al., 2012), o 2.7 y 12 mg/kg para MON y BEA, respectivamente (Leitgeb et al., 2003). Niveles sin efectos adversos observables (NOAEL) han sido establecidos para BEA (0.14-1.2 mg/kg pc/día), EN B (0.24-0.76 mg/kg pc/día) y EN B1 (0.22-0.67 mg/kg pc/día) en pollos, pavos y gallinas (EFSA, 2014).

A pesar de la ausencia de toxicidad, en la presente investigación las ENs fueron detectadas en suero de animales tratados, confirmando su absorción a nivel intestinal y permitiendo la entrada en contacto con células y otros componentes de la sangre (Manyes et al., 2014). Se han reportado alteraciones de los linfocitos en sangre periférica de rata tras la administración subaguda de EN A, y a pesar de no alterar la función del sistema inmune en situaciones fisiológicas normales, la reducción de los linfocitos podría disminuir la inmunidad humoral e innata en condiciones infecciosas (Juan et al., 2014). Niveles traza de ENs fueron detectados en suero y orina a diferentes tiempos tras la administración de un bolo oral de

una única dosis de ENs mediante cánula orofaríngea en ratas. Teniendo en cuenta que el primer tiempo de la toma de muestra fue 2 h tras la administración, los bajos niveles detectados en este caso podrían explicarse situando el punto máximo de absorción con anterioridad a la primera toma de muestras. Aunque no se dispone de datos toxicocinéticos de ENs en rata, la rápida absorción de ENs ha sido demostrada en cerdos reportando niveles máximos de concentración en suero a los 20-30 min tras la administración de un bolo oral de EN B1 (0.05 mg/kg) (Devreese et al., 2013). EN B mostró además valores mayores de absorción seguida de EN B1, EN A1, EN A, y finalmente BEA (Devreese et al., 2014b).

Las ENs se detectaron también en materiales de excreción biológicos como orina y heces, representando una muy pequeña fracción de la cantidad total de micotoxina ingerida. Las EN B y B1 se detectaron en muestras de heces a todos los tiempos estudiados tras la administración (2, 4, 6, 8h) alcanzando concentraciones máximas a las 6 h post-administración, único tiempo en el que las ENs A y A1 fueron detectadas. Los bajos niveles de ENs también detectados en orina, en el orden de μg/L, concuerdan con la literatura previa sobre micotoxinas en muestras biológicas donde, justificado por el amplio metabolismo que sufren estos compuestos, se tiende cada vez más a biomonitorizar los principales metabolitos de micotoxinas junto con la micotoxina original, cuantificando en ocasiones concentraciones mayores de los metabolitos respecto a la micotoxina inalterada (Escrivá et al., 2017a).

En cuanto a las ENs, varios estudios han confirmado su extrenso metabolismo, principalmente mediante ensayos *in vitro* con enzimas hepáticas, identificando algunos de los productos metabólicos originados que incluyen derivados monooxigenados, hidroxilados, carboxilados y

desmetilados (Faeste et al., 2011, Ivanova et al., 2011; 2014; 2017). Aunque los datos disponibles *in vitro* proporcionan una idea del proceso metabólico que sufren las micotoxinas emergentes, las vías de metabolización *in vivo* de BEA y ENs siguen estando por clarificar. La caracterización de los productos metabólicos es un paso importante en la evaluación del riesgo de compuestos tóxicos ya que los metabolitos reactivos podrían influir en el perfil tóxico general. De hecho, teniendo en cuenta la elevada biodisponibilidad oral de la EN B1 en cerdos, se ha propuesto que la escasa toxicidad de ENs observada *in vivo* podría deberse a la rápida y extensa metabolización de estas micotoxinas a compuestos con menor toxicidad (Devreese et al., 2013; 2014b). Aunque el metabolismo de las fusariotoxinas ocurre notablemente en hígado y riñones, podría producirse también en el tracto digestivo, donde la microflora intestinal realiza los procesos de desacetilación y des-epoxidación en la mayoría de las especies (World Health Organization/FAO, 2002). Metabolitos de dioxigenados y desmetilados de EN B se detectaron en hígado y colon de ratón tras la exposición a EN B (Rodríguez-Carrasco et al., 2016). Acorde con investigaciones *in vitro* que sugieren que las reacciones de oxidación de ENs se producen principalmente en cadenas laterales isopropilo de residuos valina y ácido hidroxiisovalérico (Ivanova et al., 2011), en el presente estudio de toxicidad subaguda de EN A se identificaron dos productos de degradación de la micotoxina por escisión de la molécula y pérdida de un aminoácido isoleucina y un residuo de ácido hidroxiisovalérico, respectivamente, confirmándose además su presencia en los contenidos intestinales de duodeno, yeyuno y colon de rata (Manyes et al., 2014). Sin embargo, el potencial tóxico de estos derivados, probablemente originados por fermentación microbiana intestinal, no ha sido todavía estudiado.

En lo referente a la detección de micotoxinas en órganos y tejidos, la presente investigación proporciona datos que sustentan la distribución y bioacumulación de la EN A tras su administración subaguda oral en rata Wistrar durante 28 días. Las mayores concentraciones de EN A se detectaron en hígado, probablemente debido su papel detoxificador, ya que junto a los riñones se consideran órganos particularmente susceptibles a la toxicidad siendo lugares de paso y filtración de toxinas (Di et al., 2009). Concentraciones similares de EN A se detectaron en el yeyuno, señalando éste tramo como posible punto de absorción de la micotoxina a nivel intestinal. La EN A fue también detectada en el duodeno y colon, así como en sus contenidos, sin embargo no se detectó en riñones, estómago, ni íleon (Manyes et al., 2014). Los resutados obtenidos están en consonancia con estudios posteriores de BEA y EN B en ratón, donde se confirma la presencia de las micotoxinas emergentes en suero y su bioacumulación en órganos y tejidos, principalmente hígado y tejido graso, tras su administración intraperitoneal (Rodríguez-Carrasco et al., 2016), así como su distribución en el parénquima cerebral tras el paso de las micotoxinas a través de la barrera hematoencefálica en ratones (Taevernier et al., 2016). A pesar de las diferencias en cuanto a las vías de adminitración, dosis y especie animal, los niveles detectados de ENs y BEA en ambas especies murinas alcanzan valores similares, reportándose mayores concentraciones de BEA respecto a EN B bajo las mismas condiciones de ensayo (Rodríguez-Carrasco et al., 2016).

Por tanto, en vista de los resultados y en consonancia con la literatura disponible, parece ser que la administración única de ENs vía oral ($\leq$2.2 mg/kg pc), así como la ingestión a medio plazo de EN A (20.91 mg/kg pc/día) simulando una situación de toxicidad subcrónica no son niveles

potencialmente tóxicos para ratas adultas jóvenes, a pesar de la absorción y distribución de las micotoxinas a tejidos y órganos en concentraciones considerables. Pese a que BEA y ENs son tóxicas *in vitro,* la mayoría de los datos *in vivo* indican baja o nula toxicidad (EFSA, 2014; Gruber-Dorninger et al., 2017). Son necesarias futuras investigaciones encaminadas a definir el nivel más bajo de efecto adverso observado (LOAEL) con el fin de establecer una relación dosis-respuesta para las micotoxinas emergentes, paso fundamental para evaluar el riesgo relacionado con la ingesta de estos compuestos.

4.7 Estudio Transcriptómico de Micotoxinas Emergentes

La prevención de los efectos adversos para la salud potencialmente asociados con la exposición humana a micotoxinas puede lograrse mediante la detección temprana de los efectos tóxicos. Las alteraciones globales de expresión génica que tienen lugar en sistemas biológicos en respuesta a la exposición a micotoxinas pueden manifestar eventos celulares tempranos y mecánicamente relevantes que contribuyen al inicio y la progresión de los efectos adversos inducidos por estos compuestos tóxicos (Josehp et al., 2017). Los mecanismos citotóxicos moleculares de las micotoxinas emergentes de *Fusarium* continúan planteando incógnitas y requieren profundizar en su investigación, especialmente sobre las alteraciones enzimáticas clave y las vías metabólicas implicadas en la toxicidad inducida por estas micotoxinas (Wen et al., 2016).

En la presente tesis doctoral se desarrolló una investigación toxicogenómica a nivel de transcriptoma para elucidar los mecanismos moleculares subyacentes a la toxicidad y los modos de acción de BEA y EN B

mediante el análisis de los cambios inducidos por estas micotoxinas en los patrones de expresión génica. En un intento de obtener una visión completa de los cambios de expresión del genoma humano inducidos por BEA y EN B, se aplicó la técnica de secuenciación masiva RNA-seq al análisis del material genético ribonucleico extraído de células Jurkat expuestas individualmente a ambas micotoxinas (1.5, 3 y 5 μM) durante 24 h, junto con los respectivos grupos control. Las dosis empleadas se seleccionaron en base a datos previos de citotoxicidad *in vitro* en células Jurkat, correspondiendo a valores cercanos a los IC50 e IC30 para BEA y EN B, respectivamente. A pesar de que dichas dosis suponen concentraciones superiores a las alcanzadas en suero tras su absorción, se debe considerar que dado el carácter lipófilo de BEA y ENs, la absorción podría producirse mediante el paso al sistema linfático previa entrada al torrente sanguíneo, tal y como se describe para la absorción de compuestos lipídicos (Iqbal and Hussain, 2009), lo que podría suponer el contacto directo de estas micotoxinas con la linfa y, en consecuencia, con los linfocitos en todas sus fases de maduración, justificando no sólo las dosis empleadas si no también el modelo celular seleccionado. Por otra parte, estudios previos transcriptómicos exponen varios modelos celulares a concentraciones supreriores de EN B justificado por los altos niveles de estos compuestos detectados en alimentos como cereales y sus derivados (Jonsson et al., 2016).

