VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

Departament de Medicina Preventiva i Salut Pública, Ciències de l'Alimentació, Toxicologia i Medicina Legal

Programa de Doctorat amb Menció cap a l'Excel·lència en Ciències de l'Alimentació

MICOTOXINAS EN PLANTAS MEDICINALES Y ZUMOS. ESTRATEGIAS PARA SU MITIGACIÓN

MYCOTOXINS IN MEDICINAL PLANTS AND JUICES. MITIGATION STRATEGIES

Tesi Doctoral Internacional

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4. Mycotoxins occurrence in herbal medicines dietary supplements and exposure assessment. (2020). *International Journal of Food Science and Technology. Under review. Impact factor: 2.281*
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"Hay una fuerza motriz más poderosa que el vapor, la electricidad y la energía atómica: la voluntad"

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A la meua familia,

en especial a tu, mare

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List of abbreviations

AcN	Acetonitrilo
ADMET	Absorción, Distribución, Metabolismo, Excreción y Toxicidad
ADN	Ácido desoxirribonucleico
AESAN	Agencia Española de Seguridad Alimentaria y Nutrición
AFs	Aflatoxinas
AFB1	Aflatoxina B1
AFB2	Aflatoxina B2
AFG1	Aflatoxina G1
AFG2	Aflatoxina G2
AFM1	Aflatoxina M1
ALARA	As Low As Reasonably Achievable
AME	Alternariol monometil éter
AOH	Alternariol
Aw	Actividad de agua
BBB	Barrera hematoencefálica
BEA	Beauvericina
C18	Sorbente Octadecil C18
CaCo-2	Células de adenocarcinoma de colon humano
CE	Energía de colisión
CEP	Potencial de entrada de células de colisión
CIT	Citrinina
CXP	Potencial de salida de la celda de colisión
CYP	Citocromo
DAS	Diacetoxiscirpenol
DLLME	Micro-extracción dispersiva liquid-líquido
DON	Deoxinivalenol
DON-3-Glc	Deoxinivalenol-3-glucósido
DOM-1	Deepoxi-DON
DP	Voltaje del cono
d-SPE	Dispersión en fase sólida
EA	Alcaloides del cornezuelo de centeno
EC	Comisión Europea

EFSA	Autoridad Europea de Seguridad Alimentaria
ENNs	Eniatinas
ENN A	Eniatina A
ENN A1	Eniatina A1
ENN B	Eniatina B
ENN B1	Eniatina B1
EP	Potencial de entrada
ESI	Ionización Electrospray
EtOAc	Acetato de etilo
FAO-OMS	Organización de las Naciones Unidas para la Agricultura y la Alimentación
FBs	Fumonisinas
FB1	Fumonisina B1
FB2	Fumonisina B2
FB3	Fumosina B3
FullMS–dd-MS/MS	Modo full MS/data dependiente de MS/MS
FUS	Fusaproliferina
FUS-X	Fusarenona X
GC	Cromatografía de gases
GC-MS/MS	Cromatografía de gases -Espectrometría de Masas en tándem
HAP	Hidrocarburos aromáticos policíclicos
HFBx	Fumonisinas hidrolizadas
HHP	Altas presiones hidrostáticas
HIA	Absorción intestinal humana
HPLC	Cromatografía líquida de alta resolución
HRMS	Espectrometría de masas de alta resolución

HT-2	Toxina HT-2
HyLv	Ácido hidroxivalerico
IARC	Agencia Internacional para la Investigación sobre el Cáncer
IDA	Ingesta Diaria Admisible
IDE	Ingesta Diaria Estimada
IDT	Ingesta Diaria Tolerable
IDTMP	Ingesta Diaria Tolerable Máxima Provisional
Ile	Isoleucina
INFOSAN	Red de alerta de la Organización Mundial de la Salud (OMS) y la FAO
IT	Trampa de iones
JECFA	Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios
LB	Nivel bajo de exposición
LC	Cromatografía líquida
LC-ESI-qTOF-MS	Cromatografía líquida acoplada a espectrometría de masas de alta resolución TOF
LC-MS/MS	Cromatografía líquida-Espectrometría de Masas en tándem
LOD	Límite de detección
LOQ	Límite de cuantificación
MAPA	Ministerio de agricultura, pesca y alimentación
MgSO4	Sulfato de Magnesio
ME	Efecto matriz
MeOH	Metanol
MON	Moniliformina
MPa	Megapascal
MRM	Monitorización de Reacciones Múltiples
NaCl	Cloruro de sodio
NEO	Neosolaniol
NIV	Nivalenol

OMS	Organización Mundial de la Salud
OTA	Ocratoxina A
PAT	Patulina
Pc	Peso Corporal
PCBs	Bifenilos policlorados
PEF	Pulsos eléctricos de alta intensidad
Pgp	Glucoproteína-P
Phe	Fenilalanina
p-HHP	Alta presión hidrostática pulsada
PL	Luz pulsada
PTV	Vaporización programable por temperatura
RASFF	Rapid Alert System for Food and Feed
RD	Real Decreto
ROS	Especies Reactivas de Oxígeno
RSD	Desviación estándar relativa
SCF	Comité Científico para la Alimentación
SCIRI	Sistema Coordinado de Intercambio Rápido de Información
S/N	Relación señal-ruido de fondo
SSE	Alteración de la señal por supresión o aumento
STG	Esterigmatocistina
T-2	Toxina T-2
TCs	Tricotecenos
TOF	Tiempo de vuelo
t_R	Tiempo de Retención
TWI	Ingesta semanal tolerable
UB	Nivel alto de exposición
UE	Unión Europea
UHPLC	Cromatografía líquida de ultra alta resolución
UHPLC-HRMS	Cromatografía líquida acoplada a espectrometría de masas de Alta resolución Orbitrap
US	Ultrasonidos
UV	Radiación ultravioleta
Val	Valina

ZEA	Zearalenona
α-ZAL	α-zearalanol
β-ZAL	β-zearalanol
α-ZOL	α-zearalenol
β-ZOL	β-zearalenol
3-AcDON	3-acetil deoxinivalenol
15-AcDON	15-acetil deoxinivalenol

RESUMEN

En la presente Tesis Doctoral se ha realizado una revisión bibliográfica para evaluar la presencia de micotoxinas emergentes de *Fusarium* en plantas medicinales en la materia en crudo, así como en forma de infusiones y cápsulas, así mismo se ha explorado sus posibles efectos beneficiosos y perjudiciales. Por otro lado, se ha llevado a cabo una revisión acerca del posible efecto de los tratamientos no térmicos HPP y PEF en la reducción de los contenidos de micotoxinas.

Así mismo, se han desarrollado y validado diferentes procedimientos analíticos para la determinación de micotoxinas en productos frescos, plantas medicinales y tés (en crudo o listos para su consumo) y zumos. La metodología empleada se ha basado en la determinación por cromatografía líquida y cromatografía de gases acopladas a espectrometría de masas en tándem. Por lo que respecta a la extracción de micotoxinas, se ha empleado el método QuEChERS para el análisis de matrices sólidas (plantas medicinales en diferentes formas) y el método DLLME para el análisis de muestras líquidas (infusiones de plantas medicinales y tés y zumos). La determinación analítica empleada ha sido validada en cuanto a exactitud, precisión, linealidad y límites de detección y cuantificación de acuerdo con la normativa europea.

Los resultados obtenidos tras analizar las plantas medicinales y los tés, revelan mayores contenidos de micotoxinas en crudo y en las cápsulas que en las infusiones resultantes, con contenidos que en ocasiones puntuales han sobrepasado los 1000 μg/kg y con incidencias inferiores al 34%. En las infusiones listas para el consumo los contenidos en general no han sobrepasado

100 μg/L con incidencias inferiores al 20%. En los zumos, AOH y PAT, han sido las micotoxinas detectadas con mayores incidencias, del 29 y 18%, respectivamente y mayores contenidos, con concentraciones medias de las muestras positivas de 207 y 28.18 μg/L, respectivamente.

La evaluación de la exposición a micotoxinas considerando los resultados obtenidos y los datos de consumo, revela un riesgo bajo a través del consumo de infusiones de plantas medicinales y tés, con porcentajes de IDT inferiores al 4% incluso considerando a los grandes consumidores (3 tazas/día). En cuanto a la evaluación del riego a través del consumo de cápsulas se observa un riesgo bajo en general, aunque en algunos suplementos como boldo, cardo mariano y cola de caballo se alcanzan porcentajes considerables de las IDT de hasta un 22.2%. Respecto a la evaluación del riesgo a través del consumo de zumos se alcanza un porcentaje considerable de la IDT para OTA (35.29%) y PAT (13.80%) (escenario upper bound) en niños considerando una ingesta de 200 mL diarios.

El procesamiento de los alimentos produce una reducción en los contenidos de micotoxinas y a una degradación parcial de éstas. Durante la preparación de las infusiones de plantas medicinales con tratamiento térmico se ha observado una reducción media de micotoxinas (AFs, ZEA, ENNs y BEA) del 74% al 100%, observándose una baja tendencia de las micotoxinas a migrar desde la materia prima a las infusiones resultantes. Respecto a los tratamientos no térmicos de procesado de los alimentos, como PEF y HPP, se han obtenido porcentajes de reducción que oscilan entre un 43 a 53% para micotoxinas emergentes de *Fusarium* en zumos y entre un 56 a 70% en smoothies tras el

procesado mediante PEF. Para las AFs se han obtenido porcentajes de reducción en un rango más amplio, desde un 16 a 84% tras el tratamiento por PEF. Tras la aplicación del tratamiento HPP se han observado menores porcentajes de reducción (8-29%).

Tras el tratamiento por PEF se han identificado varios productos de degradación mediante el análisis por LC-ESI-qTOF-MS. La obtención de productos de degradación de las micotoxinas tratadas, que se han originado por la pérdida de fragmentos aminoacídicos estructurales como HyLv, Val, Ile o Phe para ENNs y BEA confirma la reducción del efecto del tratamiento por PEF. Para la AFB2, se ha identificado un producto de degradación resultado de la adición de grupos OH^- y H^+ a los dobles enlaces y de la pérdida del grupo metileno ($-CH_2$). Se ha determinado la toxicidad *in silico* de los productos de degradación mediante el servidor Pro Tox-II, obteniéndose menor toxicidad que los compuestos originales, a excepción de los productos de degradación obtenidos para la ENNB.

Los datos de la evaluación de la estabilidad de ENNB en el tracto gastrointestinal humano durante la digestión *in vitro* muestra su baja estabilidad en este medio y una degradación significativa del compuesto original. Así mismo, se han identificado 5 productos de degradación de ENNB mediante UHPLC-HRMS, que pueden atribuirse a la oxidación y apertura del anillo depsipéptido.

SUMMARY

In this Doctoral Thesis, a bibliographic review has been carried out to evaluate the presence of *Fusarium* emerging mycotoxins in medicinal plants as raw materials, infusions and tablets. Their possible beneficial and harmful effects have also been explored. On the other hand, a review has been carried out about the possible effect of non-thermal treatments HPP and PEF on mycotoxin contents reduction.

Likewise, different analytical procedures have been developed and validated for the determination of mycotoxins in fresh products, medicinal plants and teas (in the form of raw material or ready to eat) and juices. The methodology used has been based on the determination by liquid chromatography and gas chromatography coupled to tandem mass spectrometry. Regarding the extraction of mycotoxins, QuEChERS method has been used for the analysis of solid matrices (medicinal plants in several forms) and DLLME method for the analysis of liquid matrices (medicinal plants, tea beverages and juices). The analytical determination used has been validated in terms of accuracy, precision, linearity and limits of detection and quantification in accordance with European regulations.

The results obtained after analyzing medicinal plants and teas, revealed higher mycotoxin contents in the raw material and tablets than in the resulting beverages, with contents that occasionally have exceeded 1000 μg/kg, with incidences lower than 34%. In ready-to-drink beverages, in general the contents have not exceeded 100 μg/L with incidences lower than 20%. In juices, AOH and PAT, are the mycotoxins detected with the highest incidences, 29 and 18%,

respectively, and contents, with mean concentrations of positive samples of 207 and 28.18 μg/L, respectively.

The evaluation of mycotoxin exposure considering the results obtained and the consumption data revealed a low risk through the consumption of medicinal plant and tea beverages, with TDI percentages lower than 4%, even considering high consumers (3 cups/day). Regarding the evaluation of the mycotoxin exposure through the consumption of tablets, low risk is observed in general, although in some supplements such as boldo, milk thistle and horsetail, considerable percentages of TDI (up to 22.2%) are reached. Regarding risk assessment through the consumption of juices, a considerable percentage of the TDI is reached for OTA (35.29%) and PAT (13.80%) (upper bound scenario) in children considering an intake of 200 mL daily.

Food processing lead to a reduction in mycotoxin contents and partial degradation. During the heat treatment of medicinal plant beverages preparation, an average reduction of mycotoxins (AFs, ZEA, ENNs and BEA) is obtained from 74% to 100%, observing a low tendency of mycotoxins to migrate from raw materials to the resulting infusions. Regarding non-thermal treatments of food processing (PEF and HPP), reduction percentages ranging from 43 to 53% are obtained for emerging *Fusarium* mycotoxins in juices and between 56 to 70% in smoothies after PEF processing. For AFs, reduction percentages are obtained in a wider range, (from 16 to 84%). After the application of the HPP treatment, lower reduction percentages are observed for both emerging mycotoxins and AFs (8-29%).

After PEF treatment, several degradation products have been identified by LC-ESI-qTOF-MS analysis. The degradation products obtained for the treated mycotoxins, which have been originated from the loss of structural amino acid fragments such as HyLv, Val, Ile or Phe for ENNs and BEA confirms the reduction effect of the treatment by PEF. For AFB2, a degradation product resulting from the addition of OH^- and H^+ groups to the double bonds and the loss of a methylene group ($-CH_2$) has been identified. *In silico* toxicity of the degradation products has been assessed by the Pro Tox-II server. Toxicity has decreased except for the degradation products obtained for the ENNB.

Evaluation of ENNB in the human gastrointestinal tract during *in vitro* digestion shows few stability in this medium and a significant degradation of the original compound. Likewise, 5 ENNB degradation products have been identified by UHPLC-HRMS, which can be attributed to oxidation and opening of the depsipeptide ring.

1.INTRODUCTION

1. INTRODUCCIÓN

1.1. Seguridad Alimentaria

Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO-OMS), existe seguridad alimentaria cuando todas las personas tienen acceso físico, social y económico de forma permanente a alimentos seguros, nutritivos y en la cantidad suficiente para poder satisfacer los requerimientos nutricionales y preferencias alimentarias, y con ellos poder llevar a cabo una vida activa y saludable. Los consumidores tienen derecho a que los alimentos sean seguros y de calidad, ya que estos constituyen la base de una dieta nutritiva. La seguridad alimentaria, además de proteger el bienestar de los consumidores debe garantizar que los productos agrícolas tengan acceso a los mercados, contribuyendo al desarrollo económico y al alivio de la pobreza. (FAO, 1996)

Las autoridades responsables de la seguridad alimentaria deben salvaguardar los intereses de los consumidores, asegurando que los alimentos que estos ingieren cumplan con las normas de seguridad alimentaria. Para ello, se requieren políticas y decisiones de gestión de riesgos, identificando las cuestiones de seguridad alimentaria de mayor preocupación e implementando medidas de control apropiadas. En la actualidad, los países se enfrentan a diversos riesgos y problemas de inocuidad alimentaria dependiendo de los patrones de consumo, los patrones de comercio, los procesos de producción, etc. La toma de decisiones sobre la inocuidad de los alimentos se desarrolla en una coyuntura donde coexisten intereses de varios sectores. Por lo tanto, se debe consultar y considerar una amplia gama de evidencias y equilibrar las

consideraciones de salud, comercio, alimentación y agricultura y seguridad alimentaria (FAO, 2017).

En la Unión Europea (EU), la Comisión Europea plasmó sus prioridades estratégicas en materia de seguridad alimentaria en el "Libro Blanco sobre Seguridad Alimentaria" (EC, 2000), en el que se plantea que la política de seguridad alimentaria debe basarse en un planteamiento global e integrado a lo largo de toda la cadena alimentaria (de la granja al consumidor).

En el Reglamento EC 178/2002 (EC, 2002) del Parlamento Europeo y del Consejo, de 28 de enero de 2002, se establecen los principios y los requisitos generales de la legislación alimentaria, se crea la Autoridad Europea de seguridad alimentaria (EFSA) y se fijan procedimientos relativos a la seguridad alimentaria.

En la actualidad, la EFSA se encarga de evaluar el riesgo y proporcionar una base científica sólida a los encargados de la gestión del riesgo alimentario. Sus áreas de actuación recogen la sanidad animal y vegetal, los riesgos biológicos, los contaminantes, la seguridad de los piensos, los ingredientes alimentarios y el envasado, la nutrición, los pesticidas y los organismos modificados genéticamente.

En el área de los contaminantes químicos, la EFSA lleva a cabo evaluaciones de riego de diferentes productos que pueden estar presentes en los alimentos y piensos, debido a la producción, distribución, envasado o consumo de alimentos, así como de aquellos que pueden estar presentes en el medio ambiente, ya sea de forma natural o como consecuencia de la actividad humana. También se encarga de recopilar datos e información acerca de la presencia de contaminantes en alimentos y piensos y de apoyar la coordinación de la

recopilación y el seguimiento de los datos por parte de los estados miembros. Esta tarea es llevada a cabo por el Panel de EFSA sobre Contaminantes en la Cadena Alimentaria (EFSA, 2020).

Debido a que los contaminantes químicos pueden tener un impacto negativo en la calidad y seguridad de los alimentos, pudiendo implicar un riesgo para la salud humana, la EU ha tomado una serie de medidas encaminadas a reducir su presencia en los alimentos. Entre estos, se incluyen las micotoxinas (aflatoxinas, ocratoxina A, toxinas de fusarium, patulina), los metales (cadmio, plomo, mercurio, estaño inorgánico), las dioxinas y PCB, los hidrocarburos aromáticos policíclicos (HAP), 3-MCPD y los nitratos (Reglamento EC 315/93 (EC, 1993) y Reglamento EC 1881/2006 (EC, 2006a)).

En España, la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN) desempeña las funciones relacionadas con la seguridad alimentaria y la nutrición saludable. Entre sus objetivos se encuentra promover la seguridad alimentaria, ofreciendo garantías e información objetiva al consumidor y a los agentes económicos del sector agroalimentario y llevar a cabo la planificación, la coordinación y el desarrollo de estrategias y actuaciones que fomentan la promoción de la salud en el ámbito de la nutrición.

Con el objetivo de proteger la salud de los consumidores y gestionar los riesgos alimentarios, la AESAN se constituye como punto nacional de contacto del sistema coordinado de alertas alimentarias, cuyos principios y funcionamiento se basan en los establecido en el artículo 25 de la Ley 17/2011, de seguridad alimentaria y nutrición y los artículos 50 a 52 del Reglamento EC

178/2002 (EC, 2002), mencionado previamente. El sistema coordinado de alertas está constituido por las siguientes redes:

- A nivel nacional, el Sistema Coordinado de Intercambio Rápido de Información (SCIRI), coordinado por la AESAN y en el que participan las autoridades competentes en materia de seguridad alimentara de las comunidades autónomas, el Ministerio de Defesa, la Comisión Europea y la Subdirección General de Sanidad Exterior, la Dirección General de Salud, Calidad e Innovación del Ministerio de Sanidad, Consumo y Bienestar, además de otros organismos y organizaciones con los que el SCIRI tiene convenios.

- A nivel de Europa, la red Rapid Alert System for Food and Feed (RASFF).

- A nivel mundial, la Red de alerta de la Organización Mundial de la Salud (OMS) y la FAO (INFOSAN).

La coordinación entre estas redes mencionadas permite mantener una vigilancia constante de cualquier riesgo o incidencia relacionada con los alimentos que pueda afectar a la salud de los consumidores, garantizando así que los productos a disposición de los consumidores sean seguros y no presenten riegos. Para ello se lleva a cabo un intercambio rápido de información entre las autoridades competentes, las empresas alimentarias y los consumidores, facilitando las actuaciones oportunas.

La red RASFF fue creada en 1979 para garantizar la seguridad alimentaria en la EU, permitiendo que la información de las alertas se envíe, reciba y responda de manera colectiva y efectiva entre sus miembros (las autoridades nacionales de seguridad alimentaria de sus estados miembros, la Comisión, la

EFSA, la ESA, Noruega, Islandia, Suiza, Noruega y Liechtenstein), evitando así muchos riesgos antes de que sean perjudiciales para los consumidores, pudiendo hacer que algunos productos sean retirados del mercado.

Según el último informe llevado a cabo por la RASFF en 2018, un total de 3699 notificaciones originales fueron transmitidas, de las cuales 569 notificaciones fueron relativas a la presencia de micotoxinas en alimentos provenientes de estados no miembros, seguidas de 370 notificaciones para microorganismos patógenos y 237 para residuos de pesticidas. Algunas de las micotoxinas implicadas fueron Aflatoxinas (AFs), Ocratoxina A (OTA), deoxinivalenol (DON), fumonisinas (FBs) y patulina (PAT) en productos alimenticios como las nueces y sus productos, las frutas y los vegetales, las hierbas y especias, el café y el cacao, los cereales y los productos de panadería y los snacks. De entre ellas, las AFs fueron las micotoxinas con un mayor número de notificaciones, 296 notificaciones en productos provenientes de estados no miembros (Annual Report RASFF, 2018). La tabla 1 muestra el ranking de las 10 primeras notificaciones en cuanto a número según el producto y el país de origen. Tal y como se puede observar en la tabla 1 más del 50% de las 10 primeras notificaciones son relativas a micotoxinas.

Tabla 1. Número de notificaciones, según el peligro y el producto/país de origen (Annual Report RASFF, 2018).

Peligro	*Tipo de producto*	*País de origen*	*Número de notificaciones*
Aflatoxinas	Nueces, productos de nueces y semillas	Estados Unidos	85
Aflatoxinas	Nueces, productos de nueces y semillas	Turquia	77
Aflatoxinas	Nueces, productos de nueces y semillas	Argentina	60
Salmonella	Carne de ave de corral y sus productos	Brasil	58
Ocratoxina A	Frutas y vegetales	Turquía	40
Mercurio	Pescados y sus productos	España	39
Aflatoxinas	Nueces, productos de nueces y semillas	China	39
Norovirus	Moluscos	Francia	35
Aflatoxinas	Nueces, productos de nueces y semillas	Egipto	35
Salmonella enterica	Carne de ave de corral y sus productos	Polonia	34

1.2. Micotoxinas

Las micotoxinas son compuestos químicos tóxicos que se producen de forma natural por algunas especies de hongos o mohos que son capaces de crecer en cultivos antes o después de la cosecha, durante el almacenamiento o en el mismo alimento si se dan unas condiciones ambientales cálidas y húmedas. Las micotoxinas aparecen en la cadena alimentaria a causa de la infección de los cultivos por mohos y la exposición humana se produce al consumir directamente alimentos contaminados o a partir del consumo de animales alimentados con piensos elaborados a partir de materia prima contaminada. La ingesta de micotoxinas de forma crónica a lo largo de toda la vida supone un riesgo potencial para la salud de las personas y de los animales (OMS, 2018). Los

mecanismos comunes de exposición a las micotoxinas incluyen, la digestión de los productos contaminados, el contacto con la piel y la inhalación de éstas.

Las micotoxinas suelen coexistir en cultivos agrícolas y a través de dietas diversificadas, por lo que la exposición a mezclas de micotoxinas es común. A pesar de la introducción de buenas prácticas agrícolas y de manufacturación en la alimentación, las micotoxinas siguen siendo un problema global, ya que pérdidas económicas significativas están asociadas con el impacto que tienen la mismas en la salud humana, el bienestar animal y su productividad y el comercio nacional e internacional.

La FAO ha establecido que el 25% o más de los cultivos alimentarios mundiales se ven afectados por hongos productores de micotoxinas (Bhat el al., 2010). Pero en la actualidad, diferentes autores sugieren una prevalencia mayor de hasta el 60-80% (Eskola et al., 2019). Los cultivos o sustratos más comúnmente contaminados consisten en cereales, nueces, semillas oleaginosas, frutos secos, café, especias y sus subproductos (Ünüsan, 2019).

Los hongos que causan una problemática de contaminación de alimentos y piensos con micotoxinas pertenecen principalmente a los géneros fúngicos *Aspergillus*, *Fusarium* y *Penicillium*. Las especies de *Aspergillus* y *Penicillium* crecen con mayor frecuencia en alimentos y piensos en las condiciones de almacenamiento, y las especies de *Fusarium* suelen afectar cultivos en crecimiento en el campo como el trigo, la cebada y el maíz y se propagan en la planta (Alshannaq & Yu 2017). Actualmente se han identificado más de 300 micotoxinas, sin embargo, las AFs, el DON, la toxina T-2 (T-2), la toxina HT-2 (HT-2), la ZEA, la OTA, las FBs, los alcaloides del cornezuelo de centeno (EA),

la PAT y la citrinina, son las que se consideran con mayor importancia desde el punto de vista reglamentario y de la inocuidad de los alimentos (Eskola et al., 2019). Debido a que el clima puede afectar profundamente el crecimiento, la distribución y la producción de micotoxinas en los hongos, el cambio climático también puede haber tenido un impacto en la mayor incidencia y puede aumentar el nivel de contaminación por micotoxinas en los próximos años (Moretti et al., 2019)

Las micotoxinas son un grupo de compuestos estructuralmente diversos con masa molecular relativamente baja. Pueden estar presentes en productos alimenticios en tres formas posibles: libre o no modificada, asociado a la matriz o como formas modificadas. Las micotoxinas libres o no modificadas describen las estructuras básicas de micotoxinas, sus estructuras químicas son muy diversas y consisten en lactonas microcíclicas como es el caso de la ZEA, lactonas pequeñas condensadas con hetero o alicíclicos como la PAT, derivados de furano como AFs y compuestos alicíclicos como la toxina T-2, entre otras. Las formas asociadas a la matriz son complejos formados con compuestos de la matriz, o están físicamente disueltas o atrapadas o están unidas covalentemente a componentes de la matriz o bien una combinación de ambos efectos. Las micotoxinas modificadas engloban a cualquier modificación de la estructura química básica de las moléculas (Marín et al., 2018). La figura 1 muestra la estructura química de AFs, TCs, OTA, ZEA, FB1 y micotoxinas emergentes.