El análisis transcriptómico tras la exposición a BEA y EN B reveló alteración del perfil genómico con predominio de la represión frente a la sobre-expresión, cuantificando un total de 5719 y 5750 genes diferencialmente expresados para BEA y EN B, respectivamente. Estos datos suponen una alteración del 27% del genoma humano, considerando que éste

contiene alrededor de 21000 genes codificantes de proteínas (HGNC database). Curiosamente, cerca de la mitad de los genes coincidieron para ambas micotoxinas, cifrando un total de 2693 dentro de los que cabe destacar genes implicados en la activación de las caspasas (CASP1, CASP2, CASP3, CASP4, CASP6, CASP8, CASP9, CASP10, CARD6, CARD9, CARD11, CARD16, BIRC2, BIRC3, CAAP1, MALT1, MAVS, NLRP1, NOL3, NOD1, CFLAR), apoptosis y muerte celular programada (BAX, BCL2, BOK, CCAR2, CIAPIN1, DDIAS, AIFM1, AIFM2, AATF, AVEN, ACIN1, AEN, BFAR, FAIM2, NAIP, NAIF1, TIAF1, PDCD2, PDCD2L, PDCD4, PDCD5, PDCD6IP, PDCD11, PIDD1, SIVA1, FAIM2, MOAP1, PAWR, ECSCR, PANO1). Se considera otro hallazgo interesante que 77 y 35 genes de los 96 implicados en la cadena respiratoria se mostraran alterados tras la exposición a BEA o EN B, revelando perturbaciones del 80 y 36%, respectivamente, de los genes involucrados en la cadena respiratoria, identificando además 32 genes coincidientes para ambas micotoxinas emergentes.

La investigación posterior se centró principalmente en los genes alterados en las tres concentraciones de estudio, tanto para BEA como EN B. Estos grupos de genes, formados por 43 en el caso de BEA y 245 para EN B, se emplearon para analizar la ontología de genes e identificar las vías metabólicas alteradas, destacando un solapamiento de 14 genes para ambas micotoxinas emergentes. Cabe destacar que 11 de estos 14 genes pertenecen al genoma mitocondrial y son, para ambas micotoxinas, los más fuertemente reprimidos/sobreexpresados apuntando ya de manera preliminar al daño mitocondrial. Algunos de estos genes, como MT-CO3, MT-ND2, MT-ND3, MT-ND5, MT-ND4, MT-ND4L, MT-ATP6, MT-ATP8, MT-CYB, están implicados en el metabolismo, cadena de transporte de eletrones, síntesis de ATP, actividad del

citocromo c oxidasa, NADH deshidrogenasa y actividad oxidoreductasa. Las alteraciones mitocondriales inducidas por las micotoxinas emergentes y la relación entre el estrés oxidativo es particularmente relevante y ofrece una importante línea de investigación para dilucidar los pasos clave del inicio de la micotoxicosis.

En este sentido, tras realizar el análisis de la ontología de genes con el total de DEGs obtenidos tanto para BEA como EN B, se observaron procesos biológicos estrechamente relacionados con la cadena respiratoria, cadena de transporte de electrones, fosforilación oxidativa, respiración celular, generación de metabolitos y energía, biogénesis y el ensamblaje de la cadena respiratoria mitocondrial, entre otros. Funciones moleculares asociadas a la actividad oxidorreductasa y NADH deshidrogenasa fueron estadísticamente más significativas, mientras que componentes celulares como la membrana mitocondrial, cadena respiratoria mitocondrial, envoltura mitocondrial, NADH deshidrogenasa, complejos oxidorreductasa, complejo de proteína mitocondrial y la cadena respiratoria se mostraron mayormente representados para el grupo de genes alterados por BEA y EN B en células Jurkat tratadas respecto a las muestras control (Escrivá et al., 2017e; Alonso-Garrido et al., 2018 under review).

El análisis de las vías metabólicas en las que los DEGs podrían estar implicados revelan 3 vías estadísticamente significativas para BEA; fosforilación oxidativa, cadena de transporte de electrones y metabolismo de nucleótidos, mientras que EN B muestra 21 vías metabólicas coincidiendo con BEA en las estadísticamente más representativas: fosforilación oxidativa y cadena de transporte de electrones. Otras vías significativamente representativas tras la exposición a EN B incluyen el metabolismo de

aminoácidos, quercetina y apoptosis celular inducida por Nf-kB/AP-1, activación transcripcional por NRF2, procesamiento de ARNm, síntesis y degradación de cuerpos cetona, ciclo de la urea y metabolismo de grupos amino.

No existen estudios previos sobre la alteración del genoma humano inducido por BEA y EN B basados en RNA-seq, lo que obliga a comparar los resultados obtenidos con estudios similares en genomas de otras especies.

El efecto sobre la expresión génica tras la exposición a BEA (30 μM; 24 h) mostró inhibición de CYP11A1 y CYP19A1 en células de la granulosa bovina (Albonico et al., 2017), alteración de la función mitocondrial mediante aumento significativo de la expresión de MT-CO1 en ovocitos porcinos, y de BCL2 en células cumulus tras la exposición a BEA (10 μM; 22 h), aunque sin activación de las vías que conducen a apoptosis o autofagia (Schoevers et al., 2016).

Respecto a EN B, los resultados concuerdan con un estudio anterior realizado mediante microarrays en células Balb 3T3, HepG2 y hepatocitos primarios expuestas a 10-20 μM durante 4 h, a pesar de las diferencias respecto a técnicas de análisis (RNA-seq vs. microarrays o RT-PCR) y genoma (humano vs. rata). Se observó alteración del perfil genómico alrededor del 5.7% del genoma de rata, con predominio de la represión respecto a la sobre-expresión. El estudio del perfil genómico reveló principalmente procesos metabólicos y catabólicos, así como relacionados con la organización de la mitocondria, identificando 37 genes perturbados con implicación mitocondrial. EN B condujo a la alteración del metabolismo energético debido a los efectos sobre la organización y función mitocondrial y el ensamblaje del complejo I de la cadena de transporte de electrones, redujo la proliferación

celular e incrementó ligeramente la proporción de células apoptóticas en Balb 3T3, originando la muerte celular necrótica en hepatocitos primarios (Jonsson et al., 2016). Acorde con estos resultados, en la presente investigación se mostraron 8 de los 37 genes también alterados en células Jurkat tras la exposición a ambas micotoxinas (BEA y EN B), incluyendo los genes apoptóticos AIFM1, BCL2L11 y TNFSF10, así como 2 y 10 genes perturbados tras la exposición a BEA y EN B, respectivamente, como CASP3, TIMM21 y TIMM8B.

Otros estudios genómicos a nivel de transcriptoma basados en RT-PCR mostraron una ligera represión de genes relacionados con la respuesta inflamatoria (quimocinas, citoquinas, interleuquinas y sus receptores) con alteración de 24 genes tras la exposición de macrófagos humanos a EN B (5 μM; 24 h). Además, se evidencian efectos sinérgicos en la co-exposición con β-glucano y lipopolisacáridos para la sobre-expresión de genes implicados en la respuesta inflamatoria, así como daño micotondrial y autofagocitosis, respectivamente (Korkalainen et al., 2017). De los 24 genes alterados, 9 se vieron también perturbados en la presente investigación tras la exposición de células Jurkat a EN B (NAMPT, TNFSF10, VEGFA, CCR2, CCR5, CSF1, IL9R) o BEA (CCR4, IL10RA).

El análisis transcripcional mediante qRT-PCR en células H295R mostró 12 de los 16 genes esteroidogénicos alterados significativamente tras la exposición a EN B (10 μM; 48 h), sin ninguna coincidencia respecto a los genes alterados en el presente trabajo. La represión de HMGR, StAR, CYP11A, 3bHSD2 y CYP17, y sobre-expresión de CYP1A1, NR0B1, MC2R, CYP21, CYP11B1, CYP11B2 y CYP19, sugiere toxicidad endocrina suprarrenal como riesgo potencial de la exposición a EN B (Kalayou et al., 2015).

La apoptosis mediada por los genes p53 y p21 fue observada tras probar una mezcla de ENs en varias células cancerosas humanas, así como la activación de las caspasas-3/7 en células H4IIE y caspasa-7 en células KB-3-1 (Watjen et al., 2009; Dornetshuber-Fleiss et al., 2015).

Finalmente, el potencial anticancerígeno de las micotoxinas emergentes ha sido recientemente propuesto (Prosperini et al., 2017). EN B ha demostrado la capacidad de resistir a la expulsión celular mediada por los transportadores ABC y de dirigirse de manera natural a las células tumorales con mayor especificidad que otros agentes quimioterapéuticos (Dornetshuber-Fleiss et al., 2015). Posteriormente, la especificidad de BEA frente a células malignas vs. células no malignas fue probada *in vivo* tras administrar la micotoxina a ratones BALB portadores de tumores CT-26 murinos o humanos injertados con células KB-3-1, respectivamente. Se observó disminución del volumen y peso tumoral en ratones tratados respecto al grupo control, sin ningún efecto adverso, con acumulación moderada de BEA en los tejidos tumorales. Además, se produjo aumento significativo de las áreas necróticas dentro de las secciones tumorales de ratones tratados cuantificando mayor número de células apoptóticas, sugiriendo a BEA como un compuesto natural prometedor para la terapia contra el cáncer (Heilos et al., 2017).

Tomados en conjunto, los resultados confirman que las micotoxinas emergentes continúan siendo motivo de preocupación en la seguridad alimentaria. A pesar del consenso científico que demuestra tanto la elevada presencia en alimentos y piensos como la toxicidad *in vitro* de BEA y ENs, no se dispone todavía de una evaluación de riesgo completa que permita defirnir límites regulatorios de estos compuestos tóxicos naturales. Pese a que los

estudios *in vivo* son escasos y parecen indicar ausencia de toxicidad, se ha demostrado que las micotoxinas emergentes se absorben y distribuyen en órganos y tejidos de organismos vivos. Los avances en la biología molecular han permitido evaluar, en los últimos años, los mecanismos moleculares subyacentes a la toxicidad de BEA y ENs, demostrando la inducción de alteraciones genómicas y su implicación en procesos biológicos a nivel mitocondrial. Son necesarios nuevos estudios con el fin de proteger y mejorar el bienestar humano y animal con respecto a la exposición a micotoxinas, con especial enfásis en las micotoxinas emergentes de *Fusarium*.

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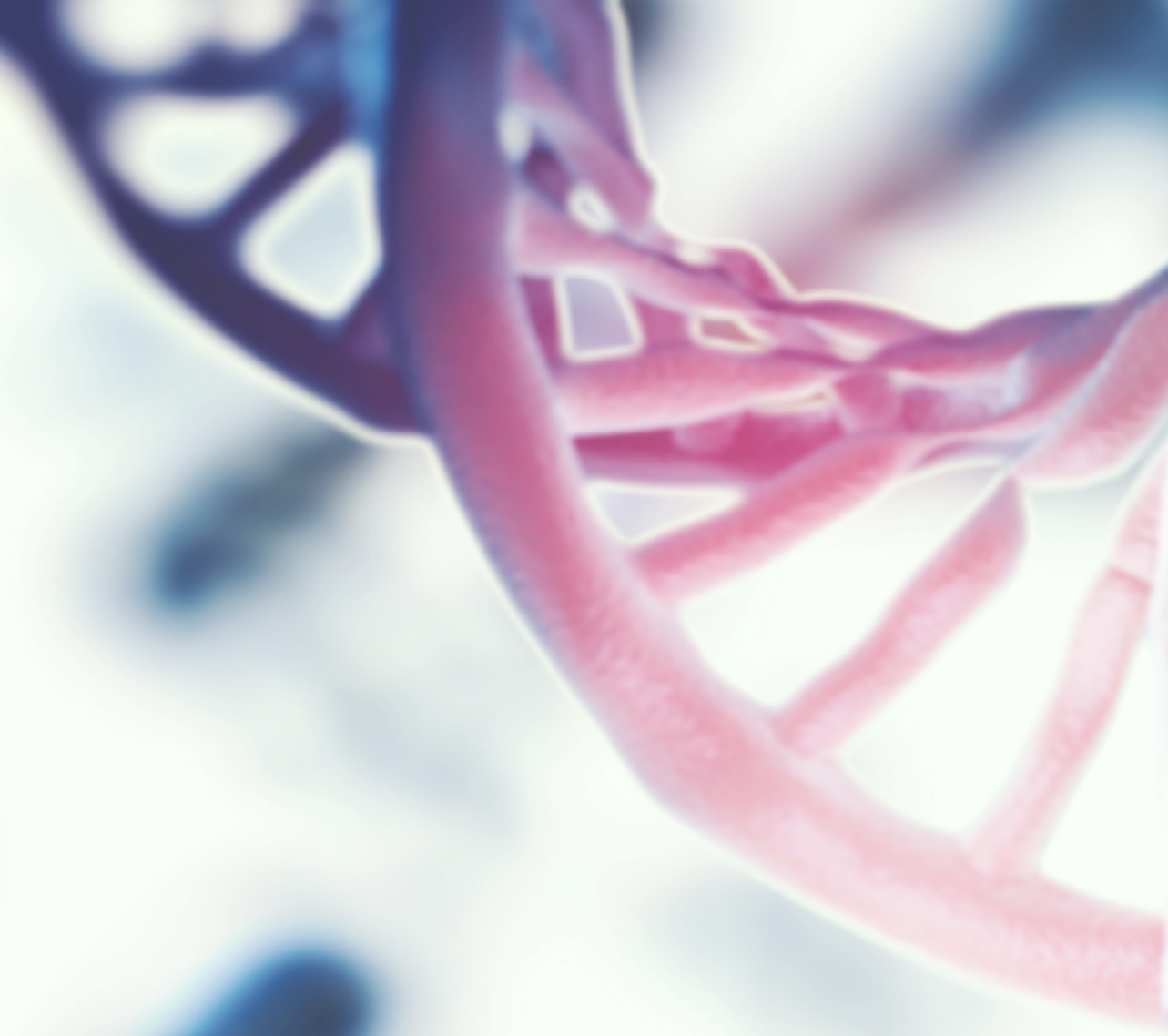


5. CONCLUSIONS

CONCLUSIONS

1. The review of *in vivo* studies of *Fusarium* mycotoxins indicated that the assays performed were mainly subacute and subchronic toxicity studies with pigs and rats by oral administration, being Fumonisin B1, Deoxynivalenol and Zearalenone the most studied fusarotoxins, as well as, mycotoxin combinations generally including Deoxynivalenol.

2. The review of mycotoxins analysis in biological samples showed that the main objectives were method development, biomonitoring studies and exposure assessment, with human urine as the most analyzed sample and Ochratoxin A, Zearalenone and Deoxynivalenol as the most analyzed mycotoxins. Despite the high frequency of mycotoxins detection the low levels quantified suggests probable daily intakes generally lower than the permited tolerable intakes.

3. The review about *Alternaria* mycotoxins analysis in food and feed indicated Alternariol, Alternariol Methyl Ether, Tentoxin, and Tenuazonic Acid as the most analized mycotoxins, reporting the highest levels in legumes, oilseeds, tomatoes, carrots, juices, wines, cereals, beer, and nuts.

4. The laboratory rat feed analysis demonstrated high percentage of multi-mycotoxin contamination with predominance of Zearalenone, Enniatins and Deoxinivalenol, indicating the need of feed quality control surveillance to ensure reliable *in vivo* test results.

5. Beauvericin and Enniatin B exposure in Jurkat cells decreased cell viability, induced cell cycle arrest, increased apoptotic/necrotic ratio and caspases

activity, with more pronounced effects for Beauvericin, which also induced DNA damage.

6. The review about Quercetin revealed the *in vitro* protective activity against the cytotoxic effect of mycotoxins such as Ochratoxin A, Deoxynivalenol, Zearalenone, Beauvericin, Enniatins, Alternariol, T-2 toxin, Patulin, and Aflatoxin B1, considering its intake in food as potential strategy to prevent the toxicity induced by these compounds.

7. Enniatins A, A1, B and B1 were detected in serum, urine and feces after single oral dose administration in Wistar rats, without observable adverse effects.

8. Subacute Enniatin A exposure (21 mg/kg bw/day) was not toxic to Wistar rats, despite the mycotoxin was detected in serum, organs and tissues demonstrating its absorption and distribution after oral administration.

9. Transcriptomic analysis revealed human gene expression perturbation induced by Beauvericin and Enniatin B in Jurkat cells with alteration of oxydative phosphorilation and electron transport chain pathways.

10. Mycotoxins contamination in food and feed is inevitable and it represents a priority objective in food safety, therefore further *in vitro, in vivo* and toxicogenomic studies are necessary to obtain a global view of the mechanisms of action and toxic effects of mycotoxins, to finally assess the potential risk of exposure to these compounds and to protect human and animal health.

CONCLUSIONES

1. La revisión de los estudios *in vivo* de las micotoxinas de *Fusarium* puso de manifiesto que los ensayos realizados fueron principalmente de toxicidad subaguda y subcrónica en cerdos y ratas por administración vía oral, siendo Fumonisina B1, Deoxinivalenol y Zearalenona las fusarotoxinas más estudiadas, así como combinaciones de micotoxinas que incluyen generalmente Deoxinivalenol.

2. La revisión del análisis de micotoxinas en muestras biológicas mostró que los objetivos principales fueron el desarrollo de métodos analíticos, estudios de biomonitorización y evaluación de la exposición, con orina humana como muestra biológica mayoritaria, siendo Ocratoxina A, Zearalenona y Deoxinivalenol las micotoxinas más analizadas. A pesar de la elevada frecuencia de micotoxinas detectadas en muestras biológicas, los bajos niveles cuantificados sugieren ingestas diarias probables generalmente inferiores a las ingestas tolerables permitidas.