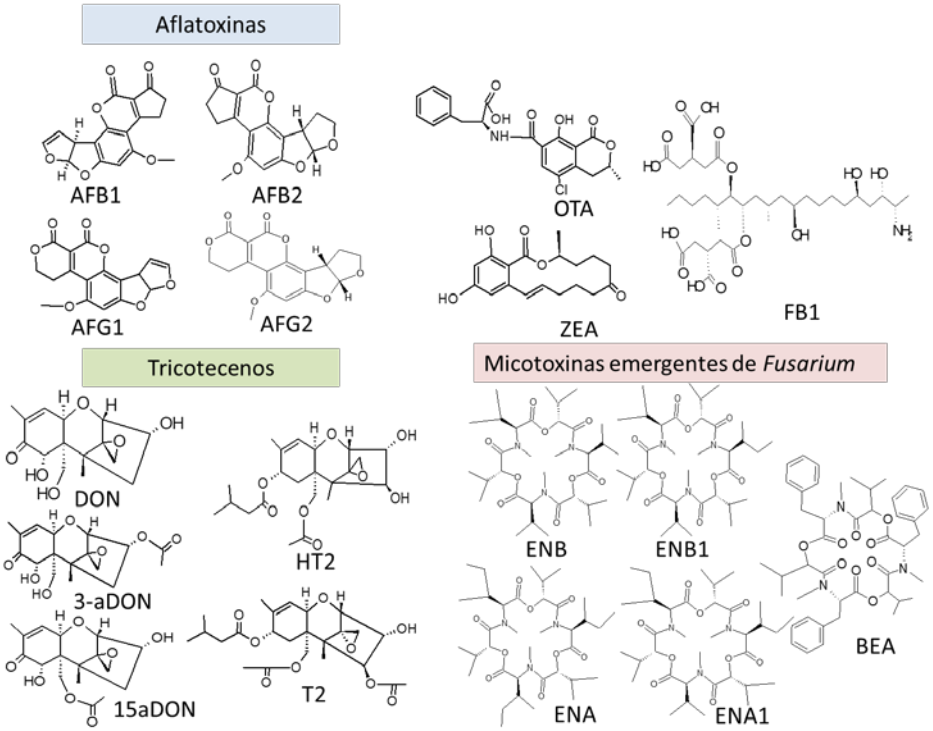

Figura 1. Estructura química de AFs, TCs, OTA, ZEA, FB1 y micotoxinas emergentes.

1.2.1. Hongos productores de micotoxinas y factores que favorecen la producción.

Actualmente se considera que los hongos están repartidos en tres reinos: Protozoa, Chromista y Fungi. Únicamente Fungi está constituido solo por hongos y es el reino donde se encuentran las especies consideradas de importancia como productoras de micotoxinas. El reino Fungi a su vez incluye cuatro divisiones: Chytridiomycota, Zygomycota, Basidiomycota y Ascomycota,

siendo esta última división donde se encuentran la gran mayoría de hongos productores de micotoxinas (Cabañes, 2007).

Dentro de los hongos, los filamentosos crecen sobre y a través del sustrato por un proceso de extensión, ramificación y anastomosis de hifas terminales, esto lleva a la producción de un micelio extensivo a través del cual se van a absorber los nutrientes y se excretarán los metabolitos secundarios, así como enzimas extracelulares que sirven para la degradación de las macromoléculas. Algunas especies han conseguido crecer en medios con actividad de agua muy baja, lo que les va a permitir colonizar sustratos como los cereales, que son demasiado secos para que puedan crecer otros microorganismos (Eley, 1992).

La mayoría de las micotoxinas que se conocen hasta el momento, han sido identificadas como metabolitos secundarios de los Fungi imperfecti, entre los que cabe destacar en particular los de los géneros *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps*, *Alternaria*, *Neotyphodium*, *Stachybotrys*, *Myrothecium*, *Phoma* y *Diploidia*. Debido a que los hongos productores de micotoxinas son cosmopolitas, las micotoxinas van a estar presentes en todas las partes del mundo induciendo efectos tóxicos por inhalación o ingestión (Martínez- Larrañaga & Anadón, 2006).

Por metabolitos secundarios se entienden aquellos compuestos que no son indispensables para el desarrollo o crecimiento de los hongos, en contraste con los metabolitos primarios, como pueden ser los aminoácidos, los ácidos grasos, los sacáridos, los ácidos nucleicos y las proteínas, que sí que lo son. Las micotoxinas van a proveer a los hongos de diversas funciones como de papel ecológico en la naturaleza, ya que el hongo va a competir con la bacteria por el sustrato, de papel regulador en el metabolismo y de papel regulador en la

diferenciación. Las micotoxinas también pueden producirse como respuesta al estrés oxidativo. Durante la colonización e la infección, los hongos pueden estar expuestos a los metabolitos de huésped, y las especies reactivas de oxígeno pueden desencadenar vías de respuesta en hongos que incluyen la producción de micotoxinas (Ponts et al., 2006; Martínez- Larrañaga & Anadón, 2006).

Entre los factores que influyen en la producción de micotoxinas por parte del hongo productor, se encuentran la temperatura, la actividad del agua, el pH, el sustrato y las interacciones microbianas. La actividad de agua (aw) junto con la temperatura van a ser los factores que más contribuyen en el desarrollo fúngico. Así, los mohos están adaptados a crecer en un intervalo amplio de temperaturas, por lo que las condiciones de producción de las materias primas en el campo pueden llevar a que se dé un problema fúngico o de micotoxinas. Además, su almacenamiento a temperatura ambiente estimula el crecimiento fúngico. La actividad del agua (aw), condiciona la presencia de algunas micotoxinas en los alimentos en la etapa de precosecha y otras en la de postcosecha, cuando los niveles de humedad son más bajos. Por ejemplo, las toxinas de *Fusarium* se suelen hallar en los cereales en el campo o durante el almacenamiento sin secado previo, mientras que las de *Aspergillus* y *Penicillum*, se suelen encontrar en los cereales ya almacenados. Por otra parte, los requerimientos mínimos de aw por parte de los hongos para sintetizar las micotoxinas suelen ser más altos que los requerimientos que presentan para su crecimiento.

Además, influye el pH, ya que el valor óptimo para el crecimiento del hongo se sitúa cercano a 5, aunque los hongos van a ser capaces de crecer en un intervalo amplio de pH (normalmente valores entre 3 y 8). Aunque el pH no es

tan importante para el desarrollo fúngico como la temperatura y la aw, a veces el pH puede provocar un cambio en la principal micotoxina sintetizada por un moho.

Por otra parte, para una determinada cepa fúngica, la capacidad para producir micotoxinas y la cantidad producida puede depender del sustrato sobre el que se desarrolla el hongo. La proporción proteínas/carbohidratos va a ser responsable de potenciar la síntesis de muchas micotoxinas, además la fuente de carbono y la cantidad de aminoácidos libres existentes en el sustrato también puede influenciar.

Así mismo, las interacciones entre los microorganismos, tanto inter como intraespecífica pueden afectar al desarrollo microbiano y tener un efecto marcado en la síntesis de micotoxinas (Sanchis et al., 2007).

1.2.2. Principales efectos tóxicos.

Debido a que las micotoxinas son sustancias tóxicas, pueden causar diversos problemas de salud en animales y humanos tanto agudos como crónicos. Las micotoxicosis agudas se producen al consumir micotoxinas a concentraciones que van desde moderadas a altas, y causan manifestaciones específicas, síndrome de enfermedad aguda e incluso la muerte, mientras que las micotoxicosis crónicas se producen por la ingestión repetida de niveles de toxinas desde valores moderados a bajos y causan enfermedades crónicas específicas.

El efecto de las micotoxinas sobre la salud puede estar influenciado por diversos factores como la edad, el sexo, el peso, la dieta, la cantidad de la micotoxina, la presencia de otras micotoxinas y la exposición a sustancias

farmacológicamente activas. Además, la gravedad de la intoxicación por micotoxinas puede verse influenciada por factores como la deficiencia de vitaminas, la baja ingesta calórica, el abuso del alcohol, la presencia de una infección o la existencia de problemas de salud (Omotayo et al., 2019).

El mecanismo de acción de las micotoxinas está determinado por su estructura química. Debido al amplio rango de estructuras químicas de las micotoxinas, sus efectos bioquímicos a nivel celular abarcan interferencias con el metabolismo energético, interacciones con las membranas celulares, interacciones con el ADN o las moléculas proteicas, inhibición de la transcripción, interacción con los receptores hormonales e interferencia con el metabolismo de las purinas (Martínez- Larrañaga & Anadón, 2006).

Las micotoxinas también se han relacionado con problemas de salud importantes debidos a la exposición a largo plazo a dosis altas tales como, mutagenicidad, carcinogenicidad, teratogenicidad, hepatotoxicidad, nefrotoxicidad, toxicidad gastrointestinal, inmunotoxicidad y neurotoxicidad. Estos efectos adversos pueden originarse debido a especies reactivas de oxígeno formadas después de la exposición de las células a las micotoxinas que inducen la activación de moléculas relacionadas con la inflamación y la apoptosis celular y por la alteración de los sistemas de reparación (Gacem et al., 2020).

1.2.3. Principales micotoxinas.

1.2.3.1. Aflatoxinas (AFs)

Las AFs son difuranocoumarinos producidos principalmente por dos especies de *Aspergillus* de la sección Flavi, *Aspergillus flavus* y *Aspergillus parasiticus*.

Aspergillus flavus, es el productor de las Aflatoxinas del grupo B y se distribuye de forma ubicua en la naturaleza, colonizando la mayoría de las partes aéreas de las plantas. *Aspergillus parasiticus* va a producir aflatoxinas B y G, adaptándose más a un ambiente del suelo, con una distribución más limitada.

Los hongos productores de AFs crecen en una amplia variedad de alimentos como los cereales (maíz, arroz, cebada, avena y sorgo), maní, nueces, pistachos, almendras y semillas de algodón. La leche también puede contaminarse con aflatoxina M1 (AFM1). En general, las AFs son compuestos muy estables, que pueden resistir a procesos, como el horneado, el tostado, la cocción y la extrusión por lo que pueden llegar a ser un problema para los alimentos procesados, los productos de panadería y los frutos secos tostados. La limpieza de los cereales o frutos secos puede resultar en una reducción del 40-80% de las AFs. Por otra parte, la molienda de los cereales va a producir una redistribución de las AFs (Marin et al., 2013; Alshannaq & Yu, 2017).

De las AFs, la Aflatoxina B1 (AFB1), se considera la más tóxica y es metabolizada principalmente por el hígado. La Aflatoxina M1 (AFM1) es el principal derivado monohidroxilado de AFB1 y se excreta en la leche.

Cuando se ingieren, inhalan o son absorbidas a través de la piel, las AFs son cancerígenas y producen efectos hepatotóxicos, teratogénicos y mutagénicos, incluso a pequeñas concentraciones. La Agencia Internacional para la Investigación sobre el Cáncer (IARC) ha clasificado las principales AFs (AFB1, AFB2, AFG1 y AFG2) en el grupo 1, como micotoxinas carcinógenas para los seres humanos. Por su parte, el potencial cancerígeno de AFM1 ha sido estimado en aproximadamente 10 veces menor que el de la AFB1 (IARC, 2012).

La incidencia de toxicidad aguda de AFs que se da actualmente en los seres humanos es muy baja y se caracteriza por vómitos, dolor abdominal, edema pulmonar y cerebral, coma, convulsiones e incluso la muerte (Alshannaq & Yu, 2017). La toxicidad crónica es la forma más común de aflatoxicosis y se ha relacionado en humanos con cáncer de hígado, efectos reproductivos negativos en hombres y efectos sobre el sistema inmune. El principal órgano diana es el hígado. También podría causar la encefalopatía con degeneración grasa de las vísceras, similar al síndrome de Reye. La exposición ocupacional a través de las vías respiratorias podría causar fibrosis intersticial (Marin et al., 2013).

1.2.3.2. Ocratoxina A (OTA)

La OTA se produce principalmente por dos géneros de hongos, *Aspergillus* y *Penicillium*. Las principales especies productoras pertenecen al *Aspergillus Circumdat*, *Aspergillus Nigr*, *Penicillium Verrucosum* y *Penicillium nordicum*. Las ocratoxinas se han encontrado en una amplia variedad de productos agrícolas como maíz, cebada, trigo, harina, café, arroz, avena, centeno, guisantes, frijoles y están notablemente presentes en el vino, el zumo de uva y las uvas desecadas. También pueden contaminar productos derivados de animales, como la carne y la leche, y se puede encontrar en la leche humana. Entre todas las fuentes, los principales contribuyentes a la ingesta de OTA son el café y los vinos. Es un compuesto muy estable que no se destruye por los procedimientos comunes de preparación de alimentos, siendo necesarias temperaturas superiores a 250ºC. (Marin et al., 2013; Alshannaq & Yu, 2017).

La OTA se ha clasificado por la IARC como posible carcinógeno para los humanos (Grupo 2B). La EFSA ha fijado la Ingesta Semanal Tolerable (TWI) en 0.12 μg/kg peso corporal (EFSA, 2006).

En cuanto a toxicidad, el riñón es el principal órgano diana de esta micotoxina, que es una nefrotoxina potente y que se ha relacionado con el cáncer urotelial del tracto urinario superior. OTA tiene una estructura similar a la del aminoácido fenilalanina (Phe) por lo que tiene un efecto inhibidor sobre las enzimas que utilicen la Phe como sustrato. Además, causa daño mitocondrial, estrés oxidativo, peroxidación de lípidos e interferencia con la fosforilación oxidativa. También ha demostrado aumentar la apoptosis de varios tipos de células (Marin et al., 2013).

1.2.3.3. Fumonisinas (FBs)

Son micotoxinas con una similitud estructural con esfinganina y están producidas principalmente por especies de *Fusarium*, siendo *Fusarium verticillioides* y *proliferatum* las principales especies productoras. Son conocidos al menos 12 análogos de las fumonisinas, de los que los más importantes son los de la serie B (FB1, FB2 y FB3). A diferencia de las otras micotoxinas, que son solubles en disolventes orgánicos, las FBs son hidrosolubles, lo que dificulta su estudio.

Las FBs están presentes principalmente en cereales como el maíz, y se forman antes de la cosecha o en la etapa temprana de almacenamiento, no soliendo aumentar la concentración durante el almacenamiento. También pueden aparecer en el sorgo, el trigo, la cebada, la soja, los espárragos, los higos, el té negro y las plantas medicinales (da Rocha et al., 2014). Son compuestos bastante estables al calor, pudiéndose reducir su contenido solo en aquellos

procesos en los que la temperatura supere los 150Cº. Durante la fermentación se produce poca degradación (Marin et al., 2013).

Desde el punto de vista toxicológico, la FB1 es la más importante. Su similitud con la esfingosina sugiere la probable intervención en la biosíntesis de los esfingolípidos. La inhibición de la biosíntesis de esfingolípidos conduce a serios problemas relacionados con la actividad celular, ya que estas sustancias son esenciales para la composición de la membrana, para la comunicación de las células, para las interacciones intracelulares y de la matriz celular, y para los factores de crecimiento.

La IARC ha clasificado la FB1 como posible carcinogénico para humanos (grupo 2B). El JECFA ha establecido una Ingesta Diaria Tolerable Máxima Provisional (IDTMP) de 2 μg/kg de peso corporal/día para las FBs (FAO/WHO, 2012). Los estudios disponibles sólo han demostrado asociaciones concluyentes entre las FBs y el cáncer relacionando la FBs con el cáncer de esófago en el Sur de África y con el cáncer de hígado en China.

1.2.3.4. Zearalenona (ZEA)

Es una micotixina producida por varias especies de *Fusarium*, principalmente por *Fusarium graminearum*. *Fusarium* crece e invade los cultivos en condiciones de campo húmedas y frías durante la floración, pero su crecimiento y la producción de toxinas también podría darse después de la cosecha si se dan unas condiciones de almacenamiento deficientes. Estas especies van a producir pequeñas cantidades de varios metabolitos, de los que α-zearalenol y β-zearalenol son los derivados más importantes. Todos los zearalenones van a ser compuestos estrogénicos, pero debido a una mayor afinidad de unión a

receptores de estrógeno el α-zearalenol va a tener un potencial estrogénico mayor que el β-zearalenol. Respecto a la estabilidad, ZEA va a ser estable hasta 150ºC y la degradación se da sólo a altas temperaturas o en condiciones alcalinas (Marin et al., 2013). ZEA se encuentra con frecuencia en maíz, trigo, cebada, sorgo, y centeno. En estados Unidos y Canadá las principales fuentes son el maíz y el trigo y en los países europeos son el trigo, el centeno y la avena (Alshannaq & Yu, 2017).

La ZEA y algunos de sus metabolitos relacionados se unen competitivamente a los receptores de estrógeno, por lo tanto, su toxicidad se asocia con problemas reproductivos en especies animales y posiblemente en seres humanos. La ingesta diaria tolerable (IDT) establecida por la EFSA ha sido calculada en 0.25 μg/kg de peso corporal (EFSA, 2014). La IARC al evaluar su carcinogenidad ha concluido que no es clasificable en cuanto a su cacinogenicidad para los humanos (grupo 3) (IARC, 1993). No obstante, se ha visto que ZEA estimula potencialmente el crecimiento de las células con receptores estrogénicos en glándulas mamarias humanas. Estos resultados podrían apoyar la hipótesis de que ZEA podría participar en la etiología del cáncer de mama. Respecto a su toxicidad aguda se disponen de pocos datos, aunque parece ser relativamente baja (Tatay et al. 2014).

1.2.3.5. Tricotecenos

De los aproximadamente 170 tricotecenos identificados se han dividido cuatro tipos (A, B, C y D). En el tipo A se encuentran HT-2 y la toxina T-2, y en el tipo B se encuentra el deoxinivalenol (DON). Los grupos C y D, son tricotecenos de menor importancia. Los tricotecenos son conocidos por su fuerte capacidad para inhibir la síntesis de proteínas eucariotas, interfiriendo en

los pasos de iniciación, elongación y terminación. Están presentes en productos como soja, patatas, semillas de girasol, maní, plátanos, y en algunos alimentos procesados derivados de cereales (pan, cereales de desayuno, fideos y cerveza). En general, son compuestos muy estables tanto durante el almacenamiento/ molienda como durante el procesamiento/cocción de los alimentos (Marin et al., 2013).

El DON es la micotoxina más comúnmente encontrada en los granos, su presencia es común en las semillas de cártamo, cebada, centeno y trigo. Se ha visto que los productos transformados, como las galletas, el pan y las pastas han sido menos contaminados que las muestras de trigo. El DON se conoce como vomitoxina o factor de rechazo de alimentos (da Rocha et al., 2014).

Por lo que a la T-2 y la HT-2 se refiere, procesar los cereales reduce sustancialmente la contaminación debido a la redistribución en las diversas fracciones (Serrano et al., 2012). En general los productos de alimentación muestran una baja incidencia y concentración, excepto los productos de avena que pueden presentar una concentración moderada. Es importante señalar también que durante el horneado y la cocción, la T-2 y la HT-2 son relativamente estables.

Respecto a la toxicidad, la toxina T-2 es un potente inhibidor de la síntesis de proteínas y la función mitocondrial tanto *in vivo* como *in vitro*, además muestra efectos inmunosupresores y citotóxicos. También se ha visto que tiene efectos muy tóxicos sobre la piel y las membranas mucosas. La toxicidad de la HT-2 está menos estudiada, pero como se sabe que la T-2 se metaboliza rápidamente hasta HT-2, se ha concluido que los efectos tóxicos de estas micotoxinas no pueden

diferenciarse (Marin et al., 2013). Así la EFSA ha fijado una IDT de 0,1 μg/kg de peso corporal para la combinación de ambas toxinas o para cada una por separado (EFSA, 2014). Este valor se modificó a 0.02 μg/kg, más recientemente (EFSA, 2017). La IARC ha clasificado la T-2 como no clasificable en cuanto a su carcinogenicidad (IARC, 1993). DON, al no haber evidenciado experimental ni epidemiológicamente propiedades mutagénicas ni carcinogénicas ha sido clasificada en el grupo 3, (IARC, 1993). La EFSA ha fijado una IDT de 1 μg/kg de peso corporal para DON (SCF, 2002). DON no es tan tóxico como la T-2 o la HT-2, pero como se ha mencionado anteriormente, es uno de los contaminantes más comunes de los cereales.

1.2.3.6. Micotoxinas emergentes de *Fusarium*

Las micotoxinas emergentes de *Fusarium* incluyen metabolitos secundarios tóxicos como la fusaproliferina, las eniatinas, la beauvericina y la moniliformina. Respecto a estas micotoxinas existen datos limitados, debido a la tardía comprensión de su papel como micotoxinas.

La Fusaproliferina (FUS) está producida por especies de *Fusarium* de la sección Liseola como *F.proliferatum* y *F.subglutinans* y es tóxica para los linfocitos B humanos.

Las Eniatinas (ENNs) y la beauvericina (BEA) son micotoxinas citotóxicas, cuya naturaleza apolar les va a permitir insertarse en las membranas celulares creando canales selectivos de cationes, perturbando así la homeostasis iónica intracelular. La especie *F.avernaceum* va a producir al menos seis eniatinas y pequeñas cantidades de beauvericina (Mallebrera et al. 2018).

La Moniliformina (MON) o ácido semicúarico está producida por varias especies de *Fusarium*, entre las que se incluyen *F.avenaceum*, *F.tricinctum*, *F.proliferatum*, *F.subglutinans* y *F. verticillioides*. Esta micotoxina va a ser un inhibidor de varias enzimas dependientes de pirofosfato de tiamina, de las que el piruvato deshidrogenasa va a ser el más estudiado, llevando su inhibición a la interrupción de la gluconeogénesis. Además, también va a inhibir a los enzimas glutatión peroxidasa y reductasa (Marin et al., 2013).

La presencia de estas micotoxinas en alimentos ha sido estudiada en estos años en algunos países de Europa (Serrano et al. 2013). La suma de BEA y ENNs en el trigo, la cebada y el maíz en Europa meridional, central y del norte ha dado como resultado altos niveles de contaminación. La ocurrencia de BEA es poco significativa en los cereales de climas más fríos, pero sí que se ha informado en concentraciones de decenas de mg/kg en el sur de Europa y Marruecos. La incidencia de FUS ha sido menos investigada, pero su presencia natural en climas más fríos parece poco frecuente. En general, la literatura actual concluye que puede haber una exposición continua a bajos niveles de estas micotoxinas en granos y alimentos y que se debe prestar más atención a los efectos toxicológicos que pueden presentar las mezclas de micotoxinas (Jestoi et al., 2009; Santini et al., 2012).

1.2.4. Legislación de Micotoxinas

Respecto a la legislación vigente, el Reglamento EC 1881/2006 (EC, 2006a) fija el contenido máximo de determinados contaminantes en determinados productos alimenticios. Este Reglamento establece límites máximos para las micotoxinas, AFs, OTA, PAT, DON, FBs, ZEA, HT-2, T-2,

citrinina y esclerocios de cornezuelo de centeno en alimentos destinados al consumo humano en un amplio rango de concentraciones, dependiendo del tipo de micotoxina, la matriz analizada y del grupo de población al que van destinados, siendo los contenidos máximos más bajos en aquellos alimentos destinados a lactantes, a niños de corta edad o alimentos dietéticos destinados a usos médicos especiales. En base a sucesivas evaluaciones de riesgo y dictámenes llevados a cabo por la EFSA, este Reglamento has sido objeto de diversas modificaciones (Tabla 2).

Tabla 2. Modificaciones del Reglamento EC 1881/2006 (EC, 2006a) llevadas a cabo por la Comisión.

Reglamento nº	**Micotoxinas**	**Alimentos**
EC 1126/2007 de la Comisión de 28 de septiembre de 2007 (EC, 2007)	Toxinas de *Fusarium*	Maíz y sus derivados
EC 105/2010 de la Comisión de 5 de febrero de 2010 (EC, 2010a)	Ocratoxina A	Especias y regaliz
EC 594/2012 de la Comisión de 5 de julio de 2012 (EC, 2012a)	Ocratoxina A	Especias
EC 1137/2015 de la Comisión de 13 de julio de 2015 (EC, 2015a)	Ocratoxina A	Especias
EC 165/2010 de la Comisión de 26 de febrero de 2010 (EC, 2010b)	Aflatoxinas	Productos alimenticios

EC 1058/2012 de la Comisión de 12 de noviembre de 2012 (EC, 2012b)	Aflatoxinas	Higos secos
EC 212/2014 de la Comisión de 6 de marzo de 2014 (EC, 2014)	Citrinina	Complementos alimenticios basados en arroz fermentado con levadura roja *Monascus purpureus*
EC 2015/1940 de la Comisión de 28 de octubre de 2015 (EC, 2015b)	Escleroios de cornezuelo de centeno	Cereales no elaborados

Además, el Reglamento EC 401/2006 (EC, 2006b) establece los métodos de muestreo y de análisis para el control oficial del contenido de micotoxinas en los productos alimenticios, evitando que se produzcan diferentes interpretaciones o variaciones en la transposición por parte de los estados miembro.

1.3. Descontaminación de micotoxinas

Las estrategias convencionales para la prevención de la contaminación de cultivos por micotoxinas a menudo requieren enfoques previos y posteriores a la cosecha. El enfoque pre-cosecha se encarga de controlar la contaminación por hongos en el campo, mientras que el enfoque post-cosecha se ocupa de las etapas posteriores. Las estrategias preventivas comprenden buenas prácticas agrícolas, la gestión de enfermedades de las plantas, y las condiciones de almacenamiento adecuadas para limitar el crecimiento de hongos productores de micotoxinas y con ello de las micotoxinas. Entre las prácticas agrícolas que se llevan a cabo se

encuentran, la utilización de variedades de cultivos resistentes, la rotación de los cultivos, la labranza del suelo, el control químico y biológico de las enfermedades de las plantas y el control de plagas. Todos estos parámetros pueden ser controlados; sin embargo, no se pueden controlar las condiciones ambientales, dónde la humedad relativa y la temperatura van a tener un efecto importante sobre la infección de los hongos y la producción de micotoxinas. Las condiciones de almacenamiento posteriores a la cosecha también van a ser importantes, por ejemplo, los granos deberían ser almacenados con menos del 15% de humedad para no favorecer el crecimiento de los hongos, además antes del almacenamiento aquellos granos visiblemente dañados o infectados deberían ser retirados. Así, las condiciones adecuadas de cosecha y almacenamiento van a ser cruciales para la prevención del crecimiento de hongos y la acumulación de micotoxinas en las cosechas. Desafortunadamente, estos dos enfoques puede que no sean suficientes y se requiera de un procesamiento adicional para la descontaminación de micotoxinas, que consiga reducir los niveles de micotoxinas presentes en los alimentos y los piensos (Jouany, 2007; Devreese et al., 2013; Pankaj et al., 2018).

Dichas medidas para la descontaminación de micotoxinas deben haber sido aceptadas por las agencias regulatorias pertinentes y deben asegurarse de reducir la exposición del consumidor a las micotoxinas. Un método eficiente para la reducción de micotoxinas debe ser capaz de eliminar o inactivar las micotoxinas de los alimentos sin producir residuos tóxicos ni afectar a las propiedades tecnológicas, nutritivas y sensoriales de los productos. Según el tipo de tratamiento aplicado, los procesos de descontaminación se clasifican en métodos físicos, químicos, biológicos, o combinaciones de los mismos (Čolović et al., 2019).

1.3.1. Métodos Químicos

Existen normas estrictas sobre la utilización de pesticidas químicos y se ejerce presión para eliminar la utilización de los productos químicos potencialmente peligrosos de los mercados.

Sin embargo, para garantizar la calidad de los alimentos los fungicidas sintéticos de baja persistencia siguen siendo relevantes en la actualidad para prevenir enfermedades en los cultivos.

En la etapa postcosecha, la eficacia de diversos productos químicos ha sido probada en la eliminación de micotoxinas, incluyendo varios ácidos, bases, sales, agentes oxidantes, agentes clorantes/ reductores y otros como el formaldehído. La Amoniación es un método ampliamente conocido que es efectivo para la desintoxicación de piensos contaminados con AFs u OTA y se ha utilizado con éxito en varios países. Se ha dado autorización regional (estado, país) limitada para emplearse en piensos en determinadas condiciones especificadas (tipo de producto, cantidad, animal).

No obstante, aunque el proceso no lleva a la formación de productos tóxicos de descomposición de micotoxinas, causa diversos cambios alterando las propiedades sensoriales y nutricionales de los alimentos, ya que produce la aparición de color marrón en los cereales tratados y disminuye los aminoácidos lisina y azufre.

En granos almacenados, el bisulfito de sodio, el ozono y el amoníaco pueden prevenir el crecimiento de hongos y en consecuencia la biosíntesis de micotoxinas (Omotayo et al., 2019). Es importante destacar que la aplicación de

estos tratamientos químicos no está permitida en la Unión Europea cuando el alimento se destina a consumo humano.

En los últimos años la búsqueda de una alternativa a los productos químicos sintéticos se ha convertido en una prioridad. Una buena opción podría ser el uso de fitoquímicos. Los fitoquímicos son sustancias naturales de origen vegetal, no nutritivas, con compuestos bilógicamente activos. Algunos fitoquímicos son nocivos para los hongos y podrían ser utilizados para proteger los cultivos, los alimentos y los piensos contra los hongos productores de micotoxinas, por lo que podrían suponer un método práctico, rentable y no tóxico para prevenir la contaminación fúngica y la carga de micotoxinas, evitando la utilización de conservantes químicos mediante el uso de extractos de plantas y aceites esenciales (Quiles et al., 2015; Assefa & Geremew, 2018).

1.3.2. Métodos Biológicos

La búsqueda de microorganismos, bacterias, mohos y levaduras capaces de biotransformar ciertas micotoxinas se está convirtiendo en una alternativa en los últimos años, así como la aplicación directa de materiales bioactivos, como las enzimas sintetizadas por estos microorganismos o disponibles comercialmente. No obstante, la mayoría de estos métodos solo han demostrado ser efectivos a escala de laboratorio y su eficacia en la cadena alimentaria aún no ha sido probada. Entre sus principales inconvenientes se encuentran el rendimiento microbiano dudoso en la degradación de múltiples micotoxinas y la posible conversión de micotoxinas en metabolitos con igual o mayor toxicidad.

Una gran variedad de bacterias, mohos y levaduras han demostrado su capacidad para biotransformar micotoxinas. Estos microorganismos son

capaces de actuar llevando a cabo reacciones de transformación biológica como acetilación, glucosilación, escisión del anillo, hidrólisis, desaminación y descarboxilación.

Por ejemplo, varias cepas de *Bacillus spp*. han demostrado ser capaces de biotransformar DON y ZEA. Otros géneros de bacterias y levaduras como *Trichosporon*, *Rhodotorula*, *Sphingomonas*, *Stenotrophomonas*, *Alcaligenaceae* y *Pichia* también han demostrado ser 100% efectivos en la reducción de OTA, ZEA y FB1 (Repečkienė et al., 2013; Nazareth et al., 2020).

Otra alternativa es la sustitución de los microrganismos por sus enzimas. Las enzimas son una herramienta muy atractiva para la biodegradación de las micotoxinas, debido a su mayor seguridad, especificidad y facilidad de manejo en comparación con los microorganismos. El desarrollo de la tecnología del ADN recombinante, el cribado basado en actividades y la ingeniería de proteínas han facilitado la búsqueda de enzimas con capacidad de biotransformar micotoxinas. Algunos estudios han aislado enzimas de bacterias y levaduras que degradan PAT, FB1 y OTA, en metabolitos menos tóxicos. Por ejemplo, Zhu et al. (2015) observaron que la levadura *Rhodosporidium paludigenum* era capaz de degradar la PAT en un producto menos tóxico mediante enzimas intracelulares.

Esta alternativa parece prometedora por lo que las perspectivas de futuro en este campo incluyen técnicas de ingeniería enzimática para mejorar la actividad de los enzimas y alterar la especificidad o estabilidad, métodos computacionales para estudiar la interacción proteína-toxina *in silico* y estudios de biodisponibilidad y toxicidad de los productos de transformación (Patriarca & Fernández Pinto, 2017).

1.3.3. Métodos Físicos

Los métodos físicos de descontaminación de micotoxinas incluyen diversos procedimientos como la limpieza, la clasificación mecánica y separación, el lavado, la molienda, la segregación, la inactivación térmica, la irradiación, las nuevas tecnologías no térmicas etc.

Los procesos de limpieza se utilizan en los cereales antes del procesamiento de los alimentos para eliminar el polvo, los granos rotos y otros materiales no deseados, pudiendo implicar la eliminación de algunas de las capas externas de los granos, siendo efectivos para la eliminación de micotoxinas.

La clasificación electrónica o por fluorescencia es otro método para el cribado de productos contaminados.

Los procedimientos de lavado empelando agua o solución de carbonato de sodio se usa a veces para disminuir la concentración de algunas micotoxinas y podría ser un procedimiento útil solo antes de la molienda húmeda (Stoev, 2013).

La eficiencia de la molienda como herramienta para la mitigación de micotoxinas se limita a aquellos productos en los que las micotoxinas se encuentran presentes en aquellas fracciones que se pueden descartar durante el procesado.

1.3.3.1. Procesado térmico

La mayoría de micotoxinas son muy estables al calor y son difíciles de eliminar durante los tratamientos térmicos convencionales de procesado. El procesado convencional de los alimentos con temperaturas hasta 100 ° C tiene poco efecto en la mayoría de las micotoxinas. No obstante, temperaturas más altas durante la fritura, el asado, el tostado y la extrusión pueden llevar a la reducción de la contaminación de micotoxinas. Por ejemplo, las AFs pueden reducirse por extrusión entre un 50 a 80% (Bullerman & Bianchini, 2007), dependiendo de la humedad y la temperatura del grano. El tostado puede reducir también los niveles de aflatoxinas entre un 50–70% en maní y nueces y en un 40–80% en maíz (Conway et al., 1978). También ha resultado ser efectivo en la eliminación del contenido de OTA al tostar el café. La extrusión también fue eficaz para reducir FBs en la sémola de maíz en un 34–95% (Bullerman & Bianchini, 2007). El aumento de la temperatura, la disminución del tornillo, la velocidad y la adición de glucosa pueden dar como resultado tasas de reducción más elevadas durante la extrusión (Karlovsky et al., 2016).

Las micotoxinas emergentes de *Fusarium* han demostrado ser más sensibles al procesado térmico. Serrano et al. (2016) observaron porcentajes de reducción de las micotoxinas durante el cocinado de la pasta, desde 98 a 100% para ENNA, 94-95% para ENNA1, 14-49% para ENNB y entre 53-65% para ENNB1. Durante el cocinado del pescado por distintos métodos (horneado, asado, cocinado por microondas y hervido), Tolosa et al. (2017) observaron porcentajes de reducción para las ENNs, desde un 30% al 100%.

Diversos factores influyen en el grado de degradación de las micotoxinas, entre ellos el nivel inicial de contaminación, el tipo y la concentración de la micotoxina, la temperatura y el tiempo empleados durante el tratamiento, el grado de penetración del calor y otros factores como el contenido en humedad, el Ph y la fuerza iónica de los alimentos.

Por tipo de micotoxina, las AFs tienen temperaturas de degradación elevadas, desde 237 hasta 306 °C. Se ha visto que son necesarias temperaturas superiores a 150 °C para conseguir una reducción parcial. La OTA, por su parte tiene un punto de fusión de 169 °C, no obstante, la información respecto al efecto del tostado del café en los niveles de OTA es contradictoria. Las FBs también son bastante estables al calor (hasta 100–120 °C), por lo que resistirán a muchos de los procesos térmicos comúnmente utilizados. DON es un compuesto muy estable, con un punto de fusión de 151-153 °C, por lo que el procesamiento térmico no va a destruir significativamente sus niveles, lográndose algunas reducciones con temperaturas más elevadas. La ZEA, es químicamente estable con un punto de fusión de 164–165 °C.

En general, aunque la mayoría de las micotoxinas son moderadamente estables al calor, se pueden lograr diversos grados de reducción con el procesamiento a altas temperaturas. También es importante estudiar los posibles efectos tóxicos de los productos de degradación de las micotoxinas en varios sistemas biológicos para saber si las reducciones observadas aparentemente durante el procesamiento se reflejan también en una pérdida de la toxicidad (Kabak, 2009).

La limitación de las tecnologías tradicionales de procesamiento de alimentos para inactivar las micotoxinas conduce a la investigación en el campo

de las nuevas tecnologías como posibles nuevas herramientas para la descontaminación de las micotoxinas.

1.3.3.2. Nuevas tecnologías no térmicas

Se han estudiado nuevos métodos para reducir el contenido de micotoxinas en los alimentos. Algunos consisten en técnicas físicas de procesamiento no térmico, como la radiación ultravioleta (UV), la luz pulsada, las altas presiones hidrostáticas (HHP), los pulsos eléctricos de alta intensidad (PEF), los ultrasonidos (US) etc.

La radiación UV es un método que se ha utilizado para la destrucción de microorganismos, pero también como medio para degradar las AFs, PAT o FBs de los alimentos. Su utilización no produce pérdidas de compuestos químicos en los alimentos o de sus propiedades sensoriales. La luz pulsada (PL) es otra técnica no térmica de conservación que se ha propuesto para la reducción de micotoxinas como PAT en productos alimenticios. Esta técnica consiste en el uso de ráfagas cortas de luz (1 s – 0.1 s) de amplio espectro con longitudes de onda, desde 200 a 1100 nm (Ioi et al., 2017).

1.3.3.2.1. Altas presiones hidrostáticas (HHP)

El procesamiento por HPP es un método no térmico, diseñado originalmente para la reducción de microorganismos, pero también se ha probado su aplicación para reducir los contenidos de micotoxinas en los alimentos.

El tratamiento con HHP consisten en colocar el producto en un recipiente a presión lleno del medio de transferencia de presión (normalmente agua en

aplicaciones alimentarias) que está comprimido por una bomba. Basado en el principio isostático, la presión hidrostática se transmite de forma uniforme a la muestra. Una de las principales ventajas de esta técnica de procesamiento frente a las convencionales, es que los efectos de la presión no van a depender del tamaño y la geometría de los productos. Los tres parámetros que caracterizan el tratamiento son la temperatura, la presión y el tiempo de exposición. En general, en el área de conservación de los alimentos se aplican tratamientos a niveles de presión entre 100 y 800 MPa, a una temperatura entre 4 a 20 ° C y con un tiempo desde varios segundos hasta varios minutos (Picart-Palmade et al., 2019). Históricamente los tratamientos de HHP se han aplicado principalmente para la conservación de alimentos, pero en los últimos años también se han estudiado distintas aplicaciones, como la recuperación de compuestos saludables, aumentar la biodisponibilidad de micronutrientes y fitoquímicos, reducir el potencial alergénico de alérgenos, preservar los lípidos saludables, reducir la ingesta de sal al aumentar la percepción de salinidad y reducir la formación de contaminantes de procesamiento (Barba et al., 2015a).

Respecto a los estudios disponibles en la bibliografía sobre el tratamiento HHP como una herramienta para reducir el contenido de micotoxinas en los alimentos, Kalagatur et al. (2018) observaron una reducción completa de las micotoxinas DON y ZEA de *Fusarium* en el maíz después del tratamiento con HHP (550 MPa) a 45 ° C durante 20 min. Tokuşoğlu et al. (2010) observaron una reducción de citrinina (CIT) del 64 al 100% en aceitunas de mesa negras tratadas por HHP a 250 MPa durante 5 minutos a 35 ± 2 ° C. Hao et al. (2016) estudiaron la degradación de PAT introducida en diferentes mezclas de zumos a una concentración de 200 μg/l, que posteriormente se sometieron a diferentes condiciones de tratamiento HHP (de 400 a 600 MPa, con un tiempo de

procesamiento de 0-300 s, y una temperatura de 11 ° C). El mayor nivel de degradación de PAT observado fue una disminución de 60 μg/l tras el tratamiento a 600 MPa durante 300 s.

Avsaroglu et al. (2015) estudiaron la reducción de PAT en zumo de manzana utilizando dos procedimientos diferentes, alta presión hidrostática (HHP) y alta presión hidrostática pulsada (p-HHP). Los tratamientos HHP consistieron en 300-500 MPa aplicados en combinación con temperaturas (30-50 ° C) durante 5 minutos, mientras que en el tratamiento p-HHP, se aplicaron dos pulsos diferentes (2 pulsos x 150 s; 6 pulsos x 50 s). Estos autores observaron porcentajes de reducción de 0 a 51.16% en el tratamiento de HHP, entre 0 y 62.11% en p-HPP a 6 pulsos x 50 s y de 0 a 45.49% en p-HHP a 2 pulsos x 150 s. Las reducciones en el contenido de patulina tras el tratamiento HHP, se pueden atribuir a la formación de aductos con compuestos que contienen grupos sulfhidrilo como el glutatión o la cisteína. Estos aductos han demostrado ser 100 veces menos tóxicos que la micotoxina inicial.

1.3.3.2.2. Pulsos Eléctricos de Alta Intensidad (PEF)

El tratamiento PEF es un método de procesamiento no térmico que se basa en la aplicación de pulsos eléctricos cortos (generalmente 1–20 μs), con una alta intensidad de campo (15-80 kv/cm) y un rango de tiempo de 50 ns a varios milisegundos a muestras colocadas entre dos electrodos. En células biológicas expuestas a un campo eléctrico suficientemente alto puede ocasionar electroporación, volviendo su membrana permeable a moléculas (Picart-Palmade et al., 2019). A parte de para la inactivación microbiana, esta técnica se ha empleado en el campo de la ciencia de los alimentos y la industria como una

herramienta para mejorar la funcionalidad y la capacidad de extraer compuestos de valor nutricional, y para mejorar la biodisponibilidad de micronutrientes en una amplia variedad de alimentos. Además, se ha utilizado como técnica para reducir los contaminantes y los pesticidas durante el procesamiento de alimentos (Barba et al., 2015b).

En el campo de la descontaminación de micotoxinas, en dos trabajos se ha estudiado la posible aplicación del tratamiento PEF para la reducción de AFs. Vijayalakshmi et al. (2018) observaron en agar de dextrosa de patata contaminado artificialmente una reducción de los contenidos de AFB1 y AFs del 77 y 97% en función de la combinación de diferentes variables estudiadas (pH, anchura del pulso y voltaje de salida). En un trabajo anterior, Vijayalakshmi et al. (2017) estudiaron la metodología del procesamiento térmico y su combinación con el tratamiento PEF para reducir los contenidos de AFs adicionadas en diferentes sistemas de agar de dextrosa de patata y encontraron que el tratamiento combinado produjo unos niveles de reducción más elevados que los procesos individuales, de 92.3 a 96.9% para AFB1 en función del pH y de 82 a 95.7% para AFs.

1.4. Biodisponibilidad y bioaccesibilidad de micotoxinas

En el estudio de su acción tóxica, debido a que la vía oral es la principal vía de exposición a micotoxinas, es importante determinar que proporción de micotoxina es movilizada desde la matriz alimentaria, no es modificada durante las reacciones gastrointestinales y finalmente está disponible para ser absorbida vía intestinal o lo que es lo mismo es bioaccesible (Prosperini et al., 2013).

La bioaccesibilidad recoge todos los eventos que tienen lugar antes del metabolismo presistemico intestinal y hepático. Se da durante la digestión de los

alimentos hasta que los macronutrientes y micronutrientes se pueden asimilar por parte de las células del epitelio intestinal. Se puede considerar como indicador de la máxima biodisponibilidad oral de las micotoxinas, para evaluar el riesgo en el consumidor en el peor de los casos. La bioaccesibilidad de una micotoxina depende de diferentes factores: la toxina, la matriz alimentaria, los cambios de pH y las actividades enzimáticas.

La biodisponibilidad de una micotoxina se define como la fracción de una micotoxina ingerida por vía oral, en una determinada matriz alimentaria, que finalmente alcanza la circulación sistémica y se distribuye por todo el cuerpo. Comprende tres procesos: la liberación de la micotoxina en el tracto gastrointestial de la matriz alimentaria (lo que sería igual a la biaccesibilidad), la absorción de la micotoxina bioaccesible por parte de las células epiteliales intestinales, transportándose al corriente sanguíneo o linfático y el metabolismo de la micotoxina de forma previa a la circulación sistémica (es decir, la biotransformación y excreción que se da por parte del epitelio intestinal o el hígado)(González-Arias et al., 2013).

Algunos protocolos *in vitro* han sido muy utilizados para evaluar la biaccesibilidad de las micotoxinas en los alimentos, entre ellos se encuentran el protocolo de Versantvoort et al. (2005) y el de Minekus et al. (2014). Estos métodos simulan los procesos de digestión comprendidos en la boca, el estómago y el intestino delgado y ofrecen algunas ventajas, como que son simples, rápidos, de bajo coste y pueden usarse para realizar los experimentos en condiciones bien controladas, no obstante tienen algunas desventajas, porque no se tienen en cuenta algunos factores fisiológicos, como la microbiota intestinal y

no pueden reproducir las condiciones de la mucosa intestinal, el ciclo enterohepático y el sistema inmune.

La combinación de estos modelos de digestión *in vitro* con otras técnicas que emplean líneas celulares intestinales, como los modelos de absorción intestinal *in vitro* con células Caco-2 o los estudios de transporte transepitelial pueden ofrecer una información más completa de lo que tiene lugar durante la digestión intestinal.

En general, respecto a lo que se ha visto en los estudios disponibles de bioaccesibilidad de micotoxinas, las AFs, FBs y ENNs presentan valores altos de bioaccesibilidad, en un rango entre 70 y 100 %. PAT, DON y BEA muestran valores intermedios del 30-70% y ZEA muestra valores más bajos. La OTA, por su parte presenta valores de bioaccesibilidad variables (González-Arias et al., 2013).

1.5. Productos de degradación y micotoxinas modificadas

Como se ha citado anteriormente, las micotoxinas pueden presentarse en tres formas posibles: libres o sin modificar, asociadas a la matriz o modificadas. Las micotoxinas libres o no modificadas recogen las estructuras básicas de micotoxinas. Las formas asociadas a la matriz son complejos formados con compuestos de la matriz, que pueden estar físicamente disueltas o atrapadas o estar unidas covalentemente a componentes de la matriz o bien una combinación de ambos efectos. Las micotoxinas modificadas recogen cualquier modificación de la estructura química básica de las moléculas. Estas modificaciones pueden ser modificaciones químicas o biológicas.

Las micotoxinas modificadas biológicamente incluyen cualquier funcionalización durante la fase 1 del metabolismo, además de los conjugados

formados durante el metabolismo de fase 2. Estas son resultados de reacciones de conjugación llevadas a cabo por plantas, como la formación de DON-3-Glc o ZEN-14-Glc, de reacciones de conjugación producidas por animales como la formación de DON-3/8/15-glucurónidos o HT2-3 / 4-glucurónidos o de conjugaciones producidas por hongos como, por ejemplo, la formación de ZEN-14-sulfato. Todas las demás modificaciones biológicas se denominan modificadas de forma diferente, este grupo incluye el metabolito intestinal del DON, deepoxi-DON (DOM-1), formado por la microbiota de animales y humanos.

Las micotoxinas modificadas químicamente, se pueden clasificar por su parte en las formadas térmicamente, durante el procesado térmico de los alimentos, y las no formadas térmicamente.

Las reacciones de degradación térmica o las modificaciones térmicas se producen durante el procesamiento de los alimentos, en procesos como hornear, asar, freír o extruir. Varios productos de degradación térmica han sido descritos para varias micotoxinas. Por ejemplo para FB1, el N- (1-desoxi-D-fructos-1-il) fumonisina B1 y el N- (carboximetil) fumonisina B1, formados en la reacción de Maillard. Para DON, se han reportado por ejemplo, los productos de degradación térmica norDON A-F y 9-hidroximetil DON lactona. Para OTA durante el tostado del café, se produce la isomerización a 14- (R) -ocratoxina A y la descarboxilación a 14-descarboxi-ocratoxina A.

Ejemplos de modificaciones no térmicas de micotoxinas son la formación de fumonisinas hidrolizadas (HFBx) o los productos de degradación norDON A – C formados en condiciones alcalinas. Otros ejemplos son las reacciones de degradación inducidas por la luz ultravioleta.

Es importante destacar que algunas veces el mismo compuesto se puede formar de distintas formas y por lo tanto estar en más de una categoría (Rychlik et al., 2014).

Es necesario disponer de métodos validados que permitan identificar y cuantificar también estas formas modificadas de las micotoxinas, ya que pueden ser hidrolizadas en el sistema digestivo dando lugar a sus precursores, cuya acción tóxica ya se conoce, o podrían ejercer efectos tóxicos comparables a las micotoxinas libres. Esto aumenta la dificultad para establecer una relación clara entre la cantidad de una micotoxina que es ingerida y los efectos tóxicos observados, y esperados, para una cantidad dada de micotoxinas. Por lo tanto, la evaluación de la exposición podría no ser lo suficientemente precisa si no se tiene en cuenta la presencia de estas formas modificadas, que en algunas ocasiones podrían hasta ser más tóxicas que su molécula original (Marín et al., 2018).

1.6. Productos frescos

La demanda y el consumo de productos frescos ha aumentado notablemente en los últimos años. Se entiende por productos frescos a cualquier fruta o verdura o una combinación de ambos en estado crudo que presentan una alteración física mayor o menor de su forma original. Varias organizaciones como la OMS, la FAO y la EFSA destacan sus beneficios para la salud y propiedades nutricionales, ya que muchos de sus ingredientes activos pueden reducir el riesgo de padecer enfermedades crónicas, como enfermedades cardíacas y ciertos tipos de cánceres (Sammugam & Pasupuleti, 2019). Estos productos vegetales constituyen una fuente importante de fitonutrientes como

carotenoides, compuestos cíclicos, polifenoles y otros metabolitos secundarios, así como de micronutrientes como vitaminas y minerales.

Los profesionales de la salud insisten en los beneficios asociados con la ingesta de frutas y verduras frescas, ya que así se mantienen los perfiles de sustancias fitoquímicas beneficiosas (terpenos, fenoles, compuestos azufrados, tocoferoles, tocotrienoles etc.) y nutrientes que pueden perderse durante su cocción u otros procesos. Sin embargo, el mayor consumo de frutas y verduras frescas puede llevar a un mayor riego de microorganismos patógenos y residuos químicos tóxicos. Por lo tanto, existe la necesidad de estrategias de control y de procesamientos innovadores que garanticen la inocuidad de los alimentos, ya que algunos de los procedimientos convencionales pueden ser perjudiciales por disminuir el contenido de flavonoides, carotenoides, sustancias fitoquímicas y micronutrientes en frutas y verduras (Bhilwadikar et al., 2019).

Debido a la creciente demanda de los consumidores de frutas frescas, varios países han optado por adoptar diversas estrategias como el empleo de envases especializados y técnicas de procesamiento mínimo y preservación natural. Dentro de las técnicas de mínimo procesamiento, el desarrollo de técnicas innovadoras como las altas presiones hidrostáticas (HHP), los pulsos eléctricos de alta intensidad (PEF), los ultrasonidos (US), la luz pulsada (PL), la luz UV y el empleo de antimicrobianos naturales permiten garantizar la seguridad y conservar las cualidades nutricionales y sensoriales de los productos (Gómez et al., 2011). Los alimentos mínimamente procesados son altamente susceptibles al crecimiento de microorganismos debido a que poseen baja actividad ácida, alta en agua y muchas porciones cortadas. Por esto, las frutas y productos vegetales mínimamente procesados deben procesarse y almacenarse a temperatura baja

para lograr una mayor vida útil, garantizando la seguridad (Vivek et al., 2019). En este sentido, la aplicación de las técnicas HHP y PEF ha sido estudiada por numerosos grupos de investigación para preservar zumos de frutas y vegetales (Rodrigo et al., 2005; Sampedro et al., 2009; Picouet et al., 2016; Mannozzi et al., 2019; Timmermans et al., 2019), siendo en la actualidad, las tecnologías no térmicas más utilizadas.

El consumo de zumos de frutas y bebidas a base de mezcla de zumos de frutas y leche fortificados con vitaminas, minerales y fibra ha aumentado en los últimos años y el mercado que se encuentra en constante expansión ofrece una gama de diferentes variedades de productos. Este consumo ayuda a alcanzar las recomendaciones diarias de frutas y verduras, establecidas por la OMS en 400 g.

A pesar de que hay muchas variedades de zumos de frutas disponibles, en realidad, hay relativamente poca diferencia entre ellos en cuanto a sus nutrientes. Sin embargo, la composición bioactiva de la planta y el perfil puede variar bastante entre las frutas, debido en gran parte a la gran variedad de colores, resultado de algunos componentes, como los carotenoides y antocianos (Caswell 2009).

Según el ministerio de agricultura, pesca y alimentación (MAPA), el consumo medio en España de zumo y néctar para el año 2018 se estima en 8.64 kg, de este consumo 1.57 kg se corresponde con zumo de fruta refrigerado, 2.19 kg con zumo concentrado, 3.95 kg con néctares y 2.62 con zumos enriquecidos (MAPA, 2018a).

El uso de productos botánicos por parte de la población se ha incrementado en los últimos años debido a la percepción actual por parte del

consumidor de que lo natural es saludable, las numerosas campañas de marketing al respecto y la creciente percepción de la necesidad de seguir una dieta saludable, por lo tanto el mercado de las plantas medicinales ha aumentado con ello, debido principalmente a la apertura de nuevas oportunidades por parte de los fabricantes en nuevos segmentos, como pueden ser los alimentos funcionales y los cosméticos (Santos et al., 2013). Según la OMS, el tratamiento con extractos de hierbas medicinales es llevado a cabo por el 80% de la población mundial. Los productos botánicos están disponibles en forma de diferentes tipos de productos, incluidos los suplementos de plantas medicinales, los productos de plantas medicinales, los productos homeopáticos, alimentos como las infusiones y jugos y los cosméticos (Abdel-Tawab, 2018). Estos botánicos constituyen una fuente importante de nutrientes y compuestos bioactivos, como vitaminas, minerales, terpenos, antioxidantes, saponinas, alcaloides y polisacáridos (Valduga et al. 2019) y se utilizan comúnmente para tratar enfermedades como dolores, resfriado, ansiedad, depresión o para perder peso. Respecto al consumo de infusiones por parte de la población española en el año 2018, el MAPA ha estimado un consumo medio de 0.12kg (MAPA, 2018b).

1.7. Referencias

-Abdel-Tawab, M. (2018). Do We Need Plant Food Supplements? A Critical Examination of Quality, Safety, Efficacy, and Necessity for a New Regulatory Framework. *Planta Medica* 84 (06–07): 372–393. https://doi.org/10.1055/s-0043-123764.

-Alshannaq, A., & Yu, J. H. (2017). Occurrence, Toxicity, and Analysis of Major Mycotoxins in Food. *International Journal of Environmental Research and Public Health* 14 (6). https://doi.org/10.3390/ijerph14060632.

-Assefa, T., & Geremew, T. (2018). Major Mycotoxins Occurrence, Prevention and Control Approaches. In . https://doi.org/10.5897/BMBR2018.0274.

-Avsaroglu, M. D., Bozoglu, F., Alpas, H., Largeteau, A., & Demazeau, G. (2015). Use of Pulsed-High Hydrostatic Pressure Treatment to Decrease Patulin in Apple Juice. *High Pressure Research* 35 (2): 214–222. https://doi.org/10.1080/08957959.2015.1027700.