3. La revisión sobre el análisis de micotoxinas de *Alternaria* en alimentos y piensos indicó que las micotoxinas más analizadas son Alternariol, Alternariol Metil Éter, Tentoxinona y Ácido Tenuazónico, detectando las concentraciones más elevadas en legumbres, semillas oleaginosas, tomates, zanahorias, zumos, vinos, cereales, cerveza y nueces.

4. El análisis de piensos de rata de laboratorio demostró un elevado porcentaje de contaminación por multi-micotoxinas con predominio de Zearalenona, Eniatinas y Deoxinivalenol, indicando la necesidad de controlar la calidad de los piensos para garantizar resultados fiables en ensayos *in vivo*.

5. La exposición de células Jurkat a Beauvericina y Eniatina B disminuyó la viabilidad celular, indujo arresto del ciclo celular, aumentó la relación de células apoptoticas/necróticas con aumento de la actividad de las capasas, siendo los efectos más pronunciados en el caso de Beauvericina, la cual produjo daño en el ADN.

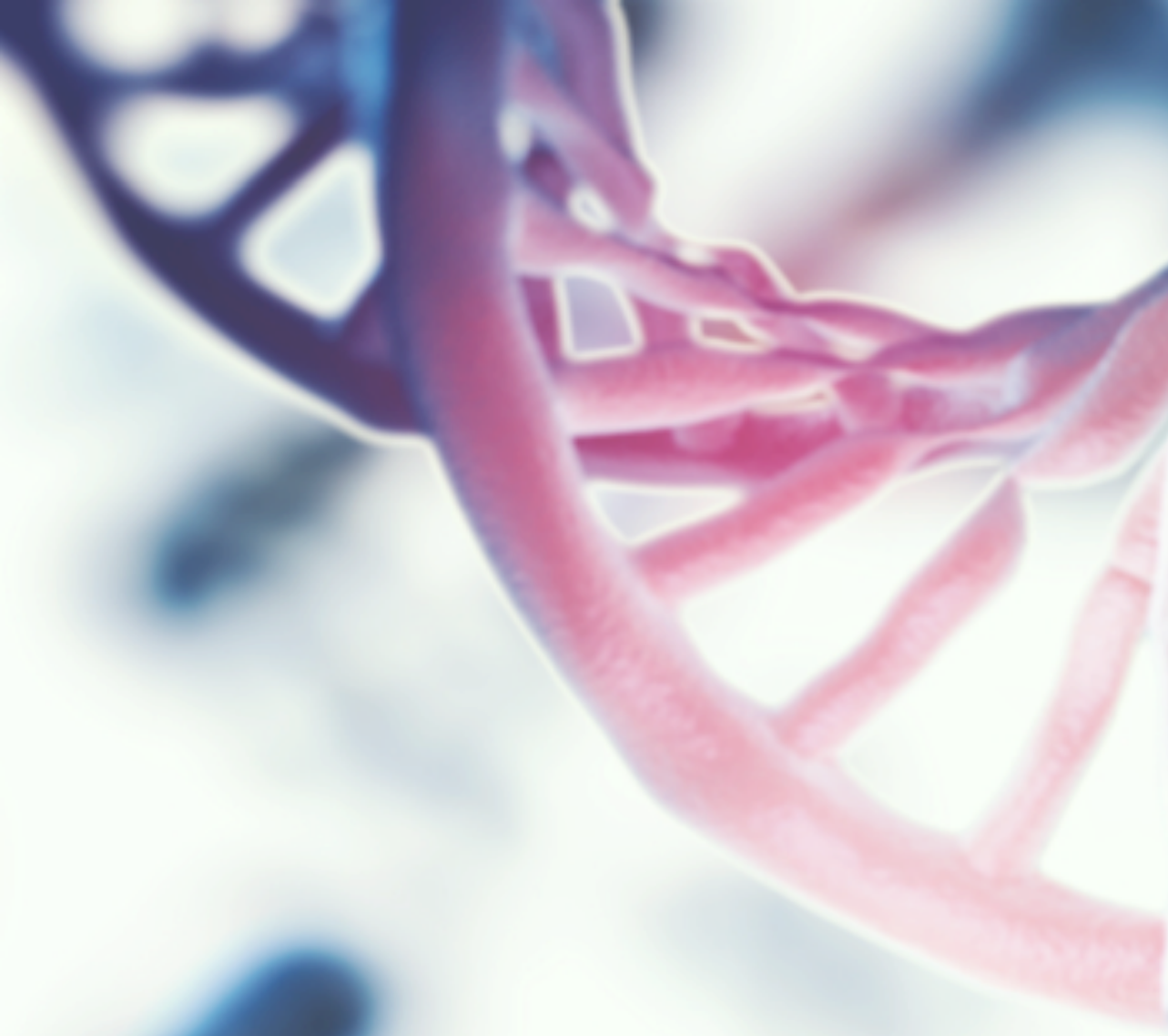
6. La revisión sobre Quercetina reveló la actividad protectora *in vitro* frente al efecto citotóxico de micotoxinas como Ocratoxina A, Deoxinivalenol, Zearalenona, Beauvericina, Eniatinas, Alternariol, Toxina T-2, Patulina y Aflatoxina B1, considerando su ingesta en alimentos como una estrategia potencial para prevenir la toxicidad inducida por estos compuestos.

7. Las Eniatinas A, A1, B and B1 se detectaron en suero, orina y heces tras su administración única por vía oral en rata Wistar, sin signos de efectos adversos observables.

8. La exposición subaguda a Eniatina A (21 mg/kg pc/día) no resultó tóxica en rata Wistar a pesar de detectar la micotoxina en suero, órganos y tejidos demostrando su absorción y distribución tras administración oral.

9. El análisis transcriptómico reveló perturbación del genoma humano inducido por Beauvericina y Eniatina B con alteración de las vías de fosforilación oxidativa y cadena de transporte de electrones en células Jurkat.

10. La contaminación de alimentos y piensos por micotoxinas es inevitable y representa un objetivo prioritario en seguridad alimentaria, por lo que son necesarios nuevos estudios *in vitro, in vivo* y toxicogenómicos que permitan obtener una visión global de los mecanismos de acción y efectos tóxicos de las micotoxinas, para finalmente evaluar el riesgo potencial de la exposición a estos compuestos y proteger así la salud humana y animal.



ANEX I. RESEARCH DIFFUSION

Toxicology Mechanisms and Methods

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RESEARCH ARTICLE

A preliminary study in Wistar rats with enniatin A contaminated feed

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Abstract

A 28-day repeated dose preliminary assay, using enniatin A naturally contaminated feed through microbial fermentation by a *Fusarium tricinctum* strain, was carried out employing 2-month-old female Wistar rats as *in vivo* experimental model. In order to simulate a physiological test of a toxic compound naturally produced by fungi, five treated animals were fed during 28 days with fermented feed. As control group, five rats were fed with standard feed. At the 28th day, blood samples were collected for biochemical analysis and the gastrointestinal tract, liver and kidneys were removed from each rat for enniatin A detection and quantitation. Digesta were collected from stomach, duodenum, jejunum, ileum and colon. Enniatin A present in organs and in biological fluids was analyzed by liquid chromatography-diode array detector (LC-DAD) and confirmed by LC-mass spectrometry linear ion trap (MS-LIT); also several serum biochemical parameters and a histological analysis of the duodenal tract were performed. No adverse effects were found in any treated rat at the enniatin A concentration (20.91 mg/kg bw/day) tested during the 28-day experiment. Enniatin A quantitation in biological fluids ranged from 1.50 to 9.00 mg/kg, whereas in the gastrointestinal organs the enniatin A concentration ranged from 2.50 to 23.00 mg/kg. The high enniatin A concentration found in jejunum liquid and tissue points to them as an absorption area. Finally, two enniatin A degradation products were identified in duodenum, jejunum and colon content, probably produced by gut microflora.

Keywords

Enniatin A, *Fusarium tricinctum*, *in vivo* study, LC-DAD, LC-MS-LIT

History

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Introduction

Enniatins (ENs) are secondary fungal metabolites that have been known for several decades (Ivanova et al., 2006). Chemically there are six-membered cyclic depsipeptides, which are commonly composed of three D-α-hydroxyisovaleric acid (Hiv) residues linked alternatively to three L-configured *N*-methyl amino acid residues to give an 18-membered cyclic skeleton (Zhukhlistova et al., 1999). ENs are produced by strains of several species of fungal genera as *Alternaria*, *Fusarium*, *Halosarpheia* and *Verticillium* (Supothina et al., 2004). ENs produced by *Fusarium subglutinans*, *Fusarium proliferatum* and *Fusarium tricinctum* are cereal contaminants, especially maize and its derivatives. ENs have been found as worldwide natural contaminants of several food and feed products (Jestoi, 2008). A few years ago, Meca et al. (2010a) reported ENs contamination of cereals available in the Spanish market and their levels ranged from 0.51 to 11.78 mg/kg.

Enniatins possess a wide range of biological activities: these substances are known as ionophores, phytotoxins, anthelmintic and antibiotics compounds (Jestoi, 2008). ENs antibiotic effects have been used in a pharmaceutical commodity with anti-inflammatory properties called fusafungine (Akbas et al., 2004). There are applications for ENs in respiratory tract infections treatment and it has been reported a positive effect on wound healing after tonsillectomy (Akbas et al., 2004). Several studies have indicated that ENs change the monovalent ion transport across membranes and disrupt the ionic selectivity of cell walls. This effect is particularly debilitating in mitochondrial membranes, resulting in the uncoupling of oxidative phosphorylation (Tonshin et al., 2010).