-Barba, F. J., Terefe, N. S., Buckow, R., Knorr, D., & Orlien, V. (2015a). New Opportunities and Perspectives of High Pressure Treatment to Improve Health and Safety Attributes of Foods. A Review. *Food Research International* 77: 725–742. https://doi.org/10.1016/j.foodres.2015.05.015.

-Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., Saraiva, J. R., Raso, J., Martin-Belloso, O. Witrowa-Rajchert, D., Lebovka, N., & Vorobiev, E. (2015b). Current Applications and New Opportunities for the Use of Pulsed Electric Fields in Food Science and Industry. *Food Research International* 77: 773–798. https://doi.org/10.1016/j.foodres.2015.09.015.

-Bhat, R., Rai, R. V., & Karim, A. A. (2010). Mycotoxins in Food and Feed: Present Status and Future Concerns. *Comprehensive Reviews in Food Science and Food Safety* 9 (1): 57–81. https://doi.org/10.1111/j.1541-4337.2009.00094.x.

-Bhilwadikar, T., Pounraj, S., Manivannan, S., Rastogi, N. K., & Negi, P. S. (2019). Decontamination of Microorganisms and Pesticides from Fresh Fruits and Vegetables: A Comprehensive Review from Common Household Processes to Modern Techniques. *Comprehensive Reviews in Food Science and Food Safety* 18 (4): 1003–1038. https://doi.org/10.1111/1541-4337.12453.

-Bullerman, L. B., & Bianchini, A. (2007). Stability of Mycotoxins during Food Processing. *International Journal of Food Microbiology* 119 (1): 140–146. https://doi.org/10.1016/j.ijfoodmicro.2007.07.035.

-Cabañes FJ, Abarca ML, Bragulat MR & Castellá G (2007). Especies productoras de micotoxinas. En: Micotoxinas en alimentos. (Soriano del Castillo JM, ed). España: Díaz de Santos. pp: 29-61.

-Caswell, H. (2009). The Role of Fruit Juice in the Diet: An Overview. *Nutrition Bulletin* 34 (3): 273–288. https://doi.org/10.1111/j.1467-3010.2009.01760.x.

-Čolović, R., Puvača, N., Cheli, F., Avantaggiato, G., Greco, D., Đuragić, O., Kos, J., & Pinotti, L. (2019). Decontamination of Mycotoxin-Contaminated Feedstuffs and Compound Feed. *Toxins* 11 (11): 617. https://doi.org/10.3390/toxins11110617.

-Conway, H. F., HF, C., RA, A., & EB, B. (1978). Detoxification of aflatoxin-contaminated corn by roasting.

-da Rocha, M. E. B., Freire, F. D. C. O., Maia, F. E. F., Guedes, M. I. F., & Rondina, D. (2014). Mycotoxins and Their Effects on Human and Animal Health. *Food Control* 36 (1): 159–165. https://doi.org/10.1016/j.foodcont.2013.08.021.

- de España, J. D. E. (2011). Ley 17/2011, de 5 de julio, de seguridad alimentaria y nutrición. *Boletín Oficial del Estado*, (160).

-Devreese, M., De Backer, P., & Croubels, S. (2013). Different Methods to Counteract Mycotoxin Production and Its Impact on Animal Health. *Vlaams Diergeneeskundig Tijdschrift* 82 (4): 181–190.

- EC 1993. European Council (1993). EEC 315/93 of 8 February 1993 laying down Community procedures for contaminants in food. *Official Journal of the European Communities*. L37, 13.2.1993, pp. 1-3.

-EC 2000. European Commission (2000). White paper on food safety of 12 January 2000 [COM/99/0719 final - Not published in the Official Journal]. Available:https://eur-lex.europa.eu/legal content/EN/TXT/HTML/?uri=LEGISSUM:l32041&from=ES

- EC 2002. European Commission (2002). Regulation (EC) 178/2002 of the European Parliament and of the Council of 28 January 2002 laying down the general principles and requirements of food law, establishing the European Food Safety Authority and laying down procedures in matters of food safety. *Official Journal of the European Communities*. L31, 1.2.2002, pp. 1-24.

-EC 2006a. European Commission (2006). Regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*. L364, 20.12.2006, pp. 5-24.

-EC 2006b. European Commission (2006). Regulation (EC) 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of mycotoxin levels in foodstuffs. *Official Journal of the European Union*. L70, 9.3.2006, pp. 12-34.

-EC 2007. European Commission (2007). Regulation (EC) 1126/2007 of 28 September 2007 amending Regulation (EC) Nº 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards *Fusarium* toxins in maize and maize products. *Official Journal of the European Union*. L255, 29.9.2007, pp. 14-17.

- EC 2010a. European Commission (2010). Regulation (EC) 105/2010 of 5 February 2010 amending Regulation (EC) Nº 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. *Official Journal of the European Union*. L35, 6.2.2010, pp. 7-8.

- EC 2010b. European Commission (2010). Regulation (EC) 165/2010 of 26 February 2010 amending Regulation (EC) Nº 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Official Journal of the European Union*. L50, 27.2.2010, pp. 8-12.

- EC 2012a. European Commission (2012). Regulation (EC) 594/2012 of 5 July 2012 amending Regulation (EC) Nº 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs. *Official Journal of the European Union*. L176, 6.7.2012, pp. 43-45.

-EC 2012b. European Commission (2012). Regulation (EC) 1058/2012 of 12 November 2012 amending Regulation (EC) Nº 1881/2006 as regards maximum levels for aflatoxins in dried figs. *Official Journal of the European Union*. L313, 13.11.2012, pp. 14-15.

-EC 2014. European Commission (2014). Regulation (EC) 212/2014 of 6 March 2014 amending Regulation (EC) No 1881/2006 as regards maximum levels of the contaminant citrinin in food supplements based on rice fermented with red yeast *Monascus purpureus*. *Official Journal of the European Union*. L67, 7.3.2014, pp. 3-4.

- EC 2015a. European Commission (2015). Regulation (EC) 1137/2015 of 13 July 2015 amending Regulation (EC) No 1881/2006 as regards the maximum

level of Ochratoxin A in *Capsicum* spp. spices. *Official Journal of the European Union*. L185, 14.7.2015, pp. 11-12.

-EC 2015b. European Commission (2015). Regulation (EC) 1940/2015 of 28 October 2015 amending Regulation (EC) No 1881/2006 as regards maximum levels of ergot sclerotia in certain unprocessed cereals and the provisions on monitoring and reporting. *Official Journal of the European Union*. L283, 29.10.2015, pp. 3-6.

-EFSA (2006). Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to ochratoxin A in food. doi:10.2903/j.efsa.2006.365.

-EFSA (2014). Scientific Opinion on the Risks for Human and Animal Health Related to the Presence of Modified Forms of Certain Mycotoxins in Food and Feed. *EFSA J* 12: 3916-4023.

- EFSA (2017). Arcella, D., Gergelova, P., Innocenti, M. L., & Steinkellner, H. Human and animal dietary exposure to T-2 and HT-2 toxin. *EFSA J* 15(8): e04972.

-EFSA (2020). Chemical contaminants. Available: http://www.efsa.europa.eu/en/topics/topic/chemical-contaminants

-Eley R (ed), (1992). Intoxicaciones alimentarias de etiología microbiana. Zaragoza: Acribia. 81,82 pp.

-Eskola, M., Kos, G., Elliott, C. T., Hajšlová, J., Mayar, S., & Krska, R. (2019). Worldwide Contamination of Food-Crops with Mycotoxins: Validity of the Widely Cited 'FAO Estimate' of 25%. *Critical Reviews in Food Science and Nutrition* 1–17. https://doi.org/10.1080/10408398.2019.1658570.

- FAO (1996). Cumbre Mundial sobre la Alimentación. 13-17 de noviembre de 1996. Roma, Italia. Disponible en: http://www.fao.org/wfs/index_es.htm

-FAO/WHO (2012). Safety evaluation of certain food additives and contaminants. Fumonisins. *WHO Food Additives Series*, 65, 325–794.

-FAO. (2017). Food safety risk management: evidence-informed policies and decisions, considering multiple factors. *Food Safety and Quality Series (FAO) eng no. 4*. ISSN 2415-1173.

-Gacem, M. A., Gacem, H., Telli, A., & Khelil, A. O. E. H. (2020). Chapter 6 - Mycotoxin-Induced Toxicities and Diseases. In *Nanomycotoxicology*, edited by Mahendra Rai and Kamel A. Abd-Elsalam, 117–154. Academic Press. https://doi.org/10.1016/B978-0-12-817998-7.00006-9.

-Gómez, P. L., Welti-Chanes, J., & Alzamora, S. M. (2011). Hurdle Technology in Fruit Processing. *Annual Review of Food Science and Technology* 2 (1): 447–465. https://doi.org/10.1146/annurev-food-022510-133619.

-González-Arias, C. A., Marín, S., Sanchis, V., & Ramos, A. J. (2013). Mycotoxin Bioaccessibility/Absorption Assessment Using *in Vitro* Digestion Models: A Review. *World Mycotoxin Journal*, *6*(2): 167-184. https://doi.org/10.3920/WMJ2012.1521.

-Hao, H., Zhou, T., Koutchma, T., Wu, F., & Warriner, K. (2016). High Hydrostatic Pressure Assisted Degradation of Patulin in Fruit and Vegetable Juice Blends. *Food Control* 62: 237–242. https://doi.org/10.1016/j.foodcont.2015.10.042.

-International Agency for Research on Cancer (IARC), (1993) . Agents Classified by the IARC Monographs.Vol 56. http://www.iarc.fr/

-International Agency for Research on Cancer (IARC), (2002) . Agents Classified by the IARC Monographs.Vol 82. http://www.iarc.fr/
-International Agency for Research on Cancer (IARC), (2012). Agents Classified by the IARC Monographs.Vol 56, 82, 100F. http://www.iarc.fr/
-International Agency for Research on Cancer (IARC), (2014). Agents Classified by the IARC Monographs.Vol 1-109. http://www.iarc.fr/
-Ioi, J. D., Zhou, T., Tsao, R., & F Marcone, M. (2017). Mitigation of Patulin in Fresh and Processed Foods and Beverages. *Toxins* 9 (5): 157. https://doi.org/10.3390/toxins9050157.
- Jestoi, M., Kokkonen, M., & Uhlig, S. (2009). What about the'other'Fusarium mycotoxins?. *World Mycotoxin Journal 2*(2): 181-192. https://doi.org/10.3920/WMJ2008.1124

-Jouany, J. P. (2007). Methods for Preventing, Decontaminating and Minimizing the Toxicity of Mycotoxins in Feeds. *Animal Feed Science and Technology* 137 (3): 342–362. https://doi.org/10.1016/j.anifeedsci.2007.06.009.

-Kabak, B. (2009). The Fate of Mycotoxins during Thermal Food Processing. *Journal of the Science of Food and Agriculture* 89 (4): 549–554. https://doi.org/10.1002/jsfa.3491.
-Kalagatur, N. K., Kamasani, J. R., Mudili, V., Krishna, K., Chauhan, O. P., & Sreepathi, M. H. (2018). Effect of High Pressure Processing on Growth and Mycotoxin Production of Fusarium Graminearum in Maize. *Food Bioscience* 21: 53–59. https://doi.org/10.1016/j.fbio.2017.11.005.
-Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I. P., Speijers, G., Chiodini, A., Recker, T., & Dussort, P. (2016). Impact of Food Processing and Detoxification Treatments on Mycotoxin

Contamination. *Mycotoxin Research* 32 (4): 179–205. https://doi.org/10.1007/s12550-016-0257-7.

-Mallebrera, B., Prosperini, A., Font, G., & Ruiz, M. J. (2018). In Vitro Mechanisms of Beauvericin Toxicity: A Review. *Food and Chemical Toxicology* 111 : 537–545. https://doi.org/10.1016/j.fct.2017.11.019.

-Marín Sillué, S., Ramos Girona, A. J., Cano Sancho, G., & Sanchís Almenar, V. (2013). Mycotoxins: Occurrence, Toxicology, and Exposure Assessment. *Food and Chemical Toxicology* 60: 218–237. https://doi.org/10.1016/j.fct.2013.07.047.

-Marín Sillué, S., Cano Sancho, G., Sanchís Almenar, V., & Ramos Girona, A. J. (2018). The Role of Mycotoxins in the Human Exposome: Application of Mycotoxin Biomarkers in Exposome-Health Studies. *Food and Chemical Toxicology* 121: 504–518. https://doi.org/10.1016/j.fct.2018.09.039.

-Martínez- Larrañaga MR & Anadón A, (2006). Micotoxinas. En: Toxicología alimentaria. (Cameán AM & Repetto M, eds). España: Díaz de Santos. pp: 289-307.

-Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T. O. R. S. T. E. N., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickham, M. S J., Weitschies, W., Brodkorb, A. (2014). A Standardised Static in Vitro Digestion Method Suitable for Food – an International Consensus. *Food & Function* 5 (6): 1113–1124. https://doi.org/10.1039/C3FO60702J.

-Ministerio de Agricultura, Pesca y Alimentación (MAPA). (2018a). Datos de consumo en hogares zumo de frutas 2018. Disponible en: https://www.mapa.gob.es/app/consumo-en-hogares/resultado1.asp

-Ministerio de Agricultura, Pesca y Alimentación (MAPA). (2018b). Datos de consumo en hogares café e infusiones 2018. Disponible en: https://www.mapa.gob.es/app/consumo-en-hogares/resultado1.asp

-Moretti, A., Pascale, M., & Logrieco, A. F. (2019). Mycotoxin Risks under a Climate Change Scenario in Europe. *Trends in Food Science & Technology* 84: 38–40. https://doi.org/10.1016/j.tifs.2018.03.008.

-Nazareth, T. D. M., Luz, C., Torrijos, R., Quiles, J. M., Luciano, F. B., Mañes, J., & Meca, G. (2020). Potential Application of Lactic Acid Bacteria to Reduce Aflatoxin B1 and Fumonisin B1 Occurrence on Corn Kernels and Corn Ears. *Toxins* 12 (1): 21. https://doi.org/10.3390/toxins12010021.

-Omotayo, O. P., Omotayo, A. O., Mwanza, M., & Babalola, O. O. (2019). Prevalence of Mycotoxins and Their Consequences on Human Health. *Toxicological Research* 35 (1): 1–7. https://doi.org/10.5487/TR.2019.35.1.001.

-OMS (2020). Mycotoxins. Available: https://www.who.int/es/news-room/fact-sheets/detail/mycotoxins.

-Pankaj, S. K., Shi, H., & Keener, K. M. (2018). A Review of Novel Physical and Chemical Decontamination Technologies for Aflatoxin in Food. *Trends in Food Science & Technology* 71: 73–83. https://doi.org/10.1016/j.tifs.2017.11.007.

-Patriarca, A., & Pinto, V. F. (2017). Prevalence of Mycotoxins in Foods and Decontamination. *Current Opinion in Food Science* 14: 50–60. https://doi.org/10.1016/j.cofs.2017.01.011.

-Picart-Palmade, L., Cunault, C., Chevalier-Lucia, D., Belleville, M. P., & Marchesseau, S. (2019). Potentialities and Limits of Some Non-Thermal Technologies to Improve Sustainability of Food Processing. *Frontiers in Nutrition* 5: 130. https://doi.org/10.3389/fnut.2018.00130.

-Picouet, P. A., Hurtado, A., Jofré, A., Bañon, S., Ros, J. M., & Guàrdia, M. D. (2016). Effects of Thermal and High-Pressure Treatments on the Microbiological, Nutritional and Sensory Quality of a Multi-Fruit Smoothie. *Food and Bioprocess Technology* 9 (7): 1219–32. https://doi.org/10.1007/s11947-016-1705-2.

-Ponts, N., Pinson-Gadais, L., Verdal-Bonnin, M. N., Barreau, C., & Richard-Forget, F. (2006). Accumulation of Deoxynivalenol and Its 15-Acetylated Form Is Significantly Modulated by Oxidative Stress in Liquid Cultures of Fusarium Graminearum. *FEMS Microbiology Letters* 258 (1): 102–7. https://doi.org/10.1111/j.1574-6968.2006.00200.x.

-Prosperini, A., Meca, G., Font, G., & Ruiz, M. J. (2013). Bioaccessibility of Enniatins A, A1, B, and B1 in Different Commercial Breakfast Cereals, Cookies, and Breads of Spain. *Journal of Agricultural and Food Chemistry* 61 (2): 456–61. https://doi.org/10.1021/jf3044023.

-Quiles, J. M., Manyes, L., Luciano, F., Manes, J., & Meca, G. (2015). Influence of the Antimicrobial Compound Allyl Isothiocyanate against the Aspergillus Parasiticus Growth and Its Aflatoxins Production in Pizza Crust. *Food and Chemical Toxicology* 83: 222–28. https://doi.org/10.1016/j.fct.2015.06.017.

- RASFF (2018). The Rapid Alert System for Food and Feed- 2018 annual report. European Commission 2018. Available:

https://ec.europa.eu/food/sites/food/files/safety/docs/rasff_annual_report_2018.pdf

-Repečkienė, J., Levinskaitė, L., Paškevičius, A., & Raudonienė, V. (2013). Toxin-Producing Fungi on Feed Grains and Application of Yeasts for Their Detoxification. https://pubag.nal.usda.gov/catalog/5205724.

-Rodrigo D., Sampedro F., Martínez A., Barbosa-Cánovas G.V., Rodrigo M. Barbosa-Cánovas, Tapia, Cano (Eds.), Application of PEF in orange juice products in CRC Press, Novel Food Processing Technologies, New York (2005), pp. 131-144.

-Rychlik, M., Humpf, H. U., Marko, D., Dänicke, S., Mally, A., Berthiller, F., Klaffke, H., & Lorenz, N. (2014) Proposal of a Comprehensive Definition of Modified and Other Forms of Mycotoxins Including 'Masked' Mycotoxins. *Mycotoxin Research* 30 (4): 197–205. https://doi.org/10.1007/s12550-014-0203-5.

-Sammugam, L., & Pasupuleti, V. R. (2019). Balanced Diets in Food Systems: Emerging Trends and Challenges for Human Health. *Critical Reviews in Food Science and Nutrition* 59 (17): 2746–2759. https://doi.org/10.1080/10408398.2018.1468729.

-Sampedro, F., Geveke, D. J., Fan, X., & Zhang, H. Q. (2009). Effect of PEF, HHP and Thermal Treatment on PME Inactivation and Volatile Compounds Concentration of an Orange Juice–Milk Based Beverage. *Innovative Food Science & Emerging Technologies* 10 (4): 463–469. https://doi.org/10.1016/j.ifset.2009.05.006.

-Sanchis, V., Marín, S. & Ramos, J. A. (2007). Factores determinantes en la producción de micotoxinas. En: Micotoxinas en alimentos. (Soriano del Castillo JM, ed). España: Díaz de Santos. pp: 63-89.

-Santini, A., Meca, G., Uhlig, S., & Ritieni, A. (2012). Fusaproliferin, beauvericin and enniatins: occurrence in food–a review. *World Mycotoxin Journal*, *5*(1), 71-81. https://doi.org/10.3920/WMJ2011.1331.

-Santos, L., Marín Sillué, S., Sanchís Almenar, V., & Ramos Girona, A. J. (2013). Mycotoxin in Medicinal/Aromatic Herbs – a Review. *Boletín Latinoamericano y del Caribe de Plantas Medicinales Aromáticas* 12(2): 119-142. https://repositori.udl.cat/handle/10459.1/60327.

-Scientific 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 by the SCF on 26 February 2002). Report SCF/CS/ CNTM/MYC/27; European Commission Scientific Committee on Food: Brussels, Belgium.

-Serrano, A. B., Font, G., Ruiz, M. J., & Ferrer, E. (2012). Co-Occurrence and Risk Assessment of Mycotoxins in Food and Diet from Mediterranean Area. Food Chemistry 135 (2): 423–429. https://doi.org/10.1016/j.foodchem.2012.03.064.

-Serrano, A. B., G. Font, J. Mañes, and E. Ferrer. (2013). Emerging Fusarium Mycotoxins in Organic and Conventional Pasta Collected in Spain. *Food and Chemical Toxicology* 51: 259–266. https://doi.org/10.1016/j.fct.2012.09.034.

-Serrano, A. B., Font, G., Mañes, J., & Ferrer, E. (2016). Development a Mitigation Strategy of Enniatins in Pasta under Home-Cooking Conditions. *LWT - Food Science and Technology* 65: 1017–1024. https://doi.org/10.1016/j.lwt.2015.09.036.

-Stoev, S. D. (2013). Food Safety and Increasing Hazard of Mycotoxin Occurrence in Foods and Feeds. *Critical Reviews in Food Science and Nutrition* 53 (9): 887–901. https://doi.org/10.1080/10408398.2011.571800.

-Tatay, E., Meca, G., Font, G., & Ruiz, M. J. (2014). Interactive effects of zearalenone and its metabolites on cytotoxicity and metabolization in ovarian CHO-K1 cells. *Toxicology in Vitro* 28(1), 95-103. https://doi.org/10.1016/j.tiv.2013.06.025.

-Timmermans, R. A. H., Mastwijk, H. C., Berendsen, L. B. J. M., Nederhoff, A. L., Matser, A. M., Van Boekel, M. A. J. S., & Groot, M. N. (2019). Moderate Intensity Pulsed Electric Fields (PEF) as Alternative Mild Preservation Technology for Fruit Juice. *International Journal of Food Microbiology* 298: 63–73. https://doi.org/10.1016/j.ijfoodmicro.2019.02.015.

-Tokuşoğlu, Ö., Alpas, H., & Bozoğlu, F. (2010). High Hydrostatic Pressure Effects on Mold Flora, Citrinin Mycotoxin, Hydroxytyrosol, Oleuropein Phenolics and Antioxidant Activity of Black Table Olives. *Innovative Food Science & Emerging Technologies* 11 (2): 250–258. https://doi.org/10.1016/j.ifset.2009.11.005.

-Tolosa, J., Font, G., Mañes, J., & Ferrer, E. (2017). Mitigation of Enniatins in Edible Fish Tissues by Thermal Processes and Identification of Degradation Products. *Food and Chemical Toxicology* 101: 67–74. https://doi.org/10.1016/j.fct.2016.12.039.

-Ünüsan, N. (2019). Systematic Review of Mycotoxins in Food and Feeds in Turkey. *Food Control* 97 : 1–14. https://doi.org/10.1016/j.foodcont.2018.10.015.

-Valduga, A. T., Gonçalves, I. L., Magri, E., & Finzer, J. R. D. (2019). Chemistry, Pharmacology and New Trends in Traditional Functional and Medicinal

Beverages. *Food Research International* 120: 478–503. https://doi.org/10.1016/j.foodres.2018.10.091.

-Versantvoort, C. H., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J., & Sips, A. J. (2005). Applicability of an in Vitro Digestion Model in Assessing the Bioaccessibility of Mycotoxins from Food. *Food and Chemical Toxicology* 43 (1): 31–40. https://doi.org/10.1016/j.fct.2004.08.007.

-Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., Kumar, S & Reddy, R. (2017). Effect of Combination Processing on Aflatoxin Reduction: Process Optimization by Response Surface Methodology. *Journal of Food Processing and Preservation* 41 (6): e13230. https://doi.org/10.1111/jfpp.13230.

-Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., & Kumar, S. S. (2018). Effect of PH and Pulsed Electric Field Process Parameters on the Aflatoxin Reduction in Model System Using Response Surface Methodology. *Journal of Food Science and Technology* 55 (3): 868–878. https://doi.org/10.1007/s13197-017-2939-3.

-Vivek, K., Singh, S. S., Ritesh, W., Soberly, M., Baby, Z., Baite, H., Mishra, S., & Pradhan, R. C. (2019). A Review on Postharvest Management and Advances in the Minimal Processing of Fresh-Cut Fruits and Vegetables. *The Journal of Microbiology, Biotechnology and Food Sciences* 8 (5): 1178. http://dx.doi.org/10.15414/jmbfs.2019.8.5.1178-1187.

-Zhu, R., Feussner, K., Wu, T., Yan, F., Karlovsky, P., & Zheng, X. (2015). Detoxification of mycotoxin patulin by the yeast Rhodosporidium paludigenum. *Food chemistry 179*: 1-5. https://doi.org/10.1016/j.foodchem.2015.01.06

2.OBJECTIVES

2. OBJETIVOS

El objetivo general de la presente Tesis Doctoral es el estudio de la presencia de micotoxinas en plantas medicinales y zumos a base de fruta y la evaluación de diferentes tratamientos para su reducción.

Para ello, se han planteado los siguientes objetivos específicos:

1. Realizar una revisión bibliográfica sobre la presencia de micotoxinas emergentes en plantas medicinales.
2. Realizar una revisión bibliográfica acerca del efecto de los tratamientos tecnológicos no térmicos HPP y PEF en el contenido de micotoxinas.
3. Validación de la metodología analítica para el análisis multi-micotoxina en plantas medicinales, tés y zumos.
4. Evaluación de la contaminación y de la exposición por micotoxinas en plantas medicinales, tés y zumos.
5. Estudio del efecto del procesado térmico del infusionado sobre los contenidos de micotoxinas.
6. Estudio del efecto de las tecnologías no térmicas de procesado de los alimentos (HPP y PEF) en los contenidos de micotoxinas.
7. Identificación de productos de degradación de micotoxinas tras los tratamientos no térmicos.
8. Estudio de la estabilidad de ENNB en el tracto gastrointestinal humano durante la digestión *in vitro*.

2. OBJECTIVES

The main objective of this Doctoral Thesis is the study of the presence of mycotoxins in medicinal plants and fruit-based juices and the evaluation of different treatments for their reduction.

For this, the following specific objectives have been set:

1. Carry out a bibliographic review on the presence of emerging mycotoxins in medicinal plants.
2. Carry out a bibliographic review about the effect of non-thermal technological treatments HPP and PEF on the mycotoxins contents.
3. Analytical methodology validation for multi-mycotoxin determination in medicinal plants, teas and juices.
4. Evaluation of mycotoxin contamination of medicinal plants, teas and juices and the corresponding risk assessment exposure.
5. Effect of the thermal infusion process on the mycotoxin contents study.
6. Effect of non-thermal food processing technologies (HPP and PEF) on mycotoxin contents study.
7. Identification of mycotoxin degradation products after non-thermal treatments.
8. Study of the stability of ENNB in the human gastrointestinal tract during *in vitro* digestion.

3.RESULTS

3.1. Emergin mycotoxins in botanicals: benefit and risk

Plant Foods for Human Nutrition (under review)

Emerging mycotoxins in botanicals: benefit and risk

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Abstract

Fusarium species are responsible of enniatins (ENNs) and beauvericin (BEA) production, emerging mycotoxins with cyclic hexadepsispeptides structures. Although these mycotoxins have not been regulated yet, their high prevalence in food and feed, as well as their potential toxic effects in humans and animals have increased the interest in these molecules the last years. Besides its inophoric properties, these mycotoxins can induce cells damages such as oxidative stress, mitochondrial modifications and the disruption on the cell cycle related to several health adverse effects such as immunotoxicity, genotoxicity, endocrine toxicity and neurotoxicity. Moreover, they showed interesting activity against various microorganisms and insects in several studies, leading to a potential use in pesticide and medicine research, as potential candidates for anticancer therapy. Their worldwide occurrence makes mandatory their study in botanicals. Botanicals can be contaminated by mycotoxins when the harvesting practices or manufacturing conditions are inadequate. This review explores emerging mycotoxins occurrence in several botanicals forms and discuss their possible prejudicial and beneficial effects. Likewise, emerging mycotoxins are involved in capacity of the multidrug transport protein in human cancer cells modulation and apoptotic cell death induction.