Several studies have evaluated the ENs cytotoxic activity *in vitro* using as experimental model rodent, monkey, porcine, insect and human cell lines (Behm et al., 2009; Dornetshuber et al., 2009; Fornelli et al., 2004; Hyun et al., 2009; Ivanova et al., 2006; Jestoi, 2008; Lee et al., 2008; Meca et al., 2010b, 2011; Vongvilai et al., 2004; Watjen et al., 2009). In the scientific literature, only few studies related to the ENs toxicity *in vivo* are available. In particular, Bosch et al. (1989) studied the toxicity of ENs, among other mycotoxins, in *Fusarium* contaminated feed on 20-day-old white female Sprague–Dawley rats, evidencing no toxic signs. To be sure about which mycotoxins where responsible of the effects, they administrated a mixture of ENs in a single oral dose

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Invited Review

In vivo toxicity studies of fusarium mycotoxins in the last decade: A review

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ABSTRACT

This review summarizes the information regarding the *in vivo* studies of *Fusarium* mycotoxins in the last decade. The most common studies are classified as subacute toxicity, subchronic toxicity, acute toxicity, toxicokinetic studies and teratogenicity in order of importance. The most used animals in *in vivo* studies are pigs, rats, chickens and mice. Fumonisin B1, deoxynivalenol, zearalenone, nivalenol and T-2 toxin are the most studied fusarotoxins. Studies with combinations of mycotoxins are also frequent, deoxynivalenol generally being one of them. The predominant route of administration is oral, administered mostly in the form of naturally contaminated feed. Other administration routes also used are intraperitoneal, intravenous and subcutaneous. *In vivo* research on *Fusarium* mycotoxins has increased since 2010 highlighting the need for such studies in the field of food and feed safety.

1. Introduction

1.1. Fusarium *genera*

The genus *Fusarium* is a large fungal form genus that is more than hundreds of years old, and globally one of the most important genera of fungi. Its species, which invade agriculturally important grains, are probably the most prevalent toxin-producing fungi of the northern temperate regions and are commonly found in cereals grown in America, Europe and Asia (Tiemann et al., 2006). Most members of the genus produce – under favorable environmental conditions – an array of secondary metabolites, which vary widely in chemical form. A number of the secondary metabolites are important as mycotoxins that are toxic and/or carcinogenic to humans and animals and may have a role in plant disease. These mycotoxins are commonly found in cereal food and feed and in other animal products consumed daily. They should be regulated in commercial and international trade. Mycotoxins possess biological activities that have been shown in many different studies and may represent a problem to both human and animal health.

The diseases that *Fusarium* species cause, the toxins they produce and the general social impact on agricultural communities are an ongoing problem (Summerell and Leslie, 2011). *Fusarium* mycotoxins are endowed with both acute and chronic toxic effects and have been shown to cause a broad variety of toxic effects in animals. The consequences of ingestion of these fungal compounds vary from acute, overt diseases with high morbidity and death to chronic disease, decreased resistance to pathogens and reduced animal

Abbreviations: ADME, absorption, distribution, metabolism and excretion; AFs, aflatoxins; ALT, alanine aminotransferase; HFB1, aminopentol; AS, aluminosilicate; AST, aspartate aminotransferase; AUC, area under curve; BEA, beauvericin; bw, body weight; CAT, catalase; CYP, cytochrome P-450; D3G, deoxynivalenol-3-β-D-glucoside; DES, diethylstilbestrol; DNA, deoxyribonucleic acid; DOM-1, deepoxy-deoxynivalenol; DON, deoxynivalenol; DONS, deoxynivalenol sulfonate; DON-3-GlcA, deoxynivalenol-3-glucuronide; DON-15-GlcA, deoxynivalenol-15-GlcA; EFSA, European Food Safety Authority; ENNs, enniatins; ENN A, enniatin A; ENN B, enniatin B; EPT, 12,13 epoxytrichothec-9-ene; EU, European Union; FAO, Food and Agricultural Organization of the United Nations; FBs, fumonisins B; FB_1, fumonisin B_1; FUS, fusaproliferin; GMA, glucomannan mycotoxin binder; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; HDL, high density lipoprotein; HT2, HT-2 toxin; HT29, human tissue colon cell line; IARC, International Agency for Research on Cancer; IP, intraperitoneal; IV, intravenous; LD50, lethal dose 50%; LDL, light density lipoprotein; LOQ, limit of quantification; LPS, lipopolysaccharides; MAPKs, mitogen-activated protein kinases; MDA, malondialdehyde; MON, moniliformin; mRNA, messenger ribonucleic acid; NIV, nivalenol; NOAEL, no-observed-adverse-effect level; PMTDI, provisional maximum tolerable daily intake; OECD, Organisation for Economic Co-operation and Development; PBMC, peripheral blood mononuclear cells; OTA, ochratoxin A; PBMC, peripheral blood mononuclear cells; PHFB1, partially hydrolyzed fumonisin B1; PUBMED, Publications of Medicine; Sa, sphinganine; Sa 1-P, sphinganine 1-phosphate; SBS, sodium metabisulfite; SC, subcutaneous; Se-GSH-Px, selenium dependent glutathione peroxidase; So, sphingosine; So 1-P, sphingosine 1-phosphate; SOD, superoxide dismutase; T2, T2 toxin; TNF, tumor necrosis factor; WHO, World Health Organization; WOS, Web of Science; ZEA, zearalenone; ZOL, zearalenol.

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RESEARCH ARTICLE

Quantitation of enniatins in biological samples of Wistar rats after oral administration by LC-MS/MS

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Abstract

The emerging *Fusarium* mycotoxins enniatins (ENNs) have diverse biological properties, mainly due to their ionophoric activity, and represent a potential risk to human and animal health since they are commonly found in food and feed. *In vivo* toxicity studies are scarce and limited to the major mycotoxins. Until now, any method for the simultaneous analysis of these compounds in plasma, serum and feces from rat has been reported. A method for the extraction and determination of ENNs A, A1, B and B1 from Wistar rat samples by liquid chromatography tandem mass spectrometry has been developed. The method was successfully validated with satisfactory recoveries (70–106%), good intraday (<10%) and interday (<20%) precision, expressed as relative standard deviation, and good linearity between limits of quantitation (LOQ) and 100 times LOQ. Limits of detection (LOD) and LOQ were $\leq$1 and $\leq$10 ng/ml, respectively. The validated method was applied for the analysis of biological Wistar rat samples that were administered a mixture of ENNs containing 1.19, 2.16, 1.03 and 1.41 mg/kg body weight of ENN A, A1, B and B1, respectively. Blood, urine and feces samples collected every 2 h during the 8-h duration of the experiment were analyzed. The administered dose of the mixture of ENNs did not cause observable adverse effects on the animals. ENNs concentrations detected in serum and urine were below LOQs. The four ENNs were detected in feces reaching the maximum concentration at 6 h after administration.

Keywords

Chromatography, emerging mycotoxins, *in vivo*, method validation

History

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Introduction

Mycotoxins, as toxic secondary metabolites produced under appropriate environmental conditions by filamentous fungi, are common contaminants of food and feed. Intake of high doses of mycotoxins may lead to acute mycotoxicoses, which are characterized by well-described clinical signs (Antonissen et al., 2014). Mycotoxins differ in structure, which explains the great variation of symptoms. The toxicological effects produced by mycotoxins can range from reproductive disorders, hepato- and nephrotoxicity, impaired immunity or dermal effects, to severe health-associated risks, including cancer and neurological disorders (Anfossi et al., 2010; Bhat et al., 2010). *Fusarium* is one of the main mycotoxin producing fungi and their mycotoxins are endowed with both acute and chronic aspects of toxicity (Afsah-Hejri et al., 2013; Cortinovis et al., 2013). Among the mycotoxins produced by *Fusarium* spp., are included the enniatins (ENNs), beauvericin (BEA), moniliformin (MON) and fusaproliferin (FUS), which are a group of bioactive compounds called emerging mycotoxins or minor *Fusarium* mycotoxins (Serrano et al., 2012). There is only limited data available about these metabolites, not only due to their late recognition but especially because of the late understanding of their role as mycotoxins (Nazaria et al., 2015). Scientific information about *in vivo* toxicity of the minor *Fusarium* mycotoxins is scarce (Escrivá et al., 2015; Santini et al., 2012). The emerging mycotoxins ENNs are cyclic hexadepsipeptides formed by alternating of the D-a-hydroxy-isovaleric acid (HyLv) and different *N*-methylamino acid residues as valine (Val) and isoleucine (Ile) (Serrano et al., 2013). Because of the widespread presence of these emerging mycotoxins in cereals and their diverse toxic effects, ENNs might pose a health risk to humans and animals (Hu et al., 2014). The most well-known ENNs reported as natural contaminants are ENN A, A1, B and B1. They have shown cytotoxic and apoptotic activity. ENNs are specific inhibitors of acyl-coenzyme A cholesterol acyltransferase and cationophoric compounds capable of transporting cations through the cell membrane, leading to toxic actions by an altered membrane potential and thereby disturbing the intracellular ionic homeostasis (Marín et al., 2013; Nazaria et al., 2015). Furthermore, their possible subclinical effects are of greater importance as these may lead to subacute effects such as feed refusal, weight loss, reduced animal productivity and increased

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Analysis of trichothecenes in laboratory rat feed by gas chromatography-tandem mass spectrometry

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ABSTRACT

A method for the determination of seven trichothecenes, neosolaniol (NEO), diacetoxyscirpenol (DAS), deoxynivalenol (DON), nivalenol (NIV), fusarenon-X (FUS-X), 3-acetyldeoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON), in laboratory rat feed by GC-MS/MS was developed. Sample extraction and purification was performed by an acidified mixture of acetonitrile/water (80–20% v/v). Limits of quantitation (LOQs) were between 1 and 10 μg kg^{-1} for all studied trichothecenes. Eight concentration levels between the LOQ and 100 × LOQ were used for the calibration curves. Matrix-matched calibration was used for quantitation purposes to compensate the detector signal enhancement obtained for all the analytes. The method accuracy was evaluated by recovery assays at three concentration levels, 25, 50 and 100 μg kg^{-1} (n = 9). Recoveries ranged from 62% to 97% and precision, expressed as intra- and inter-day relative standard deviations, was evaluated for all compounds. The validated method was successfully applied to the analysis of 35 laboratory rat feed samples showing mycotoxin contamination in 66% of the samples. DON was the most prevalent trichothecene followed by 15-ADON, NIV and 3-ADON. The maximum DON concentration reached in real samples was 2156 ± 4.3 μg kg^{-1}, while NEO, DAS and FUS-X were not detected in any sample. Multi-contamination by at least two mycotoxins was observed in 17% of the analysed feed samples.

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KEYWORDS
Trichothecenes; GC-MS/MS; laboratory rat feed

Introduction

The presence of mycotoxins in European feed and feed raw materials has been reported worldwide for decades (Streit et al. 2012; Kim et al. 2014). Fungal secondary metabolites, known as mycotoxins, are ubiquitous contaminants of crop plants and forage representing the main components of compound feeds (Zachariasova et al. 2014). Mixed feeds represent an excellent substrate for fungal growth, under favourable conditions such as appropriate moisture percentage and temperature (Monge et al. 2013). Any failure to comply with good manufacturing practice (good sorting, appropriate storage conditions avoiding air access when silage-making, etc.) usually lead to an increase in mycotoxin production (Zachariasova et al. 2014). Trichothecenes are a large family of chemically related mycotoxins produced by fungi in taxonomically unrelated genera, such as *Myrothecium*, *Stachybotrys* and in particular *Fusarium* (Borutovaa et al. 2012). The broad family of trichothecenes – over 220 – is extremely prevalent and presents a potential threat to human and animal health throughout the world since their exposure can cause feed refusal, growth retardation, vomiting, skin dermatitis, neuroendocrine changes, immunosuppression and haemorrhagic lesions (McCormick et al. 2011; Escrivá et al. 2015). Chronic intake of small amounts of trichothecenes leads to an increased susceptibility to infections (Montes et al. 2012). At a molecular level, trichothecenes would display multiple inhibitory effects on the primary metabolism of eukaryotic cells including the inhibition of proteins, DNA and RNA synthesis (Alassane-Kpembi et al. 2013). Trichothecenes contain a tricyclic 12,13 epoxytrichothec-9-ene (EPT) core structure with various side-chain substitutions, allowing their classification into four types: A, B, C and D (McCormick et al. 2011). Types A and B constitute non-macrocyclic trichothecenes and are commonly found in grain, animal feed and human food produced from contaminated grain (Döll & Dänicke 2011; Tang et al. 2015). Several surveys demonstrate that type-B trichothecenes occur more frequently and at higher concentrations compared with other types of trichothecenes (Ok et al. 2011; Rodríguez-Carrasco et al. 2014).

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RESEARCH ARTICLE

Mycotoxin contamination in laboratory rat feeds and their implications in animal research

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ABSTRACT

Compound feed is particularly vulnerable to multi-mycotoxin contamination. A method for the determination of 12 mycotoxins; enniatins A, A1, B, B1; aflatoxins B1, B2, G1, G2; OTA; ZEA; T-2 and HT-2 by liquid chromatography-tandem mass spectrometry has been developed and applied for the analysis of laboratory rat commercial feeds. The method trueness was checked by recovery assays at three different spiked levels ($n = 9$). Recoveries ranged from 73% to 112%, and the intra-day and inter-day precision were lower than 9% and 13%, respectively. Limits of quantitation were lower than 15 μg/kg. Twenty-seven laboratory rats feed samples showed multi-contamination by at least three up to six different mycotoxins. ENNs B and B1, followed by ZEA were the most prevalent mycotoxins. T-2, HT-2, and OTA were not detected. ZEA showed the highest concentration levels reaching 492 μg/kg. The results underline the importance of implementing mycotoxin regular surveillance programs for laboratory animal feeds.

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KEYWORDS
Experimental animals; feed; LC-MS/MS; method validation; mycotoxins

Introduction

Mycotoxins are fungi secondary metabolites mainly produced by *Aspergillus*, *Penicillium*, or *Fusarium* genus that can occur in all agricultural commodities in field or storage under appropriate fungi growth conditions. Mycotoxin contamination depends on climatic and environmental conditions before and after harvest (Anfossi et al., 2014). Other factors such as improper processing, packaging, drying techniques, and transport activities influence fungal growth and increase the risk of mycotoxin production (Afsah-Hejri et al., 2013).

Mycotoxins are unavoidable contaminants in crops (Zhang et al., 2009), therefore they commonly contaminate a wide range of cereals and cereal-derived products (Abysique et al., 2015). The Food and Agriculture Organization (FAO) reported that approximately 25% of crops worldwide are affected by mycotoxins (FAO/WHO, 2001). Furthermore, most mycotoxins are thermostable, resistant to milling, processing, and heating, which make difficult their elimination; therefore they readily enter the food and feed chains (Grenier & Oswald, 2011). Since fungal toxins have been detected in various food commodities from many parts of the world they have been recognized as one of the most prevalent toxic contaminants of food and feed (Bhat et al., 2010; Cheli et al., 2014).

Compound feed is particularly vulnerable to mycotoxin contamination as it typically contains a mixture of several raw materials mainly cereals and seed proteins. The high prices and scarcity of protein sources for animal feeds have led to the use of alternative protein sources such as distillers dried grains with solubles (DDGS) (Zachariasova et al., 2014). DDGS suppose at even greater risk of containing higher levels of mycotoxins, as they are about three times more concentrated than the original grains (Oplatowska-Stachowiak et al., 2015). Those procedures inevitably lead to the contamination of the final mixed feeds, manufactured by mixing different raw materials from different origins to make a totally new matrix with a new risk profile (Binder et al., 2007; Tolosa et al., 2014). In addition, as most feeds are made from annual crops, mycotoxin content may vary from year to year, and distribution of mycotoxin contamination is very heterogeneous existing highly contaminated sites among good quality material (Alonso et al., 2013). As a consequence, the available feed is frequently contaminated with mycotoxins (Bryden, 2012; Streit et al., 2013a).

On the other hand, animal feed is frequently contaminated simultaneously by several fungi, which are able to produce several mycotoxins leading to co-occurrence of mycotoxins (Bhat et al., 2010; Grenier & Oswald, 2011). Multi-mycotoxin contamination is of particular concern due to potential additive or synergistic effects (Cheli et al., 2013). Special attention should be paid to both, feed composition and contamination risk while storing (Arroyo-Manzanares et al., 2015).

In 2014, out of the 3097 original notifications counted in RASFF (Rapid Alert System for Food and Feed), 309 concerned feed, about 10% of the total, rising in number for the first time in several years. Notifications concerning feed have been increasing for a few specific categories with mycotoxin as the second most important (RASFF, 2014).

Regarding the feed manufacturing process, some mycotoxins are subject to legal regulations; aflatoxin B1 (AFB1), or

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Review

Studies on the Presence of Mycotoxins in Biological Samples: An Overview

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Abstract: Mycotoxins are fungal secondary metabolites with bioaccumulation levels leading to their carry-over into animal fluids, organs, and tissues. As a consequence, mycotoxin determination in biological samples from humans and animals has been reported worldwide. Since most mycotoxins show toxic effects at low concentrations and considering the extremely low levels present in biological samples, the application of reliable detection methods is required. This review summarizes the information regarding the studies involving mycotoxin determination in biological samples over the last 10 years. Relevant data on extraction methodology, detection techniques, sample size, limits of detection, and quantitation are presented herein. Briefly, liquid-liquid extraction followed by LC-MS/MS determination was the most common technique. The most analyzed mycotoxin was ochratoxin A, followed by zearalenone and deoxynivalenol—including their metabolites, enniatins, fumonisins, aflatoxins, T-2 and HT-2 toxins. Moreover, the studies were classified by their purpose, mainly focused on the development of analytical methodologies, mycotoxin biomonitoring, and exposure assessment. The study of tissue distribution, bioaccumulation, carry-over, persistence and transference of mycotoxins, as well as, toxicokinetics and ADME (absorption, distribution, metabolism and excretion) were other proposed goals for biological sample analysis. Finally, an overview of risk assessment was discussed.