Keywords: enniatins, beauvericin, cytotoxic, anticancer, antimicrobials, botanicals.

Introduction

According to WHO, the treatment with extracts of herbal medicine or vegetable is practiced by the 80% of world's population [1]. The use of infusions of leaves, flowers, fruits and seeds of some vegetable spices is widely practiced, and, in many situations, their consumption is associated with cultural aspects based in ethnobotanical knowledge [2]. Nowadays, the market of natural products has increased with the hope of new natural compounds obtained from plants with a commercial potential in the production of energy drinks, capsules, health supplements, energy boosters and food product materials. The phytochemicals of plants that originated interest in the industry consist in alkaloids, anthraquinones, flavonoids, glycosides, phenolics, saponins, steroids, tannins, and terpenes, among others [3,4]. More than 8000 phenolic compounds have been reported in botanicals, half of them are flavonoids presenting as aglycone, glycosides and methylated derivatives. These compounds present antioxidant, anticancer, antibacterial, cardioprotective, anti-inflammatory and immunological properties, and protect the skin from UV radiation, which make botanicals interesting candidates for pharmaceutical and medical application [5].

The attention in the quality and safety of botanicals has increased, because during the plantation, processing and storage, these matrices may be contaminated by pesticide residues, mycotoxins and heavy metals. In this sense, botanicals are susceptible to contamination by mycotoxigenic fungi, during harvesting, manufacturing, transport and storage. Mycotoxins are related to some prejudicial effects such as potential carcinogenicity, teratogenicity, immunotoxicity and neurological dysfunction [6]. The increase in consumption

of herbal products may contribute to an increase of mycotoxin intake leading to adverse human health problems [7].

Emerging mycotoxins

Mycotoxins are secondary metabolites produced by filamentous fungi. These contaminants are commonly reported in different commodities such as cereals, nuts, herbal teas, coffee or species. The contamination by mycotoxins frequently occur during field, in the post-harvest stage and throughout the food chain. Significant economic losses are associated with the impact of mycotoxins on human health, animal productivity, domestic and international trade [8, 9]. The Food and Agriculture Organization of the United Nations (FAO) has estimated that up to 25% of the world's food crops are significantly contaminated by mycotoxins [10]. While weather conditions can profoundly affect the growth, distribution and production of mycotoxins in fungi, climate change may also impact on mycotoxins incidence in the coming years [11].

Aspergillus, *Penicillium*, *Alternaria*, *Fusarium* and *Claviceps* are the principal genus involved in the mycotoxin production. *Aspergillus* is responsible of Aflatoxins (AFs) production; *Aspergillus* and *Penicillium*, both produce ochratoxin A (OTA); *Fusarium* species produce trichothecenes (HT-2, T-2, deoxynivalenol (DON) as well as nivalenol (NIV)), zearalenone (ZEA), fumonisins (FB1 and FB2) and emerging mycotoxins (fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA) and enniatins (ENs)); *Claviceps* produces ergot alkaloids; *Alternaria* species produce altenuene, alternariol, alternariol methyl ether, altertoxin, and tenuazonic acid [12]. *Fusarium* species are responsible of ENNs

and BEA production in different geographical areas, and their occurrence in some food commodities are high, at levels of mg/kg. Their presence has been highly reported in food matrixes such as maize, corn, wheat, wheat flour, durum wheat, oats but can also contaminate other products including beans, dried fruits, tree nuts, coffee, vegetables oils botanicals, and feed. Although emerging mycotoxins have not been regulated yet, and maximum levels have not been fixed in food, their high prevalence in food and feed, as well as their potential toxicity in humans and animals has increased their interest and concern. BEA and ENNs are a cyclic hexadepsispeptides structures with alternating D-α-hydroxy-isovaleryl- (2-hydroxy-3-methylbutanoic acid) and amino acid units. In BEA, the three amino acid residues are aromatic N-methyl-phenylalanines, while in ENNs the amino acid residues are aliphatic N-methyl-valine or–isoleucine or mixtures of these two [13].

BEA and ENNs cytotoxic activity

For acute toxicity, EFSA (2014) established the lethal dose (LD50) in mices upon oral administration at 100 mg/kg/bw for BEA and at 350 mg/kg/bw for a mixture of ENNs. The cytotoxicity associated with their exposure to different cell lines revealed inhibitory concentration (IC50) values at 24 h in the range from 11 to 24.6 μM for BEA and from 2.6 to 36 μM for ENNs [14].

In the last years, an increasing number of BEA and ENNs in vitro and in vivo studies were developed to understand their mechanisms of action [15].

The primary toxic mechanism of action of BEA and ENNs is related to their ionophoric properties, which make them capable of promoting the transport of mono- and divalent cations through membranes resulting in disturbances of the physiological cell cation levels [16]. These evoke changes in the ion intracellular concentration that consequently affects the cell functions. Besides it, ENNs can inhibit acyl-CoA: cholesterol acyl transferase (ACAT) activity and cause oxidative stress. It can also induce mitochondrial modifications and the disruption on the cell cycle that finally can result in apoptotic cell death [17]. In a study conducted on human colon adenocarcinoma cells (Caco-2), Prosperini et al. [18] observed that ENNA, ENNA1, ENNB and ENNB1 induced cytotoxicity involved by early ROS generation that induced LPO oxidative damage, apoptosis and necrosis via the mitochondrial pathway. Furthermore, ENNA and ENNA1 induced DNA damage, corroborated by the arrest of the cell cycle observed. In addition, ENNs produced adrenal endocrine toxicity. Kalayou et al. [19] observed a reduction of hormones and modulation of genes at the lower dose of ENNB (10 μM) in the H295R cells that could suggest that adrenal endocrine toxicity is an important potential hazard. The embryotoxicity has also been related to ENNs. The collected data obtained by Huang et al. [20] suggested that ENNB1 exerted cytotoxic effects on mouse embryos as well as oxidative stress and immunotoxicity during mouse embryo development.

Regarding possible neurotoxic effects, Krug et al. [21] studied the transport of ENN B and ENNB1 across the Blood Brain Barrier (BBB) employing a porcine brain capillary endothelial cells (PBCEC) in vitro-model

and their influence on cellular viability via cell Counting kit-8 assay (CCK-8) in three different cell types of BBB: PBCEC, human brain microvascular endothelial cells (HBMEC) and human astrocytoma cells (CCF-STTG1). The results obtained revealed high influx rates for ENNB and ENNB across BBB. The cellular viability results showed that ENNB and ENNB1 induced high cytotoxicity in CCF-STTG1 cell line. CCF-STTG1 cells were more sensitive than both endothelial cell types. Furthermore, in CCF-STTG1 especially ENNB, caused induction of apoptosis rather than necrosis.

Regarding toxicogenomic effects, Alonso- Garrido et al. [22] investigated changes in the gene expression profile induced by enniatin B exposure at concentrations of 1.5, 3 and 5 μM to human Jurkat lymphoblastic T-cells after 24 h and observed that 245 genes were differentially expressed and that mitochondria were the organelles with more related differentially expressed genes, that were involved in molecular functions and pathways related to mitochondrial metabolism and cell respiration.

BEA can disturb the normal cell cycle distribution and furthermore, can induce programmed cell death mediated by apoptosis. Moreover, BEA can induce mitochondrial transmembrane depolarization and induce immunotoxicity [23]. Wätjen et al. [24] observed in H4IIE hepatoma cells that BEA produce an inhibition of TNF-α-induced NF-κB activation without inhibiting the basal activity of NF-κB, which is an important modulator in the expression of immunoregulatory genes. BEA is related with oxidative stress, reactive oxygen species (ROS) generation and membrane lipid peroxidation (LPO) has been observed in cells after BEA exposure [23]. Prosperini et al. [25]

studied the cytotoxicity of BEA on human colon adenocarcinoma cells (Caco-2) and demonstrated that oxidative stress is one of the mechanisms involved in BEA toxicity. BEA induced cell death by mitochondria-dependent apoptotic process with loss of the mitochondrial membrane potential. Furthermore, BEA increased LPO level and reduced G0/G1 phase, with an arrest in G2/M. Moreover, DNA damage was observed. Mallebrera et al. [26] studied the injury and the mechanisms of defense in Chinese Hamster ovary (CHO-K1) cell line after exposure to BEA and observed disruption in mitochondrial enzymatic activity and cell proliferation after exposure. BEA inhibited cell proliferation by arresting cells in G0/G1 phase and increased apoptosis. At 48 and 72 h of exposure, BEA induced differentiation of CHO-K1 cells through G2/M arrest and prevented that cells entry into mitosis. After 24 h of exposure at 1 μM DNA strand breaks were observed. On the other hand, BEA exposure increased antioxidants defense mechanisms (catalase and superoxide dismutase activities) that can contribute to eliminate damages produced by BEA.

Juan-García et al. [27] studied the hepatotoxicity of BEA, ENNA1, ENNB at concentrations of 1.5 and 3 μM at 24, 48 and 72 h by flow cytometry in hepatocarcinoma cells (HepG2), and observed that ENNB1 produced a time dependent G1 blockade and that ENNA1 and BEA decreased the apoptotic-necrotic percentage of cells and produced disruptions in the mitochondrial membrane potential (MMP). In the same cell line, Juan-García et al. [28] studied individual and combined cytotoxicity effect of BEA and OTA. The cytotoxic concentrations assayed over 24, 48, and 72 h were from 0 to 25 μM for BEA, from 0 to 100 μM for OTA, and from 3.4 to 27.5 μM for BEA + OTA

combinations at a ratio of 1:10. The results obtained by these authors revealed that the toxicity observed for BEA was higher than for OTA. Furthermore, additive and synergistic effects were observed. OTA and BEA + OTA treatments produced cell cycle arrest in the GO/G1 phase, while a decrease in G0/G1 was detected for BEA, revealing induction of cell death. Finally, genotoxicity showed significant effects for BEA, OTA, and their combinations.

Fraeyman et al. [29] evaluated the cytotoxicity of ENNs and BEA towards intestinal porcine epithelial cells of the jejunum (IPEC-J2) using flow cytometric viability assays and observed that all studied mycotoxins resulted in a decline of IPEC-J2 viability, except of ENNB that resulted less cytotoxic, since the exposure at concentrations up to 100 μM resulted in 83% of viable proliferating cells. These authors suggested that ENNB may had minimal effect on intestinal morphometry.

In a work performed on Jurkat T-cells, Manyes et al. [30] studied the effects of both, BEA and ENNB at concentrations from 1 to 15 μM at 24, 48 and 72 h and observed that BEA and ENB produced several toxic responses. IC50 values obtained ranged from 3 to 7.5 μM (72 to 24 h) for BEA while for ENN B 15 μM decreased viability in the range 21-29%. BEA mediated cytotoxicity through mitochondrial alterations, while for ENNB it only occurs at high concentrations and time assayed. Furthermore, BEA affected cell cycle with apoptotic/necrotic cells increase, whereas these effects were not evident for ENNB. BEA and ENNB revealed caspase-3&7 activation, even by different profile activation. No difference in ROS production was observed for both mycotoxins. Finally, BEA produced DNA damage at high concentrations.

BEA can also affect estrogenic activity. García-Herranz et al. [31] determined the cytotoxic effects and the endocrine activities of BEA in two fish and one mammalian hepatoma cell lines and observed that BEA was as toxic to fish as to mammalians cells and showed a weak antagonistic effect at the androgen receptor.

BEA was also related to genotoxicity, internucleosomal DNA fragmentation, chromosomal condensation, membrane blebbing, cell shrinkage, apoptotic body formation and apoptotic morphological changes effects [23]. Çelik et al. [32] studied the genotoxic and cytotoxic effects of BEA on human lymphocytes in vitro culture and suggested that BEA is a genotoxic compound producing significant concentration-dependent increase in chromosomal aberrations, sister-chromatid exchanges and micronuclei. It also produced a decrease in the mitotic index at the two highest concentrations employed (5 and 10 μM). Not significant changes in the proliferative and nuclear division indices were observed.

Concerning the toxicogenomic effects, Escrivà et al. [33] investigated gene expression changes triggered by BEA exposure in Jurkat cells at concentrations of 1.5, 3 and 5 μM during 24 h through RNA-sequencing and observed a large number of differentially expressed genes mainly related to respiratory chain, apoptosis, and caspase cascade activation. Molecular functions related to mitochondrial respiratory chain and oxidoreductase activity were over-represented. 77 genes involved in the respiratory chain resulted significantly down regulated. Furthermore, 21 genes related to apoptosis and programmed cell death, and 12 genes related to caspase activity resulted significantly altered.

More recently, Escrivà et al. [34] studied the transcriptional effects of combined exposure to BEA and ENNB (1:1) at concentrations of 0.1, 0.5, 1.5 μM h in Jurkat cells at 24 h employing qPCR on 30 selected target genes (10 mitochondrial and 20 nuclear) and observed transcriptional changes, especially at mitochondrial level after BEA-ENNB co-exposure including down-regulation of gens related with antioxidant activity. Differences expression patterns were revealed between individual and combined exposures.

Regarding its possible embryotoxicity, Schoevers et al. [35] investigated the effects of BEA on porcine oocyte maturation and preimplantation embryo development and observed that BEA was toxic in embryos, oocytes and cumulus cells at concentrations >0.5 μM, and that embryos were most vulnerable after the four-cell stage. BEA toxic mechanism is suggested to involve different pathways.

BEA and ENNs bioactivity beneficial properties.

Unlike for toxic effects, beneficial properties have been described. BEA and ENNs have different biological properties, which may lead their potential use in medicinal and environmental research.

BEA is a useful tool in combination with chemotherapeutic drugs due its inhibitory capacity of the multidrug transport protein in human cancer cells and the induction of apoptotic cell death. Their anticancer properties can besides in the fact that induces extracellular translocated of Ca2+ into the cytosol, leading the increase of Ca2+ intracellular level, which activate a series of signaling pathways such as MAPK, NF-kB, etc. NF-kB is a transcription factor

related with cell survival. BEA also decreases the mitochondrial transmembrane potential, release of Cyt c, and activates caspases, finally promotes cancer cell apoptosis [36].

In this sense, Heilos et al. [37] have observed i*n vivo*, a decrease of tumor size and weight and significant increase of necrotic areas in cervix and colon carcinomas.

BEA also shows anti-inflammatory activities and inhibits inflammatory responses, due its inhibition of NF-$_k$B dependent inflammatory responses by suppressing enzymes Src and Syk. Due its anti-inflammatory properties, BEA can present a useful therapeutic role in colitis and Crohn's disease [38].

Regarding BEA antimicrobial activity, it has shown strong activity against Gram-positive and Gram-negative pathogenic bacteria. Cell organelles or enzyme systems are the targets in its antimicrobial activity [39]. Meca et al. [40] proved BEA biological activity against several pathogenic bacterias: *Escherichia coli*, *Enterococcus faecium*, *Salmonella enterica*, *Shigella dysen-teriae*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Clostridium perfringens*, *Pseudomonas aeruginosa* and two strains of *Staphylococcus aureus* at quantities from 0.1 to 1000 ng. The results revealed that BEA was effective on all pathogenic bacterias tested except of *S.aureus* strains.

Regarding their antiviral properties, Shin et al. [41] studied ENNs and BEA potential inhibitory *in vitro* against human immunodeficiency virus type-1 (HIV-1) and observed that BEA was the most effective in inhibiting the 3'-processing activity of HIV-1 integrase with an IC_{50} of 1.9±0.4 μM.

ENNB can be useful in the treatment of atherosclerosis and hypercholesterolemia due their enzyme inhibition activity of ACAT.

Furthermore, can be used in combination with chemotherapeutic drugs, because it presents an inhibitor effect of the major multidrug efflux pump $Pdr5p_6$ in *Saccharomyces cerevisiae*. ENNs can also interact with membrane-located ATP-binding cassette (ABC) transporting, so can cause potential influences on bioavailability of xenobiotics and pharmaceuticals [17].

Regarding, antibacterial, antifungal and insecticidal activities, Zaher et al. [42] observed that methanol extract of fungus *F. tricinctum* that contains the ENNs metabolites (ENNA, ENNA1, ENNB, ENNB1, ENNB2 and ENNQ) showed mild antibacterial and antifungal activities against gram-positive bacteria methicillin-resistant *Staphylococcus aureus* and *Mycobacterium intracellulare*, gram-negative bacteria *E coli* and *Pseudomonas aeruginosa* , and fungus *Candida albicans*, *C. glabrata* , *C. krusei*, *Aspergillus fumigatus* and *Cryptococcus neoformans* with IC_{50} values > 10 μg/ml. Also presented antimalarial activity against *Plasmodium falciparum* by the inhibition of PfTrxR enzyme with IC_{50} of 16.96 μg/ml and antileishmanial activity against *Leishmania donovani*. Wang et al. [43] observed anti-tuberculosis properties of ENNA1. ENNA1 showed an antibacterial effect time-concentration-dependent against *M. tuberculosis* assayed at concentration range from 4 to 64 μg/ml and displayed synergy with anti-tuberculosis drugs (rifamycin, amikacin, and ethambutol). The mechanisms of action can besides in the decreasing of membrane potential and intracellular levels of ATP. Clark et al. [44] also observed antimycobacterial activity against *M. tuberculosis* by the presence of ENNB, ENNB1, ENNB4 in addition to lateropyrone in the extract obtained after fermented *F. acuminatum* in potato dextrose.

Sebastià et al. [45] evaluated the antibiotic effect of ENNJ1 and ENNJ3 at quantities from 0.1 to 1000 ng on several pathogenic strains and lactic acid

bacteria, after purified them from the fermentation extract of *Fusarium solani* growth in wheat kamut and observed antimicrobial activity of ENNJ1 and ENNJ3, against *C. perfringens*, *E. faecium*, *E. coli*, *S. dysenteriae*, *S.aureus*, *Y. enterocolitica* and studied lactic acid bacterias, except of *B. adolescentis* that was only inhibited by enniatin J3.

Olleik et al. [46] also observed ENNs and BEA effective activity against gram-positive bacteria (*B. subtilis*, *B. subtilis NR*, *C. perfringens*, *E. faecalis*, *S. aureus*, *S. aureus MRSA*), Mycobacterium, and fungi (*C. albicans*, *F. graminearum*) due these peptides can interacted with bacterial lipids, inducing membrane depolarization and inhibition of macromolecules synthesis. Their structural side chains impact in their interaction with lipids. ENNA was found the most antimicrobial active with minimal inhibitory concentration (MIC) from 3.12 to 12.5 μM for gram positive bacterias, of 6.25 μM for *Mycobacterium* and from 1.5 to < 100 μM for fungus.

During the last years the possible effect of ENNs as anticancer agents has also been suggested. Due to the transport of mono- and divalent cations through the cell membranes can disturbance the physiological homeostasis of cell and lead to apoptotic cells death. Furthermore, present p53-dependent cytostatic and p53-independent cytotoxic activity against several cancer cell types. In lot of studies in various cancer models, after 24 h of treatment at ENNs low concentrations, DNA synthesis stop, cell cycle arrest and apoptotic cell death is induced [47,48].

Moreover, ENNs are few influenced by multidrug resistance transport proteins, leading to therapy resistance and present chemo sensitizing properties

which makes them promising compounds as constituent in preparations for cancer therapy [17].

Dornetshuber-Fleiss et al. [49] observed antiangiogenic properties of ENNB and Sorafenib against cervical cancer *in vitro* and *in vivo* due a strong inhibition of human endothelial cell migration and tube formation. The synergism is accompanied by a marked increasing in mitochondrial injury and apoptosis caused by mitochondrial membrane depolarization, caspase-7-activation, and cleavage of PARP. Furthermore, cells stopped DNA synthesis and accumulate in the phases S and G2/M of the cell cycle. The synergism is based on interference with MAPK signaling and angiogenesis inhibition. *In vivo* studies confirmed that the combination treatment is more effective than single treatments against the KB-3-1 cervix carcinoma xenograft model.

In summary, ENNs are known to be insecticidal, antifungal, antibacterial, and antihelmintic compounds. In the last years, have also been proposed as anticancer agents.

Due to their antibiotic properties ENNs can also be effective in the treatment of upper respiratory tract disease such as sinusitis, rhinitis, pharyngitis, tonsillitis, laryngitis, follicular pharyngitis and tracheitis [16].

In general, BEA has numerous biological effects related to ionophobic properties and presents anticancer, anti-inflammatory and anti-cholesterol activities. Moreover, shows insecticidal activity against many insect species, antibacterial properties including human, animal and plant pathogens, and also antiviral and antifungal activity [36].

As constituent in drug preparations, in traditional Chinese medicine, BEA is employed as constituent in anticonvulsant and antineoplastic drugs. BEA

has also been used to decrease cholesterol levels in blood. Furthermore, it can be used as chemo sensitizing agent, increasing antibiotic effectiveness, due the inhibition of the active efflux of antibiotics by membrane transport proteins [16].

Therefore, these mycotoxins may be potential candidates for be used in anticancer therapy in combination with other drugs because are cytotoxic to cancer cells, have the capacity to inhibit drug efflux pumps, and inhibit the bone resorption. Furthermore, these compounds have demonstrated interesting activity against several insects and microorganisms in different studies [36, 50].

ENNs and BEA in botanicals products

As has been mentioned above, the demand for botanicals is increasing worldwide due to the preference of the population for natural products. These products are available in the corresponding markets in several forms: the raw botanicals, consumed as infusions or as condiments, like essential oils and like food supplements. Few information is available in literature about the presence of emerging mycotoxins (ENNs and BEA) in botanicals, but the interest in these compounds is growing because their high prevalence in several foods and feed. In order to provide information about emerging mycotoxin contamination in botanicals, this review is focused in ENNs and BEA presence in botanicals as ready for human consumption, such as aqueous infusions, tablets, or capsules.

About the presence of emerging mycotoxins of *Fusarium* in botanical raw materials, Hu & Rychlik, [51] studied ENNs and BEA in 60 Chinese medicinal herbs and observed that 25% of analyzed samples were contaminated with one

or more of the ENNs and BEA, with total contents ranging from 2.5 to 751 μg/kg. The mean concentrations of positive samples were 28.9 μg/kg (ENNA), 28.4 μg/kg (ENNA1), 32 μg/kg (ENNB), 3.9 μg/kg (ENNB1) and 33 μg/kg (BEA). Reinholds et al. [52] investigated the presence of 12 mycotoxins in 60 botanicals purchased from Latavia and observed that the 57% of samples were contaminated by emerging mycotoxins (ENNs and BEA). More than one ENNs were found in 13 samples with total contamination levels from 0.35 to 28.4 μg/kg. BEA was detected at concentrations from 4.50 to 5.25 μg/kg. Pallarés et al. [53] analyzed the presence of AFs, ZEA, ENNs and BEA in 224 samples of medicinal plants raw materials and observed for ENNs and BEA incidences between 1 and 15% with mean concentrations ranging from <LOQ (BEA) to 42.43 μg/kg (ENNB), being ENNB the most reported emerging mycotoxins.

In botanical infusions, Pallarés et al. [53] after preparing the resulting beverages from 224 medicinal plants samples (belonging to 56 different species of herbs), observed that ENNB was the only emerging mycotoxin detected at levels > LOQ (with mean concentration of 0.005 μg/L). Also, Pallarés et al. [54] analyzed the multimycotoxin (AFs, 3aDON, 15aDON, NIV, HT-2, T-2, ZEA, OTA, ENNs, and BEA) presence in 44 samples of tea beverages (belonging to black, red, green and green mint tea) and observed that regarding ENNs and BEA, only two samples of green tea resulted positive for ENNB at level <LOQ. Pallarés et al. [55] studied the presence of the 16 mycotoxins mentioned above in 52 samples of botanical beverages belonging to chamomile, chamomile with honey, chamomile with anise, linden, pennyroyal with mint, thyme, valerian and

horsetail. For emerging mycotoxins, only two samples of horsetail showed positive, but at levels <LOQ.

In botanical dietary supplements, Veprikova et al. [56] studied the presence of 57 mycotoxins in 69 samples of botanical dietary supplements employed to improve liver function (32) (based on milk thistle), reduce the menopause effects (9) (red clover, flax seed, and soya) and support health in general (28) (green barley, nettle, goji berries, yucca, etc.). The mainly detected mycotoxins were *Fusarium (*trichothecenes, zearalenone, enniatins) and *Alternaria* mycotoxins. In milk thistle-based supplements ENNs were one of the most detected mycotoxins with incidences of (84-91%) and maximum concentrations ranging from 2340 to 10940 μg/kg. BEA was detected with maximum concentration of 2730 μg/kg. In supplements for reduce menopausal effects, ENNs were also one of the most frequently mycotoxins found with incidences from 67 to 78% and maximum concentrations between 89 and 1230 μg/kg. BEA was detected with maximum concentration of 131 μg/kg. In supplements for general health improvement BEA was detected with higher maximum concentration (215 μg/kg) than ENNs (13-136 μg/kg). Narváez et al. [57] studied the presence of 16 mycotoxins in 10 samples of Cannabidiol botanical supplements made of *Cannabis sativa* L. The results obtained by these authors revealed ENNs presence. One sample was contaminated by ENNB1, ENNA and ENNA1 at levels of 11.6, 4.2 and 5.8 ng/g, respectively. ENNB1 was detected in two other samples at levels below the LOQ (1.56 ng/g). Contrary to these results Di Mavungu et al. [58] not detected BEA in 62 samples of botanical supplements made of soy, St John's wort, garlic, Ginkgo biloba and black radish.

Risk assessment is an important scientific tool that contributes to risk analysis in the area of food safety. Perform the risk assessment is not possible to emerging mycotoxins, due no TDI value has been set yet. However, some authors have performed an approximate estimation of the risk assessment comparing the EDIs obtained for emerging mycotoxins with the TDIs established for other *Fusarium mycotoxins*.

In teas beverages, Pallarés et al. [54] obtained an EDI of 0.038 ng/kg bw/day for ENNB, that reached less than 0.05% of the TDI established for other *Fusarium* mycotoxins, like DON (1 μg/ kg bw/ day) or the sum of T-2 and HT-2 toxins (0.1 μg/ kg bw/ day). In other study, considering the consumption on botanical beverages of the Spanish population, Pallarés et al. [55] obtained an EDI for ENNB that represented less than 0.05% of the TDI established for the other *Fusarium* mycotoxins. In medicinal plants beverages, Pallarés et al. [53] calculated and EDI for ENNs (ENNB+ENNB1) that reached less than 0.1% of the TDI established for other *Fusarium* mycotoxins, however the percentage increase to 1.42% when were considered high consumers of infusions (3 cups/day).

Comparing the percentages of TDI obtained in these matrixes with those obtained in wheat-based products, Stanciu et al. [59] observed percentages of TDI up to 8% for the sum of ENNS, which are higher than those observed in botanicals ready for consumption. In general, not risk was observed for population to emerging mycotoxins through the consumption of botanicals.