Keywords: mycotoxins; biological samples; extraction; determination; chromatography-mass spectrometry; bioaccumulation

1. Introduction

Mycotoxins are secondary metabolites of low molecular weight, approximately of <1000 Da, produced both pre- and post-harvest by several fungus species [1]. From about 200 identified filamentous fungi, the most prevalent toxigenic species belong to the genera *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria*. *Fusarium* and *Alternaria* usually represent a high mycotoxicological risk at pre-harvest level or in freshly harvested products on drying, whereas *Aspergillus* and *Penicillium* toxigenic species pose a higher risk for stored food and feed products or other sorts of processing [2]. It is difficult to reduce mycotoxin exposure risks because they occur naturally under certain temperature and moisture conditions, contaminating the food throughout the food chain, in process, transport or storage [3]. The reason for mycotoxins production is not yet known since they seem not to be necessary for growth nor the development of fungi. Moreover, it is genotypically specific but not limited to one species or one toxin per species [4]. Several factors such as environmental and ecological conditions—temperature, relative humidity, substrate and use of fungicides—contribute to mycotoxin presence or production in food and feed, however, the interrelations between all these factors are not yet well understood and toxin production cannot reasonably be predicted [2,5].

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REVIEW ARTICLE

Effects of Quercetin Against Mycotoxin Induced Cytotoxicity: A Mini-Review

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Abstract: ***Introduction*:** Quercetin (QUER) is a bioactive phytochemical belonging to a large group of polyphenolic flavonoid substances and one of the most abundant flavonoids in the human diet. The antioxidant and pro-oxidant activity of QUER has been extensively investigated because of contradictory findings about its ability to protect mammalian cells from cytotoxicity. QUER is concomitant with mycotoxins in numerous food and foodstuffs. In the 80s, experimental approaches started to evaluate *in vitro* its protective effects against cytotoxicity related to mycotoxin ingest.

***Objective*:** The aim of this mini-review was to analyze how QUER treatment modifies mycotoxin effects in *in vitro* experimental models.

***Methods*:** A literature research was performed using the keyword "quercetin", "mycotoxin" and "*in vitro*", individually or all together, in Scopus, Web of Science and Pubmed.

***Results*:** In literature, the tested mycotoxins are described as follows: ochratoxin A, zearalenone and its metabolites α- and β-zearalenol, alternariol, alternariol monomethyl ether, T-2 toxin, patulin, aflatoxin B-1, deoxyni-valenol, enniatins and beauvericin. Regarding the experimental models, human hepatocellular (HepG2) and colorectal carcinoma (HCT116 and Caco-2), human peripheral blood mono-nuclear cells (hPMBC), embryonic kidney (HEK293), chinese hamster ovary (CHO-K1) and african green monkey kidney (Vero) cell lines were used.

***Conclusion*:** QUER showed promising results that made researchers move up to *in vivo* experiments. As future implementation, a combination of dietary non-enzymatic antioxidant products in food and feed could reduce the effects of mycotoxins on humans and animals.

Keywords: Antioxidant, cytotoxicity, *in vitro*, mycotoxins, polyphenol, quercetin.

1. INTRODUCTION

Flavonoids are polyphenolic compounds integrated in the human diet produced exclusively by plants, widely distributed in fresh fruits, berries, black tea, red wine, purple grape juice or medicinal herbals [1]. The protective effect of these compounds includes antiproliferative and anti-inflammatory activity and stimulation of the immune system, mainly due to a wide variety of mechanisms, including lightening oxidative stress by scavenging free radicals, promoting cellular survival by modulating intracellular signals, regulating gene expression or enzymes activity, *etc*. [2].

The flavonol 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one (QUER; Fig. **1**) is a secondary metabolite in higher plants, commonly found glycosylated in vegetables and fruits, showing exceptional high concentration in onions, apples, tea, broccoli, peppers, berries, cherries, grapes, and red wine. QUER is a major polyphenol ingested with a dietary intake of 20-100 mg, which may increase to 200-500 mg in case of high intake of fruits and vegetables [3, 4]. Principally, QUER exhibits a broad spectrum of properties, such as anti-inflammatory, immunomodulatory, antitumoral, antiplatelet, and vasoprotector effect, and modulator of enzyme activities and gene expression through both direct interaction or indirect modification of signal transduction [5]. Moreover, the regular intake of QUER is suggested to exert beneficial health effects including protection against osteoporosis, certain forms of cancer, pulmonary and cardiovascular diseases, possibly due to its ability to scavenge Reactive Oxygen Species (ROS) and free radicals [6].

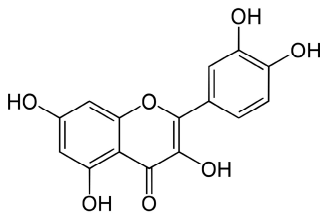

Fig. (1). Chemical structure of quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one).

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Mycotoxin Analysis of Human Urine by LC-MS/MS: A Comparative Extraction Study

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Abstract: The lower mycotoxin levels detected in urine make the development of sensitive and accurate analytical methods essential. Three extraction methods, namely salting-out liquid–liquid extraction (SALLE), miniQuEChERS (quick, easy, cheap, effective, rugged, and safe), and dispersive liquid–liquid microextraction (DLLME), were evaluated and compared based on analytical parameters for the quantitative LC-MS/MS measurement of 11 mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, BEA, EN A, EN B, EN A1 and EN B1) in human urine. DLLME was selected as the most appropriate methodology, as it produced better validation results for recovery (79–113%), reproducibility (RSDs < 12%), and repeatability (RSDs < 15%) than miniQuEChERS (71–109%, RSDs <14% and <24%, respectively) and SALLE (70–108%, RSDs < 14% and < 24%, respectively). Moreover, the lowest detection (LODS) and quantitation limits (LOQS) were achieved with DLLME (LODs: 0.005–2 μg L^{-1}, LOQs: 0.1–4 μg L^{-1}). DLLME methodology was used for the analysis of 10 real urine samples from healthy volunteers showing the presence of ENs B, B1 and A1 at low concentrations.

Keywords: mycotoxins; urine; optimization; method validation; LC-MS/MS

1. Introduction

Toxic fungal secondary metabolites, known as mycotoxins, frequently contaminating food and feed are of concern due to their association with a wide array of adverse health effects [1,2]. The diversity of mycotoxins leads to a broad range of acute and chronic toxic effects in animals and humans, such as vomiting, hematotoxicity, immunosuppression, hepatotoxicity, nephrotoxicity, teratotoxicity, immunotoxicity, and hormonal or reproductive effects, although potencies vary depending on species and sex [3]. Humans are often simultaneously exposed to mycotoxins mixtures along with other contaminants such as pesticides or heavy metals, making multi-mycotoxin exposure study relevant from a public health perspective. Actual exposure to mycotoxins is difficult to measure using an indirect approach based on mycotoxin occurrence in food combined with data on food consumption. Individual exposure is influenced by the heterogeneous distribution of mycotoxins, under- and overestimation of food consumption data, the presence of masked mycotoxins, and individual differences in absorption, distribution, metabolism and excretion (ADME) [4]. To overcome these disadvantages, detecting the presence of mycotoxins in biological fluids such as blood and urine could be useful and reliable in short- and long-term exposure assessment, and may make it possible to predict future adverse health consequences [2]. Urine is the body fluid most often used to measure mycotoxin exposure due to large amounts being easily and non-invasively collected, although blood (serum, plasma) has also been used [4]. Hence, detection of mycotoxins in human or animal urine allows more accurate and objective exposure assessment at an individual level since it covers exposure

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Review Article

Alternaria Mycotoxins in Food and Feed: An Overview

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Alternaria is one of the major mycotoxigenic fungal genera with more than 70 reported metabolites. *Alternaria* mycotoxins showed notably toxicity, such as mutagenicity, carcinogenicity, induction of DNA strand break, sphingolipid metabolism disruption, or inhibition of enzymes activity and photophosphorylation. This review reports on the toxicity, stability, metabolism, current analytical methods, and prevalence of *Alternaria* mycotoxins in food and feed through the most recent published research. Half of the publications were focused on fruits, vegetables, and derived products—mainly tomato and apples—while cereals and cereal by-products represented 38%. The most studied compounds were alternariol, alternariol methyl ether, tentoxin, and tenuazonic acid, but altenuene, altertoxins (I, II, and III), and macrosporin have been gaining importance in recent years. Solid-liquid extraction (50%) with acetonitrile or ethyl acetate was the most common extraction methodology, followed by QuEChERS and dilution-direct injection (both 14%). High- and ultraperformance liquid chromatography coupled with tandem mass spectrometry was the predominant determination technique (80%). The highest levels of alternariol and alternariol methyl ether were found in lentils, oilseeds, tomatoes, carrots, juices, wines, and cereals. Tenuazonic acid highest levels were detected in cereals followed by beer, while alternariol, alternariol methyl ether, tenuazonic acid, and tentoxin were found in legumes, nuts, and oilseeds

1. Generalities of Alternaria

1.1. Introduction. *Alternaria* is a fungal genus that includes saprophytic and pathogenic species and that is widespread in nature. They can infect a wide variety of crops in the field and in the postharvest stage causing considerable losses due to fruit and vegetable decay [1]. The most common *Alternaria* species include *A. alternata*, *A. tenuissima*, *A. arborescens*, *A. radicina*, *A. brassicae*, *A. brassicicola*, and *A. infectoria*. They colonize a range of plants including cereals, tomatoes, apples, grapes, oil crops, oilseeds, sunflower seeds, oranges, lemons, melons, cucumbers, cauliflowers, peppers, and tangerines [2].