Conclusion

Although emerging mycotoxins cause cytotoxic effects inducing oxidative stress, mitochondrial modifications, disruptions on the cell cycle, related to several health adverse effects such as immunotoxicity, genotoxicity, endocrine toxicity, neurotoxicity, several studies suggested their potential use in medicinal and pesticide research. Emerging mycotoxins showed potential use in anticancer therapy in combination with other drugs, as they are cytotoxic to cancer cells, also they inhibit drug efflux pumps. Furthermore, they show insecticidal activity, antibacterial properties against human, animal and plant pathogens, and also antifungal and antiviral activities. Their occurrence in raw materials and botanical tablets is reported by several researchers at low incidences and levels, however sometimes their concentrations reached 1000 μg/kg. The levels of these emerging mycotoxins are at unconcerning in botanicals beverages, due the little tendency of these mycotoxins to migrate from raw materials. The risk assessment approaches revealed that the population is not much exposed to mycotoxins through botanicals consumption. More studies are required to explore their prejudicial effects and their possible applications in medicinal and pesticide research.

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References

1. Sen T, Samanta SK (2015) Medicinal plants, human health and biodiversity: a broad review. Adv Biochem Eng Biotechno. 147:59–110. https://doi.org/10.1007/10_2014_273

2. Lannicelli J, Guariniello J, Pitta Alvarez S, Escandon AS (2018) Traditional uses, conservation status and biotechnological advances for a group of aromatic/medicinal native plants from America. Bol. latinoam. Caribe plantas med. aromát. 17 (5): 453 – 491.

3. Patra JK, Das G, Lee S, Kang SS, Shin HS (2018) Selected commercial plants: A review of extraction and isolation of bioactive compounds and their pharmacological market value. Trends Food Sci Tech. 82: 89-109. https://doi.org/10.1016/j.tifs.2018.10.001.

4. Doughari JH (2012) Phytochemicals: extraction methods, basic structures and mode of action as potential chemotherapeutic agents. In Phytochemicals-A global perspective of their role in nutrition and health. InTechOpen

5. Tungmunnithum D, Thongboonyou A, Pholboon A, Yangsabai, A (2018). Flavonoids and other phenolic compounds from medicinal plants for pharmaceutical and medical aspects: An overview. Medicines 5(3): 93. https://doi.org/10.3390/medicines5030093.

6. Li L, Fu QL, Achal V, Liu YA (2015). Comparison of the potential health risk of aluminum and heavy metals in tea leaves and tea infusion of commercially available green tea in Jiangxi, China. Environ. Monit. Assess.187(5): 228. https://doi.org/10.1007/s10661-015-4445-2.

7. Ashiq S, Hussain M, Ahmad B (2014). Natural occurrence of mycotoxins in medicinal plants: a review. Fungal Genet. Biol. 66: 1-10. https://doi.org/10.1016/j.fgb.2014.02.005.

8. Ünüsan, N (2019). Systematic review of mycotoxins in food and feeds in Turkey. Food Control 97: 1-14. https://doi.org/10.1016/j.foodcont.2018.10.015.

9. Food and Agriculture Organization (FAO) (2020). Mycotoxins. Available: http://www.fao.org/food/food-safety-quality/a-z-index/mycotoxins/es/ (Accessed February 2020).

10. Bhat R, Rai RV, Karim AA (2010). Mycotoxins in food and feed: present status and future concerns. Compr. Rev. Food Sci. Food Saf. 9(1): 57-81. https://doi.org/10.1111/j.1541-4337.2009.00094.x.

11. Moretti A, Pascale M, Logrieco AF (2019). Mycotoxin risks under a climate change scenario in Europe. Trends Food Sci. Technol. 84: 38-40. https://doi.org/10.1016/j.tifs.2018.03.008.

12. Marin S, Ramos AJ, Cano-Sancho G, Sanchis, V (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. Food Chem. Toxicol. 60: 218–237. https://doi.org/10.1016/j.fct.2013.07.047.

13. Agriopoulou S, Stamatelopoulou E, Varzakas T (2020). Advances in Occurrence, Importance, and Mycotoxin Control Strategies: Prevention and Detoxification in Foods. Foods 9(2): 137. https://doi.org/10.3390/foods9020137.

14. EFSA Panel on Contaminants in the Food Chain (CONTAM) (2014). Scientific Opinion on the risks to human and animal health related to the

presence of beauvericin and enniatins in food and feed. EFSA Journal 12(8): 3802.

15. Cimbalo A, Alonso-Garrido M, Font G, Manyes L (2020). Toxicity of mycotoxins in vivo on vertebrate organisms: A review. Food and Chem. Toxicol, 111161. https://doi.org/10.1016/j.fct.2020.111161.

16. Jestoi M (2008). Emerging Fusarium-mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin—A review. Crit Rev Food Sci Nutr. 48(1): 21-49. https://doi.org/10.1080/10408390601062021.

17. Prosperini A, Berrada H, Ruiz MJ, Caloni F, Coccini T, Spicer LJ, Perego MC, Lafranconi A (2017). A review of the mycotoxin enniatin B. Front. Public Health 5: 304. https://doi.org/10.3389/fpubh.2017.00304.

18. Prosperini A, Juan-García A, Font G, Ruiz MJ (2013). Reactive oxygen species involvement in apoptosis and mitochondrial damage in Caco-2 cells induced by enniatins A, A1, B and B1. Toxicol. Lett. 222(1): 36-44. https://doi.org/10.1016/j.toxlet.2013.07.009.

19. Kalayou S, Ndossi D, Frizzell C, Groseth PK, Connolly L, Sørlie M, Verhaegen S, Ropstad E (2015). An investigation of the endocrine disrupting potential of enniatin B using in vitro bioassays. Toxicol. Lett. 233(2): 84-94. https://doi.org/10.1016/j.toxlet.2015.01.014.

20. Huang CH, Wang FT, Chan WH (2019). Enniatin B1 exerts embryotoxic effects on mouse blastocysts and induces oxidative stress and immunotoxicity during embryo development. Environ Toxicol. 34(1): 48-59. https://doi.org/10.1002/tox.22656.

21. Krug I, Behrens M, Esselen M, Humpf HU (2018). Transport of enniatin B and enniatin B1 across the blood-brain barrier and hints for

neurotoxic effects in cerebral cells. PloS one 13(5). https://doi.org/10.1371/journal.pone.0197406.

22. Alonso-Garrido M, Escrivá L, Manyes L, Font G (2018). Enniatin B induces expression changes in the electron transport chain pathway related genes in lymphoblastic T-cell line. Food and Chem. Toxicol. 121: 437-443. https://doi.org/10.1016/j.fct.2018.09.018.

23. Mallebrera B, Prosperini A, Font G, Ruiz MJ (2018). In vitro mechanisms of Beauvericin toxicity: A review. Food Chem. Toxicol. 111: 537-545. https://doi.org/10.1016/j.fct.2017.11.019.

24. Wätjen W, Debbab A, Hohlfeld A, Chovolou Y, Proksch P (2014). The mycotoxin beauvericin induces apoptotic cell death in H4IIE hepatoma cells accompanied by an inhibition of NF-κB-activity and modulation of MAP-kinases. Toxicol. Lett. 231(1): 9-16. https://doi.org/10.1016/j.toxlet.2014.08.021.

25. Prosperini A, Juan-García A, Font G, Ruiz MJ (2013). Beauvericin-induced cytotoxicity via ROS production and mitochondrial damage in Caco-2 cells. Toxicol. Lett. 222(2): 204-211. https://doi.org/10.1016/j.toxlet.2013.07.005.

26. Mallebrera B, Juan-Garcia A, Font G, Ruiz MJ (2016). Mechanisms of beauvericin toxicity and antioxidant cellular defense. Toxicol. Lett. 246: 28-34. https://doi.org/10.1016/j.toxlet.2016.01.013.

27. Juan-García A, Ruiz MJ, Font G, Manyes L (2015). Enniatin A1, enniatin B1 and beauvericin on HepG2: Evaluation of toxic effects. Food Chem. Toxicol. 84: 188-196. https://doi.org/10.1016/j.fct.2015.08.030.

28. Juan-García A, Tolosa J, Juan C, Ruiz MJ (2019). Cytotoxicity, Genotoxicity and Disturbance of Cell Cycle in HepG2 Cells Exposed to OTA and BEA: Single and Combined Actions. Toxins 11(6): 341. https://doi.org/10.3390/toxins11060341.

29. Fraeyman S, Meyer E, Devreese M, Antonissen G, Demeyere K, Haesebrouck F, Croubels S (2018). Comparative in vitro cytotoxicity of the emerging Fusarium mycotoxins beauvericin and enniatins to porcine intestinal epithelial cells. Food Chem. Toxicol. 121: 566-572. https://doi.org/10.1016/j.fct.2018.09.053.

30. Manyes L, Escrivá L, Ruiz MJ, Juan-García A (2018). Beauvericin and enniatin B effects on a human lymphoblastoid Jurkat T-cell model. Food Chem. Toxicol. 115: 127-135. https://doi.org/10.1016/j.fct.2018.03.008.

31. García-Herranz V, Valdehita A, Navas JM, Fernández-Cruz ML (2019). Cytotoxicity against fish and mammalian cell lines and endocrine activity of the mycotoxins beauvericin, deoxynivalenol and ochratoxin-A. Food Chem. Toxicol. 127: 288-297. https://doi.org/10.1016/j.fct.2019.01.036.

32. Çelik M, Aksoy H, Yılmaz S (2010). Evaluation of beauvericin genotoxicity with the chromosomal aberrations, sister-chromatid exchanges and micronucleus assays. Ecotox Environ Safe 73(7): 1553-1557. https://doi.org/10.1016/j.ecoenv.2010.07.036.

33. Escrivá L, Jennen D, Caiment F, Manyes L. (2018). Transcriptomic study of the toxic mechanism triggered by beauvericin in Jurkat cells. Toxicol. Lett. 284: 213-221. https://doi.org/10.1016/j.toxlet.2017.11.035.

34. Escrivá L, Alonso-Garrido M, Font G, Manyes L (2019). Transcriptional study after Beauvericin and Enniatin B combined exposure in Jurkat T cells. Food Chem. Toxicol. 130: 122-129. https://doi.org/10.1016/j.fct.2019.05.018.

35. Schoevers EJ, Santos RR, Fink-Gremmels J, Roelen BA (2016). Toxicity of beauvericin on porcine oocyte maturation and preimplantation embryo development. Reprod Toxicol. 65: 159-169. https://doi.org/10.1016/j.reprotox.2016.07.017.

36. Caloni F, Fossati P, Anadón A, Bertero A (2020). Beauvericin: The beauty and the beast. Environ. Toxicol. Pharmacol 75: 103349. https://doi.org/10.1016/j.etap.2020.103349.

37. Heilos D, Rodríguez-Carrasco Y, Englinger B, Timelthaler G, Van Schoonhoven S, Sulyok M, Boecker S, Süssmuth RD, Heffeter P, Lemmens-Gruber R, Dornetshuber-Fleiss R, Berger W (2017). The natural fungal metabolite beauvericin exerts anticancer activity in vivo: a pre-clinical pilot study. Toxins 9(9): 258. https://doi.org/10.3390/toxins9090258.

38. Wu XF, Xu R, Ouyang ZJ, Qian C, Shen Y, Wu XD, Gu YH, Xu Q, Sun Y (2013). Beauvericin ameliorates experimental colitis by inhibiting activated T cells via downregulation of the PI3K/Akt signaling pathway. PloS one 8(12). https://dx.doi.org/10.1371%2Fjournal.pone.0083013.

39. Wu Q, Patocka J, Nepovimova E, Kuca K (2018). A review on the synthesis and bioactivity aspects of beauvericin, a fusarium mycotoxin. Front. Pharmacol. 9: 1338. https://doi.org/10.3389/fphar.2018.01338.

40. Meca G, Sospedra I, Soriano JM, Ritieni A, Moretti A, Manes J (2010). Antibacterial effect of the bioactive compound beauvericin produced by

Fusarium proliferatum on solid medium of wheat. Toxicon 56(3): 349-354. https://doi.org/10.1016/j.toxicon.2010.03.022.

41. Shin CG, An DG, Song HH, Lee C (2009). Beauvericin and enniatins H, I and MK1688 are new potent inhibitors of human immunodeficiency virus type-1 integrase. J. Antibiot. 62: 687–690. https://doi.org/10.1038/ja.2009.102.

42. Zaher AM, Makboul MA, Moharram AM, Tekwani BL, Calderón AI (2015). A new enniatin antibiotic from the endophyte Fusarium tricinctum Corda. J Antibiot. 68(3): 197-200. https://doi.org/10.1038/ja.2014.129.

43. Wang G, Dong W, Lu H, Lu W, Feng J, Wang X, Chen H, Liu M, Tan C (2019). Enniatin A1, A Natural Compound with Bactericidal Activity against Mycobacterium tuberculosis In Vitro. Molecules 25(1): 38. https://doi.org/10.3390/molecules25010038.

44. Clark TN, Carroll M, Ellsworth K, Guerrette R, Robichaud GA, Johnson JA, Gray CA (2018). Antibiotic mycotoxins from an endophytic Fusarium acuminatum isolated from the medicinal plant Geum macrophyllum. Nat. Prod. Commun. 13(10): 1934578X1801301017. https://doi.org/10.1177%2F1934578X1801301017.

45. Sebastià N, Meca G, Soriano JM, Mañes J (2011). Antibacterial effects of enniatins J1 and J3 on pathogenic and lactic acid bacteria. Food Chem. Toxicol. 49(10): 2710-2717. https://doi.org/10.1016/j.fct.2011.06.070.

46. Olleik H, Nicoletti C, Lafond M, Courvoisier-Dezord E, Xue P, Hijazi A, Baydoun E, Perrier J, Maresca M (2019). Comparative Structure–Activity Analysis of the Antimicrobial Activity, Cytotoxicity, and Mechanism of Action of the Fungal Cyclohexadepsipeptides Enniatins and Beauvericin. Toxins 11(9): 514.

47. Dornetshuber R, Heffeter P, Kamyar MR, Peterbauer T, Berger W, Lemmens-Gruber R (2007). Enniatin exerts p53-dependent cytostatic and p53-independent cytotoxic activities against human cancer cells. Chem. Res. Toxicol 20(3): 465-473. https://doi.org/10.1021/tx600259t.

48. Wätjen W, Debbab A, Hohlfeld A, Chovolou Y, Kampkötter A, Edrada RA, Ebel R, Hakiki A, Mosaddak M, Totzke F, Kubbutat MHG, Proksch P (2009). Enniatins A1, B and B1 from an endophytic strain of Fusarium tricinctum induce apoptotic cell death in H4IIE hepatoma cells accompanied by inhibition of ERK phosphorylation. Mol. Nutr. Food Res. 53(4): 431-440. https://doi.org/10.1002/mnfr.200700428.

49. Dornetshuber-Fleiss R, Heilos D, Mohr T, Richter L, Süssmuth RD, Zlesak M, Novicky A, Heffeter P, Lemmens-Gruber R, Berger W (2015). The naturally born fusariotoxin enniatin B and sorafenib exert synergistic activity against cervical cancer in vitro and in vivo. Biochem. Pharmacol. 93(3): 318-331. https://doi.org/10.1016/j.bcp.2014.12.013.

50. Tedjiotsop Feudjio F, Dornetshuber R, Lemmens M, Hoffmann O, Lemmens-Gruber R, Berger W (2010). Beauvericin and enniatin: emerging toxins and/or remedies? World Mycotoxin J. 3(4): 415-430. https://doi.org/10.3920/WMJ2010.1245.

51. Hu L, Rychlik, M (2014). Occurrence of enniatins and beauvericin in 60 Chinese medicinal herbs. Food Addit. Contam. A 31(7): 1240-1245. https://doi.org/10.1080/19440049.2014.913813.

52. Reinholds I, Bogdanova E, Pugajeva I, Bartkevics V (2019). Mycotoxins in herbal teas marketed in Latvia and dietary exposure assessment. Food Addit. Contam. B 12(3): 199-208. https://doi.org/10.1080/19393210.2019.1597927.

53. Pallarés N, Berrada H, Fernández-Franzón M, Ferrer E (2020). Risk Assessment and Mitigation of the Mycotoxin Content in Medicinal Plants by the Infusion Process. Plant Foods Hum. Nutr. https://doi.org/10.1007/s11130-020-00820-4.

54. Pallarés N, Font G, Mañes J, Ferrer E (2017). Multimycotoxin LC–MS/MS analysis in tea beverages after dispersive liquid–liquid Microextraction (DLLME). J. Agr. Food Chem. 65(47): 10282-10289. https://doi.org/10.1021/acs.jafc.7b03507.

55. Pallarés N, Tolosa J, Mañes J, Ferrer E (2019). Occurrence of Mycotoxins in Botanical Dietary Supplement Infusion Beverages. J. Nat. Prod. 82(2): 403-406. https://doi.org/10.1021/acs.jnatprod.8b00283.

56. Veprikova Z, Zachariasova M, Dzuman Z, Zachariasova A, Fenclova M, Slavikova P, Vaclavikova M, Mastovska K, Hengst D, Hajslova J (2015). Mycotoxins in plant-based dietary supplements: hidden health risk for consumers. J. Agr. Food Chem. 63(29): 6633-6643. https://doi.org/10.1021/acs.jafc.5b02105.

57. Narváez A, Rodríguez-Carrasco Y, Castaldo L, Izzo L, Ritieni A (2020). Ultra-High-Performance Liquid Chromatography Coupled with Quadrupole Orbitrap High-Resolution Mass Spectrometry for Multi-Residue Analysis of Mycotoxins and Pesticides in Botanical Nutraceuticals. Toxins 12(2): 114. https://doi.org/10.3390/toxins12020114.

58. Di Mavungu JD, Monbaliu S, Scippo ML, Maghuin-Rogister G, Schneider YJ, Larondelle Y, Callebaut A, Robbens J, Peteghem CV, De Saeger S (2009). LC-MS/MS multi-analyte method for mycotoxin determination in

food supplements. Food Addit. Contam. 26(6): 885-895. https://doi.org/10.1080/02652030902774649.

59. Stanciu O, Juan C, Miere D, Loghin F, Mañes J (2017). Presence of enniatins and beauvericin in Romanian wheat samples: From raw material to products for direct human consumption. Toxins 9(6): 189. https://doi.org/10.3390/toxins9060189.

3.2. The potential of HPP for minimizing pesticides and toxins in food products

Chapter 7 in Book: Present and Future of High Pressure Processing (under review)

The potential of HPP for minimizing pesticides and toxins in food products

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Abstract

High-pressure (HP) treatment has emerged as a novel, additive-free food preservation technology. It has been scientifically and commercially proven that HP can produce microbially safe and stable products with improved quality characteristics such as enhanced flavor and color. Recent studies have focused on the effects of HP on health attributes and allergenic potential of foodstuff to develop the next generation of convenience foods. The contamination of produced food in various conditions along the production chain always is the point of concern. In this context, food safety is among the main health concerns worldwide on the occurrence of food contaminants such as heavy metals, histamine, mycotoxin, polycyclic aromatics, pesticides, and allergens. Moreover, the prevalence of some fungal species in processed food products can be addressed as a warning signal of mycotoxin contamination, which can result in lower product quality and failure to meet the required criteria for consumption by humans and animals. The detoxification of mycotoxin can be carried out through different methods; however, based on the one approved category, they could divide to physical, chemical, and biological methods. However, the application of emerging technologies such as HP in detoxification of toxins such as mycotoxins reserves notable attention. In the regard, this chapter provides an overview of the current knowledge, challenges, and new perspectives regarding the application of HP treatment to minimize pesticides and toxins in food products.

Keywords: High-pressure processing, Pesticides, Mycotoxins, Mycotoxigenic fungi.

1. General Introduction

Food safety is among the main health concern worldwide according to the published reports on the occurrence of food contaminants such as heavy metals (Fathabad et al., 2018), histamine (Rahmani et al., 2018), mycotoxin (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018; Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Mousavi Khaneghah, Fakhri, Raeisi, Armoon, & Sant'Ana, 2018; Mousavi Khaneghah, Fakhri, & Sant'Ana, 2018), polycy- clic aromatic hydrocarbons (PAHs) (Yousefi et al., 2018), and pesticides (Amirahmadi et al., 2017; Razzaghi et al., 2018; Yadolahi, Babri, Sharif, & Khaneghah, 2012) among others.

Among them, mycotoxins are toxic secondary metabolites produced by fungi mainly be- longing to genera *Aspergillus*, *Penicillium*, and *Fusarium* (Khaneghah, Chaves, & Akbarirad, 2017; Mousavi Khaneghah, Ismail, Raeisi, & Fakhri, 2018). Mycotoxigenic fungi under physiological conditions (like temperature or humidity), grow and synthesize mycotoxins (Khaneghah et al., 2018; Mousavi Khaneghah, Fakhri, Raeisi, et al., 2018; Mousavi Khaneghah, Fakhri, & Sant'Ana, 2018).

Mycotoxin production can occur during fungi colonization or infection if fungi are ex- posed to host metabolites. Moreover, reactive species can trigger response pathways of myco- toxins production. Species of *Aspergillus* genera produce aflatoxins (AFs); genera *Aspergillus* and *Penicillium* are responsible for ochratoxin A (OTA) production. *Fusarium* species produce trichothecenes [HT-2, T-2, deoxynivalenol (DON), and nivalenol (NIV)], zearalenone (ZEA), fumonisins FB1 and FB2, and emerging mycotoxins [fusaproliferin (FUS),

moniliformin (MON), beauvericin (BEA), and enniatins (ENNS)] (Amirahmadi, Shoeibi, Rastegar, Elmi, & Mousavi Khaneghah, 2018; Heshmati, Zohrevand, Khaneghah, Mozaffari Nejad, & Sant'Ana, 2017; Majeed, Khaneghah, Kadmi, Khan, & Shariati, 2018).

The chemical structures of these compounds are diverse; microcyclic lactones like ZEA, small lactones condensed with hetero- or alicycles like patuline (PAT), furan derivatives like AFs, and alicyclic compounds like T-2 among others. Mycotoxins can be present in food commodities in three possible forms: (i) free or unmodified, (ii) associated to matrix or as (iii) modified forms (Marín, Cano-Sancho, Sanchis, & Ramos, 2018; Oteiza et al., 2017; Rastegar et al., 2017).

The most commonly contaminated crops or substrates consist of cereals, nuts, oilseeds, dried fruits, coffee, spices, and their by-products. The contamination by mycotoxins occurs throughout the food chain, i.e., during the field and/or in the postharvest stage (Fakhri et al., 2019; Nabizadeh et al., 2018). Significant economic losses are associated with the impact of mycotoxins on human health, animal productivity, and domestic and international trade. AFs, OTA, ZEA, trichothecenes (TCs), FBs, and PAT are some of the mycotoxins with higher agroeconomic impact (FAO, 2018; Ünüsan, 2019).

Mycotoxins are responsible for different health adverse effects, such as cancer induction, mutagenicity and estrogenic, gastrointestinal, urogenital, vascular, kidney, and nervous disorders. AFs produce genotoxicity, teratogenicity, and immunosuppressive activity. Moreover, they are among the most powerful mutagens and carcinogens known, furthermore the International Agency for Research on Cancer (IARC) has classified them as human

carcinogens (group 1) (Campagnollo et al., 2016; Mahmood Fashandi, Abbasi, & Mousavi Khaneghah, 2018).

Toxic effects of OTA consist of nephrotoxic, teratogenic, genotoxic, neurotoxic, hepatotoxic, and immunosuppressive activities. FBs are associated with human esophageal cancer, hepatotoxicity, nephrotoxicity, and immunosuppressive activity. ZEA is related to reproductive tract alterations that may result in severe reproductive disorders, infertility, and changes in serum progesterone levels. TCs inhibit protein synthesis and mitochondrial function *in vitro* and *in vivo* and also exhibit cytotoxic and immunosuppressive effects. Regarding emerging mycotoxins, FUS is toxic for human B lymphocytes, MON inhibits several enzymes and ENNs and BEA are cytotoxic with the capacity to disrupt intracellular ionic homeostasis (Marin et al., 2013). The structure of some of the mycotoxins is shown in Fig. 1. Although most of the operation units are not designed to reduce the concentration of mycotoxins in food products (Campagnollo et al., 2016; Kaushik, 2015; Milani & Maleki, 2014), the chemical structure of mycotoxins, process conditions; temperature as well as the duration of the process, present or absent of humidity are among the several factors which influence the mycotoxin degradation during processing steps of food products (Karlovsky et al., 2016; Lancova et al., 2008).

In this regard, food processing can have an impact on mycotoxins levels, but the details remain unclear. Most publications are focused on the effect of processing techniques like cleaning and milling of grains, microbiological fermentation, or thermal processes such as cooking, boiling, and extrusion among other (Cano-Sancho, Sanchis, Ramos, & Marín, 2013; Khaneghah et al.,

2018; Mousavi Khaneghah, Fakhri, Raeisi, et al., 2018; Mousavi Khaneghah, Fakhri, & Sant'Ana, 2018).

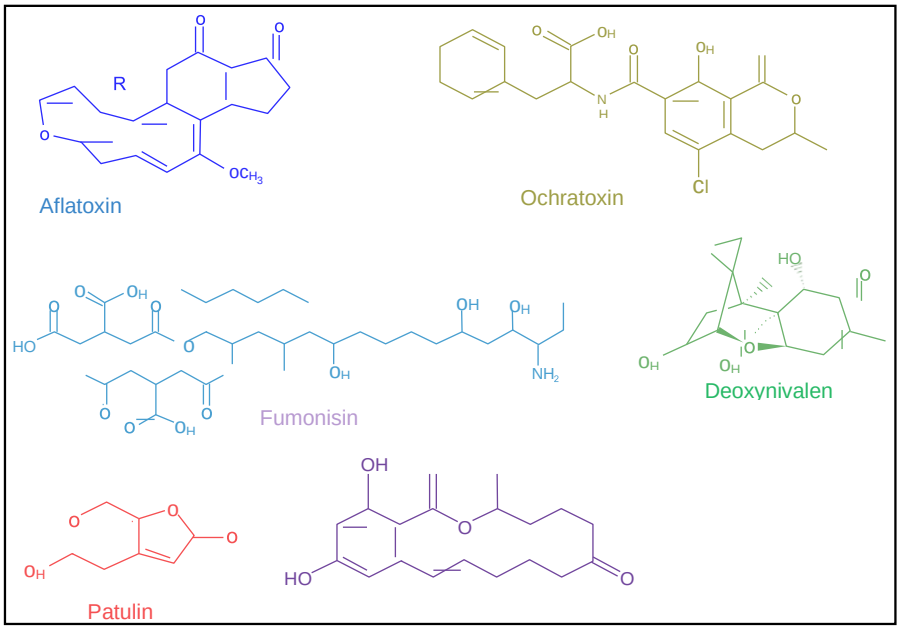

FIG. 1 Chemical structures of the most commonly identified mycotoxins.

Less information is available about the effect of emerging technologies in food processing [high hydrostatic pressure (HHP or HPP), pulsed electric fields (PEFs), ultrasound (US), and cold plasma] on mycotoxins levels (Gavahian & Mousavi Khaneghah, 2019).

Over the last years, HPP has emerged as a promising decontamination technique in both the agriculture and food industry. HPP is highly acceptable for inhibiting the growth and toxins of foodborne pathogens in food, without

causing an impact on nutrients, flavor, taste, and freshness of food (Kalagatur et al., 2018).

The efficacy of high-pressure (HP) treatment is governed by Le Chatelier's principle, which implies that reactions or phase transitions associated with a decrease in the volume are favored, while those accompanied with a volume increase are inhibited. Low molecular weight molecules like aroma compounds, vitamins, and minerals are rarely affected as such by HP because of the very low compressibility of covalent bonds. On the other hand, macromolecules, such as proteins and starch, can change their native structure during HP similar to thermal treatments (Gross & Jaenicke, 1994; Heinz & Buckow, 2010).

High-pressure process (HPP) or HP treatment is characterized by three processing parameters: temperature T, pressure P, and exposure time t. The three processing parameter allows for great variability in the design of the process (Barba, Ahrné, Xanthakis, Landerslev, & Orlien, 2018). Generally, the pressure is transmitted instantaneously and uniformly throughout the food system independent of the size and geometry of the food product, unlike heat processing, where heat is gradually transferred through the food system. The momentary pressure transmission can reduce processing time, processing energy, and the risk of overprocessing of some parts of voluminous products. Previously conducted studies revealed some of the benefits and drawbacks of HPP as an emerging food processing technique (Table 1) (Barba, Esteve, & Frígola, 2012; Barba, Koubaa, do Prado-Silva, Orlien, & Sant'Ana, 2017; Barba, SantAna, Orlien, & Koubaa, 2018).

TABLE 1 General benefits and drawbacks of using high-pressure processing (HPP) in the food industry.

Benefits	Drawbacks
• Rapid and uniform distribution of hydrostatic pressure all over the product • Reduced thermal exposure • Rapid heating and subsequent cooling upon depressurization • Appropriate for foods with high moisture content • Appropriate for both pumpable and liquid products • The processing time is independent of product size and shape • HPP accelerates inactivation of microorganisms at some pressure-thermal boundary conditions • The possibility of food detoxification and pesticide removal	• Usually limited to batch or semicontinuous units • Preheating step is needed for pressure-assisted thermal processing • Inappropriate for products with dissimilar compressibility components (e.g., marshmallows) • Variable efficiency in inactivation of food enzymes • The inability of pressure alone, i.e., only pressure, in inactivation of the spores of bacteria • Higher capital costs compared to conventional techniques

TABLE 2 Effect of high-pressure processing (HPP) on pesticide removal from food products.

Matrix	Compound	HPP treatment	Main findings	References
Brussels sprouts	Chlorpyrifos	(0.1–400 MPa/5 or 25°C/30 min)	≈ 89% removal under 200 MPa/5°C/30 min + ethanol (10% *v*/v)	Iizuka and Shimizu (2014a)
Cherry tomatoes	Chlorpyrifos	(0.1–400 MPa/5 or 25°C/30 min)	≈ 75% removal under 75 MPa/5°C/30 min	Iizuka, Maeda, and Shimizu (2013)
		(0.1–400 MPa/5 or 25°C/30 min) various HPP time periods (0, 0.5, 1, 6, and 12 h)	Increased HPP time decreased the levels of chlorpyrifos. Ethanol helped removing pesticides	Iizuka and Shimizu (2014b)

TABLE 3 Effect of high-pressure processing (HPP) on toxin formation in food products.

Matrix	Targeted compound/ fungi	HPP treatment	Main findings	References
Apple juice	*Neosartorya fischeri* ascospores	HPP 600 MPa + ultrasound processing (24 kHz, 0.33 W/ mL) + 75°C	HPP at 75°C resulted in 3.3 log reduction after 10 min, vs no inactivation after applying either US treatment at 75°C or thermal treatment (75°C) alone	Evelyn, Kim, and Silva (2016)
Apple, celery, cucumber, kale, lemon mixture juice parsley, Romaine, and spinach	Patulin	(400–600 MPa, 0–300 s, 11°C)	Up to 60 μg/L decrease after 600 MPa for 300 s	Hao, Zhou, Koutchma, Wu, and Warriner (2016)
maize	*Fusarium graminarum* Deoxynivalenol and Zearalenone	(380 MPa/60°C/30 min) and (500 MPa/45°C/20 min)	Spore germination inactivation of *F. graminarum* after HPP (380 MPa/60°C/30 min) in peptone water. Complete reduction in CFU was observed after applying HPP (500 MPa/45°C/20 min) Complete reduction of DON and ZEA in maize after HPP treatment (550 MPa/45°C/20 min)	Kalagatur et al. (2018)
Strawberry puree	*Byssochlamys nivea* ascospores	HPP 600 MPa + ultrasound processing (24 kHz, 0.33 W/ mL) + 75°C	HPP at 75°C resulted in 1.4 log reduction after 10 min, vs no inactivation after applying either US treatment at 75°C or thermal treatment (75°C) alone	Evelyn and Silva (2015)
Table olives	*Penicillium* spp. Citrinin	(250 MPa/5 min/35°C)	≈ 90% reduction of mold flora. Citrinin reduction from 64% to 100%	Tokuşoğlu, Alpas, and Bozoğlu (2010)

It is frequently claimed that HP can be used to produce healthy and fresh-like foods due to its minimal effects on nutritional and aroma compounds (Rastogi, Raghavarao, Balasubramaniam, Niranjan, & Knorr, 2007). For the last two decades, HP treatment has been mainly applied to increase food safety and/or extend the shelf life of refrigerated foods of high value (Barba et al., 2012; Barba, Criado, Belda-Galbis, Esteve, & Rodrigo, 2014; Barba, SantAna, et al., 2018).

The present chapter is intended to highlight several opportunities for HP treatment application to minimize pesticides and toxins in food products. Some examples are listed in Tables 2 and 3.

2. The effectiveness of HPP in pesticide removal

The potential of HP treatment as a method for washing food products, thus reducing pesticide levels has been studied by different authors (Table 2). In this line, the effects of HP (0.1–400 MPa/5°C or 25°C/30 min) on the reduction of pesticide (chlorpyrifos, CP) levels of cherry tomatoes were evaluated (Iizuka, Maeda, & Shimizu, 2013). It was found that under the optimum conditions (75 MPa/5°C/30 min), approximately 75% of pesticides were removed. More recently, it was observed that a significant reduction of chlorpyrifos concentration when HP treatment (0.1–400 MPa/5 or 25°C/30 min) was applied to cherry tomatoes (Iizuka & Shimizu, 2014b). Moreover, these authors also evaluated the effects of HP treatment at various time periods (0, 0.5, 1, 6, and 12 h), thus observing that increased time decreased the levels of chlorpyrifos. In addition, they also found a significant improvement in removal efficiency of chlorpyrifos when ethanol was used instead of water for all pressure

levels. Similar to these findings, the same authors also found a significant reduction of chlorpyrifos levels when they evaluated the effects of HP treatment (0.1–400 MPa/5 or 25°C/30 min) to reduce chlorpyrifos content from Brussels sprouts. They found the best results (89% removal rate) when HP treatment at the optimum conditions (200 MPa/5°C/30 min) was combined with ethanol (10% v/v) (Iizuka & Shimizu, 2014a) (Fig. 2).

2.1. Mechanisms involved in pesticide removal by HP

The mechanisms involved in pesticide removal from food material through HP may vary depending on many parameters including the type of pesticide, the type of food material, and HP conditions. However, previously conducted research hypothesized various pathways for removal of some pesticides from food commodities. According to a study conducted by Iizuka, Maeda, et al. (2013) on chlorpyrifos removal from cherry tomato through HPP, it was revealed that HPP did not generate any toxic pesticide intermediates in the samples. While it was traditionally believed that pesticides can be remained in the HPP-treated food sample without changing into other materials, these authors showed that HPP broke the tomatoes surface and facilitated the penetration of chlorpyrifos into the product which was concurrent with a decrease in the apparent removal rate of the pesticide. This was confirmed when the authors assessed the pesticide residue in the cuticle layer of HPP-treated tomatoes. Therefore, one of the potential mechanisms involved in the reduction of the pesticide concentration by HPP is the movement of the pesticide compounds from the outer layer of the product into the food sample. This

hypothesis can be considered as a reason against the applicability of HPP for pesticide removal from food products.

Iizuka, Yahata, and Shimizu (2013) also explained another potential mechanism involved in decreasing the pesticide concentration after HPP. According to the authors, pressure can affect the solubility of pesticides. Therefore, the reduced removal rate is reattachment under decompression from high pressure or lower solubility under high pressure. In this case, the chemical structure of the pesticide and the solution can affect the effectiveness of pesticide removal by HPP. For example, Iizuka, Yahata, et al. (2013) evaluated the effects of HPP on a hydrophobic pesticide, i.e., chlorpyrifos, from tomatoes using a relatively hydrophobic solution, i.e., 70% ethanol solution compared to the hydrophilicity of water to block the possible reattachment phenomenon. They observed that almost all the pesticide remains in the cuticle layer of the tomatoes samples after HPP. The authors suggested that it takes a long time to dissolve pesticides in water under high pressure. Particularly, pressurization duration can be considered a crucial parameter in this mechanism as a high removal rate was observed when an extended period of 400 MPa HPP, i.e., 24 h, was applied without ethanol solution. Therefore, HPP optimization in terms of process time, pressure value, and the type of solution can affect the effectiveness of HPP in pesticide removal from food materials. It should be noted that only limited number of research has been conducted in this area of the science and more comprehensive studies can reveal the details of the mechanism(s) that can be involved in the removal of various types of pesticides from food material by HPP.

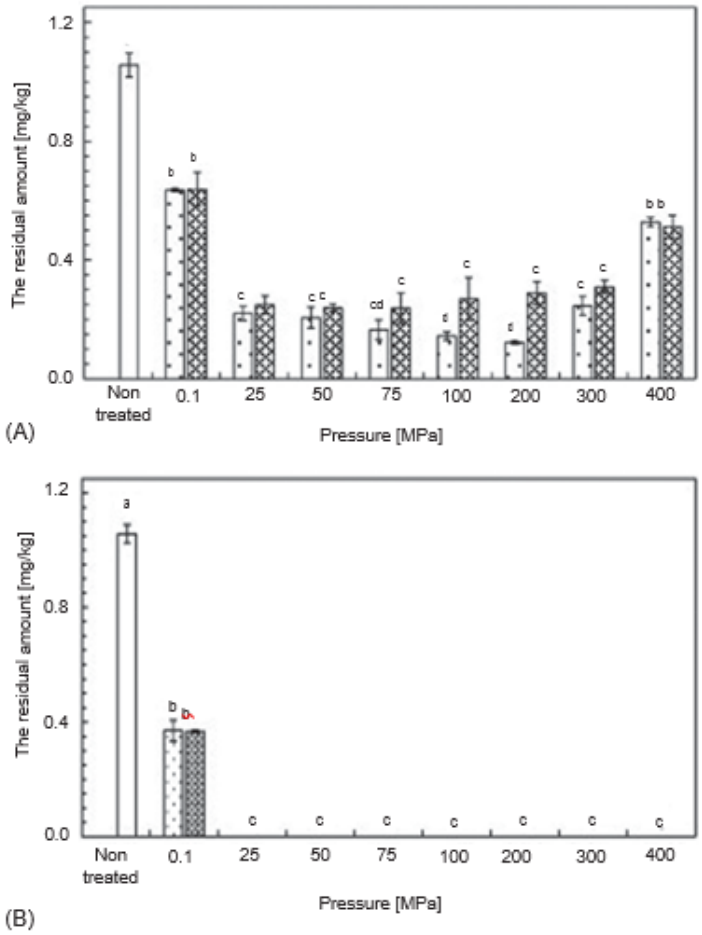

FIG. 2 Residual amounts (mg/kg) of chlorpyrifos after HP-assisted [0.1–400 MPa, 5°C ( ) and 25°C ( ) for 30 min] removal (A) with water and (B) with ethanol. *From Iizuka, T., & Shimizu, A. (2014). Removal of pesticide residue from Brussels sprouts by hydrostatic pressure.* Innovative Food Science & Emerging Technologies, 22*, 70–75, with permission.*

3. The effectiveness of HPP in reducing mycotoxin

Some studies have evaluated the effect of HPP in the inactivation of spores and growth of mycotoxigenic fungi (Table 3). For instance, Evelyn and Silva (2015) investigated HPP (600 MPa) and ultrasound processing (US) (24 kHz, 0.33 W/mL) in combination with temperature (75°C) for the inactivation of 4 weeks old *Byssochlamys nivea* (B. nivea) ascospores in strawberry puree for more than 30 min and compared these processes with thermal treatment alone at 75°C. *B. nivea* is a heat-resistant mold that can produce mycotoxins. HPP at 75°C resulted in 1.4 log reduction after 10 min, while no inactivation was observed after applying either US treatment at 75°C or thermal treatment (75°C) alone. These authors also observed faster inactivation at higher temperatures for all processes tested. Moreover, lower temperatures in combination with HPP were more effective in spore inactivation than heat treatment alone at higher temperatures, because temperatures above 90°C were required for an efficient inactivation when thermal treatment was applied alone.

In another study, Evelyn et al. (2016) studied the effect of HPP at 600 MPa and US processing (24 kHz, 0.33 W/mL) at 75°C on 4-week-old *Neosartorya fischeri* (N. fischeri) ascospores inactivation in apple juices. *N. fischeri* is a heat-resistant mold that can produce mycotoxins. These treatments combined with thermal treatment (75°C) were compared with the thermal process (75°C) alone. The authors observed that HPP at 75°C was also the most effective technique, resulting in 3.3 log reductions after 10 min while no inactivation was found after applying the US and thermal process at 75°C. The effect of different temperatures on mold inactivation was also investigated, evidencing faster

inactivation on ascospores at higher temperatures for all the technologies proved.

More recently, Evelyn and Silva (2017) investigated the potential of HPP (600 MPa) and US (24 kHz, 0.33 W/mL) at 75°C for the inactivation of 4–12 weeks old spores of *B. nivea* in strawberry puree and *N. fischeri* in apple juice. These authors observed a reduction of 2.7 log for 4 weeks and 2 log for 12 weeks spores using HPP treatment in *B. nivea*, and 2 log difference between the inactivation of 8- and 12-week-old *N. fischeri* spores. The results showed that the resistance of mold spores differed according to the species evaluated as well as age, increasing the spore resistance with the age. Furthermore, HPP treatment was more effective than US treatment similar to that was observed in previous studies.

Kalagatur et al. (2018) studied a predictive model for the reduction of *F. graminarum* as a function of variables pressure, temperature, and pressure holding. Then the experiment was carried out and responses were verified with the predicted values. HPP being an effective treatment for inhibiting spore germination, spore germination inactivation of *F. graminarum* caused by HPP (380 MPa/60°C/30 min) in peptone water was observed. In maize, a complete reduction in CFU was observed after applying HPP (500 MPa/45°C/20 min) of pressure holding time. Furthermore, propidium iodide staining revealed that the inactivation was produced by damage in cells membranes.

Tokuşoğlu et al. (2010) observed that the dominant mold flora in black table olives (*Penicillium spp.*) was reduced on average of 90% at 25°C of incubation and 100% at 4°C based on Rose- Bengal Chloramphenicol Agar (RBCA) after the application of HPP (250 MPa/5 min/35°C).

Regarding the application of HPP processing technique as a promising tool for mycotoxins decontamination in food, Tokuşoğlu et al. (2010) observed citrinin (CIT) reduction from 64% to 100% in black table olives treated by HPP (250 MPa/5 min/35°C). After spiked blank olives sample with concentrations of 1, 1.25, 2.5, 10, 25, and 100 µg/kg and treated them with HPP, reduction averages of 100%, 98%, 55%, 37%, 9%, and 1.3% were found, respectively. In all, 1 µg/kg of less amount of CIT contamination was inhibited as 100%.

Kalagatur et al. (2018) also observed a complete reduction of DON and ZEA in maize after HPP treatment (550 MPa/45°C/20 min) of pressure holding time. So, HPP was also effective in minimizing DON and ZEA levels in maize grains. The decrease of the producer fungi *F. graminearum* could be the reason for the reduction of mycotoxins.

Hao et al. (2016) studied the degradation of PAT introduced in different juices composition at the concentration of 200 µg/L. Then these juices were subjected to different HPP conditions (400–600 MPa, 0–300 s, 11°C). The highest level of PAT degradation found was a decrease of 60 µg/L in romaine, celery, cucumber, apple, spinach, kale parsley, and lemon mixture juice treated at 600 MPa for 300 s. Furthermore, these authors observed that PAT degradation was dependent on juice constituents, suggesting that the degradation was directly related to the concentration of sulfhydryl groups.

In another study, Avsaroglu, Bozoglu, Alpas, Largeteau, and Demazeau (2015) performed a study about the reduction of PAT in apple juices spiked with 5, 50, and 100 µg/L, using two different pressure procedures, high hydrostatic pressure (HPP) and pulsed-high hydrostatic pressure (p-HPP) treatments. HPP treatments were applied at 300–500 MPa in combination with temperatures (30

°C–50 °C) and holding time of 5 min for each treatment, in p-HPP, two different pulses were applied (2 pulses × 150 s; 6 pulses × 50 s). These authors observed that HPP treatment produced a decrease of PAT ranging from 0% to 51.16%, p-HPP at 6 pulses × 50 s between 0% and 62.11% and p-HPP at 2 pulses × 150 s between 0% and 45.49%. Comparing HPP with p-HPP applications, HPP was effective in high PAT concentrations while p-HPP in low PAT concentrations. In this study also it was suggested that the adduct of PAT with other compounds containing sulfhydryl groups present in apple juice as a possible explication of PAT reduction.

4. Conclusions

The removal of pesticide residues from food material through HPP is an emerging concept in the food industry. While previously conducted research showed a substantial reduction in pesticide concentration of some food commodities after HPP, others showed that the pesticide may transfer from the outer layer of the product into the inner parts, resulting in a misleading data interpretation. In other words, HPP can be ineffective in pesticide degradation. However, researchers showed that using an appropriate solution, in terms of hydrophobicity, and HPP optimization, in terms of pressure and duration can result in pesticide removal through blocking the possible reattachment of pesticide into the food material, considering the effect of high pressure on the solubility of materials. Moreover, it is known that HPP did not produce toxic intermediates from pesticide residues, suggesting that pesticide removal from food material by HPP is mainly through the effect of this process on the solubility of the pesticide which can dissolve the pesticide in either food product

or the surrounding solvent. On the other hand, the use of HPP is a promising tool to reduce mycotoxin in food products, either by reducing the mycotoxigenic molds or decreasing the amount of mycotoxins in the food products. Although, it seems it is necessary to combine HPP with conventional heat treatments or other alternative processing techniques (e.g., ultrasound) to increase the effectiveness of this technology against both mycotoxigenic molds and mycotoxins. Therefore, it can be concluded that an optimized HPP has the potential of reducing pesticide concentration, mycotoxigenic molds, and mycotoxins of the food commodities. Further experiments are required in this area of the research.

References

Amirahmadi, M., Kobarfard, F., Pirali-Hamedani, M., Yazdanpanah, H., Rastegar, H., Shoeibi, S., & Mousavi Khaneghah, A. (2017). Effect of Iranian traditional cooking on fate of pesticides in white rice. Toxin Reviews, 36(3), 177–186.

Amirahmadi, M., Shoeibi, S., Rastegar, H., Elmi, M., & Mousavi Khaneghah, A. (2018). Simultaneous analysis of my- cotoxins in corn flour using LC/MS-MS combined with a modified QuEChERS procedure. Toxin Reviews, 37(3), 187–195.

Avsaroglu, M. D., Bozoglu, F., Alpas, H., Largeteau, A., & Demazeau, G. (2015). Use of pulsed-high hydrostatic pres- sure treatment to decrease patulin in apple juice. High Pressure Research, 35(2), 214–222.

Barba, F. J., Ahrné, L., Xanthakis, E., Landerslev, M. G., & Orlien, V. (2018). Innovative technologies for food preservation: Inactivation of spoilage and pathogenic microorganisms. 25–51. https://doi.org/10.1016/B978-0-12-811031-7.00002-9.

Barba, F. J., Criado, M. N., Belda-Galbis, C. M., Esteve, M. J., & Rodrigo, D. (2014). Stevia rebaudiana Bertoni as a nat- ural antioxidant/antimicrobial for high pressure processed fruit extract: Processing parameter optimization. Food Chemistry, 148, 261–267.

Barba, F. J., Esteve, M. J., & Frígola, A. (2012). High pressure treatment effect on physicochemical and nutritional properties of fluid foods during storage: A review. Comprehensive Reviews in Food Science and Food Safety, 11(3), 307–322.

Barba, F. J., Koubaa, M., do Prado-Silva, L., Orlien, V., & Sant'Ana, A. D. S. (2017). Mild processing applied to the inactivation of the main foodborne bacterial pathogens: A review. Trends in Food Science and Technology, 66, 20–35.

Barba, F. J., SantAna, A. S., Orlien, V., & Koubaa, M. (2018). Innovative technologies for food preservation: Inactivation of spoilage and pathogenic microorganisms. 1–315.

Campagnollo, F. B., Ganev, K. C., Khaneghah, A. M., Portela, J. B., Cruz, A. G., Granato, D., … Sant'Ana, A. S. (2016). The occurrence and effect of unit operations for dairy products processing on the fate of aflatoxin M1: a review. Food Control, 68, 310–329.

Cano-Sancho, G., Sanchis, V., Ramos, A. J., & Marín, S. (2013). Effect of food processing on exposure assessment studies with mycotoxins. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30(5), 867–875.

Evelyn, Kim, H. J., & Silva, F. V. M. (2016). Modeling the inactivation of Neosartorya fischeri ascospores in apple juice by high pressure, power ultrasound and thermal processing. Food Control, 59, 530–537.

Evelyn, & Silva, F. V. M. (2015). Inactivation of Byssochlamys nivea ascospores in strawberry puree by high pressure, power ultrasound and thermal processing. International Journal of Food Microbiology, 214, 129–136.

Evelyn, & Silva, F. V. M. (2017). Resistance of Byssochlamys nivea and Neosartorya fischeri mould spores of different age to high pressure thermal processing and thermosonication. Journal of Food Engineering, 201, 9–16.

Fakhri, Y., Rahmani, J., Oliveira, C. A. F., Franco, L. T., Corassin, C. H., Saba, S., … Mousavi Khaneghah, A. (2019). Aflatoxin M1 in human breast milk: A global systematic review, meta-analysis, and risk assessment study (Monte Carlo simulation). Trends in Food Science and Technology, 88, 333–342.

FAO. (2018). Food safety and quality. http://www.fao.org/food/food-safety-quality/a-z-index/mycotoxins/en/. [Accessed February 10, 2019].

Fathabad, A. E., Shariatifar, N., Moazzen, M., Nazmara, S., Fakhri, Y., Alimohammadi, M., … Mousavi Khaneghah, A. (2018). Determination of heavy metal content of processed fruit products from Tehran's market using ICP-OES: A risk assessment study. Food and Chemical Toxicology, 115, 436–446.

Gavahian, M., & Mousavi Khaneghah, A. (2019). Cold plasma as a tool for the elimination of food contaminants: Recent advances and future trends. Critical Reviews in Food Science and Nutrition, 1–12. https://doi.org/10.1080/10408398.2019.1584600.

Gross, M., & Jaenicke, R. (1994). Proteins under pressure. The influence of high hydrostatic pressure on structure, function and assembly of proteins and protein complexes. European Journal of Biochemistry, 221(2), 617–630.

Hao, H., Zhou, T., Koutchma, T., Wu, F., & Warriner, K. (2016). High hydrostatic pressure assisted degradation of patulin in fruit and vegetable juice blends. Food Control, 62, 237–242.

Heinz, V., & Buckow, R. (2010). Food preservation by high pressure. Journal Fur Verbraucherschutz Und Lebensmittelsicherheit, 5(1), 73–81.

Heshmati, A., Zohrevand, T., Khaneghah, A. M., Mozaffari Nejad, A. S., & Sant'Ana, A. S. (2017). Co-occurrence of aflatoxins and ochratoxin A in dried

fruits in Iran: Dietary exposure risk assessment. Food and Chemical Toxicology, 106, 202–208.

Iizuka, T., Maeda, S., & Shimizu, A. (2013). Removal of pesticide residue in cherry tomato by hydrostatic pressure. Journal of Food Engineering, 116(4), 796–800.

Iizuka, T., & Shimizu, A. (2014a). Removal of pesticide residue from Brussels sprouts by hydrostatic pressure. Innovative Food Science & Emerging Technologies, 22, 70–75.

Iizuka, T., & Shimizu, A. (2014b). Removal of pesticide residue from cherry tomatoes by hydrostatic pressure (part 2). Innovative Food Science & Emerging Technologies, 26, 34–39.

Iizuka, T., Yahata, M., & Shimizu, A. (2013). Potential mechanism involved in removal of hydrophobic pesticides from vegetables by hydrostatic pressure. Journal of Food Engineering, 119(1), 1–6.

Kalagatur, N. K., Kamasani, J. R., Mudili, V., Krishna, K., Chauhan, O. P., & Sreepathi, M. H. (2018). Effect of high pres- sure processing on growth and mycotoxin production of Fusarium graminearum in maize. Food Bioscience, 21, 53–59.

Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., … Dussort, P. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. Mycotoxin Research, 32(4), 179–205.

Kaushik, G. (2015). Effect of processing on mycotoxin content in grains. Critical Reviews in Food Science and Nutrition, 55(12), 1672–1683.

Khaneghah, A. M., Chaves, R. D., & Akbarirad, H. (2017). Detoxification of aflatoxin M1 (AFM1) in dairy base bev- erages (Acidophilus milk) by using

different types of lactic acid bacteria-mini review. Current Nutrition & Food Science, 13(2), 78–81.

Khaneghah, A. M., Martins, L. M., von Hertwig, A. M., Bertoldo, R., & Sant'Ana, A. S. (2018). Deoxynivalenol and its masked forms: Characteristics, incidence, control and fate during wheat and wheat based products processing—A review. Trends in Food Science and Technology, 71, 13–24.

Lancova, K., Hajslova, J., Kostelanska, M., Kohoutkova, J., Nedelnik, J., Moravcova, H., & Vanova, M. (2008). Fate of trichothecene mycotoxins during the processing: Milling and baking. Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 25(5), 650–659.

Mahmood Fashandi, H., Abbasi, R., & Mousavi Khaneghah, A. (2018). The detoxification of aflatoxin M 1 by Lactobacillus acidophilus and Bifidobacterium spp.: A review. Journal of Food Processing and Preservation, 42(9), https://doi.org/10.1111/jfpp.13704.

Majeed, M., Khaneghah, A. M., Kadmi, Y., Khan, M. U., & Shariati, M. A. (2018). Assessment of ochratoxin a in com- mercial corn and wheat products. Current Nutrition & Food Science, 14(2), 116–120.

Marín, S., Cano-Sancho, G., Sanchis, V., & Ramos, A. J. (2018). The role of mycotoxins in the human exposome: Application of mycotoxin biomarkers in exposome-health studies. Food and Chemical Toxicology, 121, 504–518.

Marin, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. Food and Chemical Toxicology, 60, 218–237.

Milani, J., & Maleki, G. (2014). Effects of processing on mycotoxin stability in cereals. Journal of the Science of Food and Agriculture, 94(12), 2372–2375.

Mousavi Khaneghah, A., Fakhri, Y., Raeisi, S., Armoon, B., & Sant'Ana, A. S. (2018). Prevalence and concentration of ochratoxin A, zearalenone, deoxynivalenol and total aflatoxin in cereal-based products: A systematic review and meta-analysis. Food and Chemical Toxicology, 118, 830–848.

Mousavi Khaneghah, A., Fakhri, Y., & Sant'Ana, A. S. (2018). Impact of unit operations during processing of cereal-based products on the levels of deoxynivalenol, total aflatoxin, ochratoxin A, and zearalenone: A systematic review and meta-analysis. Food Chemistry, 268, 611–624.