The optimum growth temperatures for *Alternaria* range from 22 to 30°C; however, minimum growth temperatures ranging from 2.5 to 6.5°C and, even lower, from 0 to −5°C in cooler regions were reported [3]. Their ubiquitous occurrence and ability to grow and produce toxins even under unfavorable conditions (low temperatures and low water activity) make the *Alternaria* genus responsible for the spoilage of several commodities during transport and storage, even if they are refrigerated [1]. Therefore, *Alternaria* species has been shown to be a significant contaminant of refrigerated fruits, vegetables, and stored feedstuffs [4]. *A. alternata* is the most important within the genus as regards to mycotoxin contamination of fruits and vegetables. However, the production of host specific toxins from pathogenic *A. alternata* strains seems not to be a real problem in terms of food safety while a much more important problem is its saprotrophic strains, which colonizes harvested plant products and can produce reasonable amounts of certain mycotoxins, which exert poisonous effects after consumption by humans [5].

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Beauvericin and enniatin B effects on a human lymphoblastoid Jurkat T-cell model

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ABSTRACT

Several mycotoxins exert their effect on the immunological system; some are classified as immunotoxic. Jurkat T-cells were used to study toxic effects of beauvericin (BEA) and enniatin B (ENN B). Both are not legislated mycotoxins with increasing presence in feed and food. Concentrations studied were from 1 to 15 μM at 24, 48 and 72 h. Cell death by increasing the percentage of apoptotic/necrotic cells was: BEA > ENN B. IC50 values ranged from 3 to 7.5 μM for BEA. ENN B 15 μM decreased viability (21-29%). The percentage of apoptotic/necrotic cells was BEA > ENN B at 24 h but not at 48 h. Caspase-3&7 activation profile varied, although both mycotoxins increased this activation. No difference in ROS production for any mycotoxin was observed. Arrest in S phase for both mycotoxins was obtained. BEA increased the percentage of DNA in the tail (18% and 20%) with respect to the control, whereas not for ENN B. In summary, cytotoxicity of BEA involved mitochondrial alterations; while ENN B only at highest concentrations and time assayed. BEA had cell cycle disturbances and apoptotic and apoptotic/necrotic cells increased; for ENN B these were not evident. Different toxic responses in Jurkat T-cells may be involved in BEA and ENN B toxicity.

1. Introduction

Beauvericin (BEA) and enniatin B (ENN B) are secondary metabolites synthesized by various toxigenic fungi, including several *Fusarium* species. Both have chemical structure of cyclic hexadepsipeptides and are able to grow in maize, wheat, rice and other commodities (Juan et al., 2016; Covarelli et al., 2015). The observed levels of contamination in food chain demonstrate that a reduction of mycotoxin levels in food is needed to ensure food safety to humans and animals (EFSA, 2014; Juan et al., 2014).

Toxicological effects attributed to either BEA and ENN B have been studied in literature (Mallebrera et al., 2014, 2016; Prosperini et al., 2013a, 2013b; Juan-García et al., 2015), nevertheless a classification of toxic responses from the mechanistic point of view is difficult. BEA, as ionophore compound, increases ion permeability in biological membranes by forming a complex with essential cations (Ca^{2+}, Na^{+}, K^{+}) consequently affecting the ionic homeostasis and uncoupling the process of oxidative phosphorylation (Kouri et al., 2003; Tonshin et al., 2010). Additionally, disruption in mitochondrial activity and cell proliferation has been observed after BEA exposure, which can lead or be consequence of cell death (Mallebrera et al., 2016). ENN B has been described as phytotoxin, with a wide range of toxicological effects, such as antibacterial, antifungal, insecticidal, phytotoxic and cytotoxic properties (Kamyar et al., 2004). ENN B cytotoxicity involved early reactive oxygen species (ROS) generation that induced lipid peroxidation (LPO), apoptosis and necrosis via the mitochondrial pathway in Caco-2 cells (Prosperini et al., 2013a).

The number of *in vitro* studies reporting cytotoxic effects of ENNs on several cell types is high, as reviewed by Jestoi (2008) (EFSA, 2014) and it has increased in the last years, but the lack of data of their effects at immunological system is a fact especially when several mycotoxins have been classified as immunotoxic (Milićević et al., 2010). Among that, literature are scarce when searching for mycotoxins effect on Jurkat-T cells; in fact, only few mycotoxins have been tested on those cells as follows: aflatoxins B1 and M1 (AFB1 and AFM1) (Luongo et al., 2014), satratoxin H (Nielsen et al., 2009), deoxynivalenol (DON) (Katika et al., 2012; Taranu et al., 2010) and nivalenol (NIV) (Taranu et al., 2010), fumonisin B1 (FB1), alpha-zearalenol (alpha-ZEA) (Luongo et al., 2008, 2006), 4 beta-acetoxyscirpendiol (4-MAS), 15-acetoxyscirpenol (15-MAS), 4,15-diacetoxyscirpenol (4,15-DAS), and 30-acetyldiacetoxyscirpenol (TAS) (Lee et al., 2006). To notice that none emerging mycotoxin, neither enniatins (ENNs) nor BEA, have been studied *in vitro* in human malignant immunologic cells lines.

Cytotoxicity produced by toxicants can involve early ROS generation; an excess of which can lead to oxidative stress; oxidized macromolecular structures such as membrane lipids, proteins and, DNA; cell

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Transcriptomic study of the toxic mechanism triggered by beauvericin in Jurkat cells

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ABSTRACT

Beauvericin (BEA), an ionophoric cyclic hexadepsipeptide mycotoxin, is able to increase oxidative stress by altering membrane ion permeability and uncoupling oxidative phosphorylation. A toxicogenomic study was performed to investigate gene expression changes triggered by BEA exposure (1.5, 3 and 5 μM; 24 h) in Jurkat cells through RNA-sequencing and differential gene expression analysis. Perturbed gene expression was observed in a concentration dependent manner, with 43 differentially expressed genes (DEGs) overlapped in the three studied concentrations. Gene ontology (GO) analysis showed several biological processes related to electron transport chain, oxidative phosphorylation, and cellular respiration significantly altered. Molecular functions linked to mitochondrial respiratory chain and oxidoreductase activity were over-represented (q-value < 0.01). Pathway analysis revealed oxidative phosphorylation and electron transport chain as the most significantly altered pathways in all studied doses (z-score > 1.96; adj *p*-value < 0.05). 77 genes involved in the respiratory chain were significantly down-regulated at least at one dose. Moreover, 21 genes related to apoptosis and programmed cell death, and 12 genes related to caspase activity were significantly altered, mainly affecting initiator caspases 8, 9 and 10. The results demonstrated BEA-induced mitochondrial damage affecting the respiratory chain, and pointing to apoptosis through the caspase cascade in human lymphoblastic T cells.

1. Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi species with low-molecular weight and high variety of structural compounds. They represent one of the most important categories of biologically produced natural toxins relative to human health and economic impact worldwide, being found in numerous commodities of plant origin, especially cereals grains (Ruiz et al., 2011). Mycotoxins are practically unavoidable contaminants in food and feed representing among the natural food contaminants a major issue in food safety, and actually posing critical challenges in food toxicology (Dellafiora and Dall'Asta, 2017). Mycotoxin ingestion may induce various chronic and acute effects on humans and animals, such as cytotoxic, hepatotoxic, neurotoxic, genotoxic, immunosuppressive, estrogenic, nephrotoxic, mutagenic, teratogenic, and/or carcinogenic effects (Smith et al., 2016). Biochemically, the modes of action of mycotoxins can be divided into four categories: interactions with deoxyribonucleic acid (DNA), inhibition of different steps in protein synthesis, effects on cell membranes, and interfering on energy metabolism (Celik et al., 2010). From a molecular perspective, the primal mechanisms of toxic action commonly affect the integrity, functionality, and turn over of biological macromolecules −such as DNA, RNA, and proteins- or the biochemistry of the multitude of low-molecular-weight molecules (e.g. the production of reactive chemicals in cells) (Dellafiora and Dall'Asta, 2017).

Among the thousands of fungal secondary metabolites currently known, only a few groups of mycotoxins are important from the safety and economic points of view; namely aflatoxins, ochratoxin A, zearalenone, fumonisins and trichothecenes. However, several *Fusarium* species can produce a group of lesser-studied toxins called emerging mycotoxins, which includes beauvericin (BEA), enniatins, moniliformin, and fusaproliferin (Escrivá et al., 2015).

BEA is a cyclic hexadepsipeptide mycotoxin, which contains three D-hidroxyisovaleryl and three N-methylphenylalanil residues in an alternating sequence (Mallebrera et al., 2014). It shows insecticidal and antiviral properties, strong antibacterial activity against human, animal and plant pathogenic bacteria, and immunosuppressive effects (Wang and Xu, 2012). BEA, as lipophilic and ionophoric compound, was reported to increase ion permeability in biological membranes by forming a complex with essential cations (Ca^{2+}, Na^{+}, K^{+}) and cation-selective

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