Mousavi Khaneghah, A., Ismail, E.ş., Raeisi, S., & Fakhri, Y. (2018). Aflatoxins in cereals: State of the art. Journal of Food Safety, 38(6), https://doi.org/10.1111/jfs.12532.

Nabizadeh, S., Shariatifar, N., Shokoohi, E., Shoeibi, S., Gavahian, M., Fakhri, Y., … Mousavi Khaneghah, A. (2018). Prevalence and probabilistic health risk assessment of aflatoxins B1, B2, G1, and G2 in Iranian edible oils. Environmental Science and Pollution Research, 25(35), 35562–35570.

Oteiza, J. M., Khaneghah, A. M., Campagnollo, F. B., Granato, D., Mahmoudi, M. R., Sant'Ana, A. S., & Gianuzzi, L. (2017). Influence of production on the presence of patulin and ochratoxin A in fruit juices and wines of Argentina. LWT - Food Science and Technology, 80, 200–207.

Rahmani, J., Miri, A. L. I., Mohseni-Bandpei, A., Fakhri, Y., Bjørklund, G., Keramati, H., … Khaneghah, A. M. (2018). Contamination and prevalence of histamine in canned tuna from Iran: A systematic review, meta-analysis, and health risk assessment. Journal of Food Protection, 81(12), 2019–2027.

Rastegar, H., Shoeibi, S., Yazdanpanah, H., Amirahmadi, M., Khaneghah, A. M., Campagnollo, F. B., & Sant'Ana, A.S. (2017). Removal of aflatoxin B1 by roasting with lemon juice and/or citric acid in contaminated pistachio nuts. Food Control, 71, 279–284.

Rastogi, N. K., Raghavarao, K.S.M.S., Balasubramaniam, V. M., Niranjan, K., & Knorr, D. (2007). Opportunities and challenges in high pressure processing of foods. Critical Reviews in Food Science and Nutrition, 47(1), 69–112.

Razzaghi, N., Ziarati, P., Rastegar, H., Shoeibi, S., Amirahmadi, M., Conti, G. O., … Mousavi Khaneghah, A. (2018). The concentration and probabilistic health risk assessment of pesticide residues in commercially available olive oils in Iran. Food and Chemical Toxicology, 120, 32–40.

Tokuşoğlu, Ö., Alpas, H., & Bozoğlu, F. (2010). High hydrostatic pressure effects on mold flora, citrinin mycotoxin, hydroxytyrosol, oleuropein phenolics and antioxidant activity of black table olives. Innovative Food Science and Emerging Technologies, 11(2), 250–258.

Ünüsan, N. (2019). Systematic review of mycotoxins in food and feeds in Turkey. Food Control, 97, 1–14.

Yadolahi, M., Babri, M., Sharif, A. A. M., & Khaneghah, A. M. (2012). Pesticide residue determination in Shahr-e-Rey tomatoes using quechers method. Advances in Environmental Biology, 6(8), 2434–2438.

Yousefi, M., Shemshadi, G., Khorshidian, N., Ghasemzadeh-Mohammadi, V., Fakhri, Y., Hosseini, H., & Mousavi Khaneghah, A. (2018). Polycyclic aromatic hydrocarbons (PAHs) content of edible vegetable oils in Iran: A risk assessment study. Food and Chemical Toxicology, 118, 480–489.

3.3. The potential of pulsed electric fields to reduce pesticides and toxins

Chapter 7 in book: Pulsed Electric Fields to Obtain Healthier and Sustainable Food for Tomorrow (2020)

The potential of pulsed electric fields to reduce pesticides and toxins

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Abstract

Several studies in the available literature have demonstrated the feasibility of pulsed electric fields (PEFs) for different applications in food industry. PEF technology is a valuable tool to improve functionality, extractability, and recovery of nutritionally valuable compounds as well as bioavailability of micronutrients and components in a diverse variety of foods. Moreover, some studies have shown the potential of PEF treatments to reduce the formation of food processing contaminants, pesticides, and toxins. This opens the doors to new PEF applications in the food industry. Recently, scientists explored the possibility of removing harmful chemical, such as pesticides and toxins, from food commodity by PEF treatment. This chapter will focus on the impact of PEF on pesticides and toxins.

Keywords: Pulsed electric fields; pesticides; toxins

1. Introduction

Among the contaminants that could threaten our health, the proposed risks by pesticides, as one of the most used chemical compounds, are a matter of concern (Amirahmadi et al., 2017; Razzaghi et al., 2018; Yadolahi, Babri, Sharif, & Khaneghah, 2012). For instance, huge concentration of pesticides are widely used to protect crops against the pest infestation, resulting in residues on harvested crops, which can lead to health concerns among consumers (Banias, Achillas, Vlachokostas, Moussiopoulos, & Stefanou, 2017). In this context, in addition to serious environmental issues correlated with the use of pesticides, other types of chemical compounds such as herbicides, and fungicides have been also identified as a public concern (Nicolopoulou-Stamati, Maipas, Kotampasi, Stamatis, & Hens, 2016) (Fig. 1). According to available data, around 5.6 billion pounds of pesticide are consumed worldwide (Alavanja, 2009), which are associated with negative effects on human health (e.g., growth retardation) (Nicolopoulou-Stamati et al., 2016) (Fig. 2).

On the other hand, the mycotoxins, as a serious concern for both food and feed, can be defined as secondary metabolites secreted by some fungal species belonging mainly to the *Aspergillus*, *Fusarium*, *Penicillium*, and *Alternaria* genera (Khaneghah, Martins, von Hertwig, Bertoldo, & Sant'Ana, 2018; Majeed, Khaneghah, Kadmi, Khan, & Shariati, 2018; Marin, Ramos, Cano-Sancho, & Sanchis, 2013; Mousavi Khaneghah, Fakhri, Raeisi, Armoon, & Sant'Ana, 2018; Mousavi Khaneghah, Fakhri, & Sant'Ana, 2018).

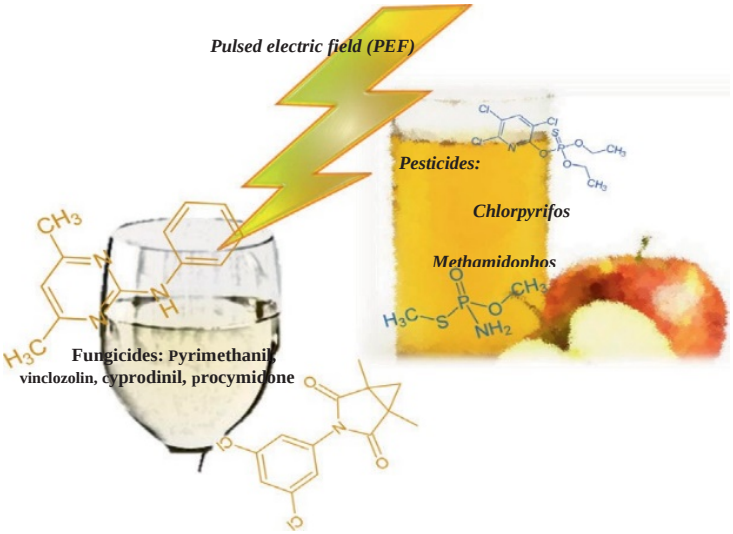

Figure 1. Chemical structures of the most commonly evaluated pesticides and fungicides in apple juice and wine after PEF treatments. PEF, Pulsed electric field.

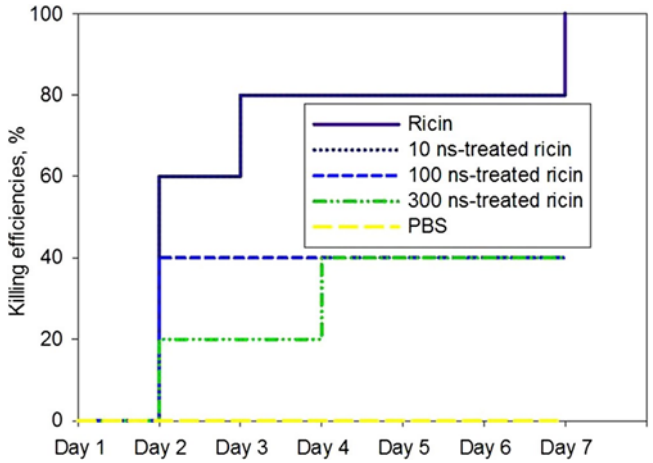

Figure 2. The killing efficiency of 0.5 mL of PEF-treated and -untreated ricins at the initial concentration of 20 μg/mL on mice. PEF, Pulsed electric field.

Source: Wei, K., Li, W., Gao, S., Ji, B., Zang, Y., Su, B., .Wang, J. (2016). Inactivation of ricin toxin by nanosecond pulsed electric fields including evidences from cell and animal toxicity. Scientific Reports, 6. doi:10.1038/srep18781, with permission. This work is licensed under a Creative Commons Attribution 4.0 International License.

During different stages of processing as well as during storage, the growing of mycotoxigenic mold in contaminated foods can result in the formation of mycotoxins (Khaneghah, Chaves, & Akbarirad, 2017; Mousavi Khaneghah, Ismail, Raeisi, & Fakhri, 2018; Rastegar et al., 2017). Among 300 identified secondary metabolites, ochratoxin A, aflatoxins, fumonisins, zearalenone, patulin, and deoxynivalenol can be considered highly significant mycotoxins with adverse effects on food safety, the economy as well as human health (Marin et al., 2013).

Besides the huge economic losses, mycotoxins could pose some adverse effects on human and animal's health such as immunosuppression, neurotoxicity, estrogenicity, dermatotoxicity, teratogenicity, hepatotoxicity, carcinogenicity, and mutagenicity (Amirahmadi, Shoeibi, Rastegar, Elmi, & Mousavi Khaneghah, 2018; Heshmati, Zohrevand, Khaneghah, Mozaffari Nejad, & Sant'Ana, 2017). However, although mycotoxin contamination can be detected in a wide variety of food products, the prevalence of mycotoxins is mostly reported in agricultural crops such as barley, wheat corn, and rice (Amirahmadi et al., 2017; Majeed et al., 2018).

The most commonly contaminated crops or substrates consist of cereals, nuts, oilseeds, dried fruits, coffee, spices, and their byproducts. The contamination by mycotoxins occurs throughout the food chain, during field and/or in the postharvest stage. Significant economic losses are associated with the impact of mycotoxins on human health, animal productivity, and domestic and international trade. AFs, OTA, ZEA, trichothecens, FBs, and PAT are some of the mycotoxins with higher agroeconomic impact (FAO, 2018; U¨ nu¨ san, 2019).

Food processing can have an impact on pesticide and mycotoxin levels, but the details remain unclear. Most publications are focused on the effect of processing techniques such as cleaning and milling of grains, microbiological fermentation or thermal processes such as cooking, boiling, and extrusion, among others (Cano-Sancho, Sanchis, Ramos, & Marín, 2013). Less information is available about the effect of emerging technologies in food processing such as high hydrostatic pressure, pulsed electric fields (PEFs), or ultrasound on pesticides and mycotoxin levels.

Some authors have evaluated the impact of nonthermal processing such as gamma irradiation (Di Stefano, Pitonzo, & Avellone, 2014; Jalili, Jinap, & Noranizan, 2010), ozone gas (El- Desouky, Sharoba, El-Desouky, El-Mansy, & Naguib, 2012), among others. The impact of PEF could constitute an effective tool to reduce pesticides and mycotoxin levels of food matrices.

PEF is an emerging nonthermal technology in the food industry that has been shown to maintain the sensory and nutritional properties of the food materials better than those of conventional thermal treatments (Barba, Koubaa, do Prado- Silva, Orlien, & Sant'Ana, 2017; Barba et al., 2015; Gabric´ et al., 2018; Misra et al., 2017; Zulueta, Barba, Esteve, & Frígola, 2010).

This innovative processing technique was also proved to be superior to traditional processing techniques in terms of processing time required (Yang, Huang, Lyu, & Wang, 2016) and, therefore, can reduce the process time and production cost while improving the process efficiency. In a PEF process an electric field is applied across the samples through PEF electrodes for some microseconds (Pue´rtolas & Barba, 2016; Pue´rtolas, Koubaa, & Barba, 2016; Zhu et al., 2016). This technique is different from that of ohmic heating

(Gavahian, Farahnaky, Javidnia, & Majzoobi, 2012) and moderate electric field (Gavahian, Chu, & Sastry, 2018) mainly due to the applied frequencies and the treatment time. This technique has been successfully used for microbial decontamination (Puligundla, Pyun, & Mok, 2018) and the extraction process (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Koubaa et al., 2016; Lang & Jun, 2017; Pue´rtolas et al., 2016). Researchers have also explored the feasibility of PEF for pesticides and toxins removal from food materials. Some of the main findings are reported in the following sections.

2. Pesticides

The potential of PEF to reduce the concentration of pesticides in food products has been a matter of interest for several authors (Table 1). For instance, Chen et al. (2009) studied the impact of PEFs on the reduction of methamidophos and chlorpyrifos from apple juice. They found that PEF treatments at electric field strengths of 8-20 kV/cm and pulse number from 6 to 26 pulses led to significant degradations of both pesticides, being chlorpyrifos much more labile to PEF than methamidophos. They also observed that the degradation of both pesticides was increased when the electric field was augmented. They attributed this fact to the ability of high voltage increase to induce the vibration and rotation of polar molecules, thus facilitating the degradation of pesticides.

In another study, the effects of PEF treatments on the degradation of pesticides diazinon and dimethoate from apple juice were studied (Zhang et al., 2012). These authors found a significant degradation of both pesticides after PEF treatments, with a significant decrease when electric field strength and

treatment time were increased, observing the maximum degradation of both diazinon (47.6%) and dimethoate (34.7%) after 260 μs PEF at electrical field strength of 20 kV/cm.

More recently, the impact of PEF on four residual fungicides (pyrimethanil, vinclozolin, cyprodinil, and procymidone) in white wines was evaluated (Delsart et al., 2015). It was found that PEF significantly induced the degradation of these fungicides. Moreover, they observed that the effect of the strength and energy of PEF treatment on the degradation of these fungicides was higher than that of the treatment time.

Table 1. Effect of pulsed electric fields (PEFs) on pesticides from food products.

Matrix	Compound	PEF treatment	Main findings	References
Apple juice	Chlorpyrifos, methamidophos	8, 12, 16, and 20 kV/cm/40°C/6, 9, 12, 19, and 26 pulses/60—260 μs	Significant degradation of both pesticides being chlorpyrifos more labile than methamidophos. Increased degradation with enhanced electric field and time.	Chen et al. (2009)
	Diazinon, dimethoate	8, 12, 16, and 20 kV/cm/15°C—23.5°C/60, 90, 120, 190, and 260 μs	Significant degradation of both pesticides. Electric field strength and time had a significant effect.	Zhang et al. (2012)
Wine	Pyrimethanil, vinclozolin, cyprodinil, procymidone	5—20 kV/cm/0.5—2 ms/10—160 kJ/L	Significant degradation fungicides. Increased with enhanced PEF strength and energies.	Delsart et al. (2015)

3. Toxins

Mycotoxins are a type of toxins produced by fungi. Mycotoxins can be a problem at the level of human health. The composition regarding mycotoxins differs according to the food matrix studied. For example, the mycotoxins that are most frequently found in cereals are aflatoxins, ochratoxin A, fumonisins, deoxynivalenol, and zearalenone. These mycotoxins are not completely destroyed during the processing of cereals and can contaminate other final products. This is why over the last decades there have been numerous efforts to find different processes that can reduce the content of mycotoxins. In fact there are several processes that can have an effect on mycotoxins such as sorting, trimming, cleaning, milling, brewing, cooking, baking, frying, roasting, canning, flaking, alkaline cooking, nixtamalization, and extrusion.

Generally, the greatest reduction is when higher temperatures are used, although normally they are not completely eliminated. In fact, different inactivation mechanisms are observed according to the type of mycotoxin evaluated and the type of treatment used (Bullerman & Bianchini, 2007).

On the other hand, extrusion and roasting processes have been proved to be effective in reducing the concentrations of mycotoxin concentrations. It should be noted that this reduction is correlated with the elevated temperatures involved in these processes. For example, a previously conducted study showed that the extrusion process at the temperatures 150○C effectively reduced the concentrations of zearalenone, aflatoxins, deoxynivalenol, and fumonisins. On the other hand, running the process at an elevated temperature, that is, 160○C resulted in better elimination of fumonisins. The same enhanced detoxification effect was observed when the extrusion process was conducted in the presence

of glucose. When the corn grits contaminated with fumonisin B1, mixed with 10% glucose, and subjected to the extrusion process, only 15% 25% of the fumonisin remained in the processed product. According to the authors, extrusion process also resulted in the formation of several degrades products of fumonisin such as N-(1-deoxy-D-fructos-1-yl) fumonisin B1, hydrolyzed fumonisin B1, and N-(carboxymethyl)—fumonisin B1. Moreover, rats were used to evaluate the toxicity of the extruded corn grits, and the results confirmed that extrusion process can reduce the toxicity of the fumonisin contaminated products (Bullerman & Bianchini, 2007).

It is now accepted that PEF treatment can decrease the afla-toxins B1 and G1 produced by *Aspergillus flavus* (Eisa, Ali, El- Habbaa, Abdel-Reheem, & Abou-El-Ella, 2003) (Table 2).

Researchers observed 75.6% and 82.8% when 4-day-old *A. flavus* cultures were subjected to PEF treatments up to 24 h at the frequencies of 0.50 and 50 Hz, respectively. The rising frequency of the PEF treatment from 0.1 to 0.4 kHz decreases the population of *A. flavus* slightly. On the other hand, an elevated frequency, that is, 0.8 kHz significantly enhanced the decontamination effects of PEF treatment. Moreover, multiple-exposure mode at different frequencies of PEF ranging from 0.5 to 0.8 kHz reduced the aflatoxin production rate by up to 99%. It should be noted that aflatoxin B1 was not detectable at different combined PEF strengths. Multiple-exposure of yellow corn grains for 3 weeks to a combined treatment decreased the aflatoxin concentration, in both *A. flavus* inoculated and noninoculated grains as compared to that of the control sample. In addition, the study revealed that only slight changes occurred in the changes

were observed in carbohydrates and protein contents of the PEF- treated samples (Eisa et al., 2003).

In another study the effect of previously optimized heat treatment alone or in combination with PEF on artificially spiked aflatoxin in potato dextrose agar was evaluated and compared (Subramanian, Shanmugam, Ranganathan, Kumar, & Reddy, 2017). First, the authors optimized heat processing using a response surface methodology with temperatures of 110○C- 119.8○C and times ranging from 10 to 20 min. They also optimized pH (from 4 to 10). After that, they evaluated the effect of PEF treatment (1 kJ/Pulse frequency of 50 Hz)/burst (10), for a time of 10 s) on aflatoxins combined with the optimal conditions for heat treatment and compared the results to those obtained for control samples (without PEF treatment). The authors observed a decrease in aflatoxins content in potato dextrose agar after using the combined treatment (thermal+ PEF) compared to the treatments performed individually.

More recently, Vijayalakshmi, Nadanasabhapathi, Kumar, and Sunny Kumar (2018) assessed the effectiveness of a PEF process in decreasing the concentrations of toxic compounds in model systems of potato dextrose agar that were artificially contaminated with aflatoxin. In this regard, the authors examined the concentrations of aflatoxins in the PEF-treated and -untreated samples by means of high-performance liquid chromatography technique. The authors also tried to optimize the decontamination effects of PEF treatment [output voltage (20% 65%), pulse width (10 26 μs), and pH (4 10)] through the response surface methodology by adjusting effective process parameters in aflatoxin reduction. The authors observed that pH was the main responsible of the changes in aflatoxin contents. Moreover, they also found the factors involved

in aflatoxin B1 and total aflatoxin reduction fitted the 2FI polynomial model and quadratic model respectively, being of great importance to control moisture content when the experiments will be carried out in real food matrices. This study highlighted the importance of PEF process optimization for a successful aflatoxin removal from food materials. According to the authors, pulse width, food sample pH, and the applied voltage (voltage intensity) were among the crucial parameters that should be considered for maximizing the decontamination effects of PEF processes (Vijayalakshmi et al., 2018).

On the other hand, in a study conducted by Wei et al. (2016), these authors evaluated the effect of PEF (30 kV/cm, 10 300 ns per pulse) to inactivate ricin up to 4.2 μg/mL. In order to evaluate the effectiveness of PEF treatments, cells and mice were used. Then, ricin (without treatment or PEF-treated) was injected intraperitoneally directly and the mice were also exposed to inhalation to ricin. While 40% of the mice exposed to ricin previously treated by PEF survived, 100% of the mice exposed to ricin without previous treatment had to be sacrificed, thus demonstrating the efficacy of the PEF. The authors attributed this positive effect of the PEFs in the decrease in the toxicity of ricin to a modification in the secondary structure (beta-sheet and beta-turn underwent transition) of ricin after treatment, which was confirmed after carrying out an electrophoresis analysis in polyacrylamide gel with dodecyl sulfate of sodium (SDS—PAGE) and circular dichroism.

Table 2. Effect of pulsed electric fields (PEFs) on toxins from food products.

Matrix	Compound	PEF treatment	Main findings	References
Potato dextrose agar	Artificially spiked aflatoxin	PEF: Pulse frequency (50 Hz), burst (10), energy (1 kJ), and for a time of 10 s Thermal treatment: 110°C—119°C/	Decrease after combined application of PEF 1 thermal treatment.	Subramanian et al. (2017)
	Aflatoxins	Output voltage (20%—65%), pulse width (10—26 μs), pH (4—10)	Increased pulse width and voltage decreased aflatoxin content.	Vijayalakshmi et al. (2018)
Model solution	Ricin	30 kV/cm, 10—300 ns per pulse	Decrease in ricin toxicity or modification in the secondary structure (beta-sheet and beta-turn underwent transition) after PEF treatment. Reduced toxicity in mice of the PEF-treated ricin compared to the untreated one.	Wei et al. (2016)

4. Conclusion

PEF has shown good potential for application in the food industry, especially for liquid food pasteurization and potato. Toxin and pesticide removal is an emerging application of this nonthermal process. It was shown that PEF can successfully reduce the concentrations of some toxins (e.g., aflatoxin) and pesticides, such as methamidophos and chlorpyrifos, from food materials. Furthermore, it was proved that this decontamination can be performed with limited negative effects on the product quality parameters as compared to those of traditional thermal technologies. Besides, researchers have highlighted the importance of PEF optimization in terms of process duration, pulse width, food sample pH, and the applied voltage (voltage intensity) for a successful decontamination process. However, further fundamental studies are required to understand the details of the mechanisms involved in pesticide and toxin removal from food samples by PEF.

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References

Alavanja, M. C. R. (2009). Introduction: Pesticides use and exposure extensive worldwide. Reviews on Environmental Health, 24(4), 303—309.

Amirahmadi, M., Kobarfard, F., Pirali-Hamedani, M., Yazdanpanah, H., Rastegar,H., Shoeibi, S., & Mousavi Khaneghah, A. (2017). Effect of Iranian traditional cooking on fate of pesticides in white rice. Toxin Reviews, 36(3), 177—186.

Amirahmadi, M., Shoeibi, S., Rastegar, H., Elmi, M., & Mousavi Khaneghah, A. (2018). Simultaneous analysis of mycotoxins in corn flour using LC/MS-MS combined with a modified QuEChERS procedure. Toxin Reviews, 37(3), 187—195.

Banias, G., Achillas, C., Vlachokostas, C., Moussiopoulos, N., & Stefanou, M. (2017). Environmental impacts in the life cycle of olive oil: A literature review.Journal of the Science of Food and Agriculture, 97(6), 1686—1697.

Barba, F. J., Koubaa, M., do Prado-Silva, L., Orlien, V., & Sant'Ana, A. D. S. (2017). Mild processing applied to the inactivation of the main foodborne bacterial pathogens: A review. Trends in Food Science and Technology, 66, 20—35.

Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N., & Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. Food Research International, 77(4), 773—798.

Barba, F. J., Zhu, Z., Koubaa, M., Sant'Ana, A. S., & Orlien, V. (2016). Green alternative methods for the extraction of antioxidant bioactive

compounds from winery wastes and by-products: A review. Trends in Food Science and Technology, 49, 96—109.

Bullerman, L. B., & Bianchini, A. (2007). Stability of mycotoxins during food processing. International Journal of Food Microbiology, 119(1—2), 140—146.

Cano-Sancho, G., Sanchis, V., Ramos, A. J., & Mar´ın, S. (2013). Effect of food processing on exposure assessment studies with mycotoxins. Food Additives and Contaminants — Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30(5), 867—875.

Chen, F., Zeng, L., Zhang, Y., Liao, X., Ge, Y., Hu, X., & Jiang, L. (2009). Degradation behaviour of methamidophos and chlorpyrifos in apple juice treated with pulsed electric fields. Food Chemistry, 112(4), 956—961.

Delsart, C., Franc, C., Grimi, N., de Revel, G., Vorobiev, E., & Mietton Peuchot, M. (2015). Effects of pulsed electric fields on four residual fungicides in white wines. In 1st world congress on electroporation and pulsed electric fields in biology, medicine and food & environmental technologies. Portoroz˘, Slovenia.

Di Stefano, V., Pitonzo, R., & Avellone, G. (2014). Effect of gamma irradiation on aflatoxins and ochratoxin A reduction in almond samples. Journal of Food Research, 3(4), 113—118.

Eisa, N. A., Ali, F. M., El-Habbaa, G. M., Abdel-Reheem, S. K., & Abou-El-Ella, M. F. (2003). Pulsed electric field technology for checking aflatoxin production in cultures and corn grains. Egypt Journal of Phytopathology, 31, 75—86.

El-Desouky, T. A., Sharoba, A. M. A., El-Desouky, A. I., El-Mansy, H. A., & Naguib, K. (2012). Effect of ozone gas on degradation of aflatoxin B1 and Aspergillus flavus fungal. Journal of Environmental and Analytical Toxicology, 2, 128.

FAO. Food safety and quality. (2018). ,http://www.fao.org/food/food-safety- quality/a-z-index/mycotoxins/en/. Accessed 10.02.19.

Gabric´, D., Barba, F., Roohinejad, S., Gharibzahedi, S. M. T., Radojc´in, M., Putnik, P., & Bursac´ Kovac´evic´, D. (2018). Pulsed electric fields as an alternative to thermal processing for preservation of nutritive and physicochemical properties of beverages: A review. Journal of Food Process Engineering, 41(1), e12638. Available from https://doi.org/10.1111/jfpe.12638.

Gavahian, M., Chu, Y.-H., & Sastry, S. (2018). Extraction from food and natural products by moderate electric field: Mechanisms, benefits, and potential industrial applications. Comprehensive Reviews in Food Science and Food Safety, 17(4), 1040—1052.

Gavahian, M., Farahnaky, A., Javidnia, K., & Majzoobi, M. (2012). Comparison of ohmic-assisted hydrodistillation with traditional hydrodistillation for the extraction of essential oils from Thymus vulgaris L. Innovative Food Science and Emerging Technologies, 14, 85—91.

Heshmati, A., Zohrevand, T., Khaneghah, A. M., Mozaffari Nejad, A. S., & Sant'Ana, A. S. (2017). Co-occurrence of aflatoxins and ochratoxin A in dried fruits in Iran: Dietary exposure risk assessment. Food and Chemical Toxicology, 106, 202—208.

Jalili, M., Jinap, S., & Noranizan, A. (2010). Effect of gamma radiation on reduction of mycotoxins in black pepper. Food Control, 21(10), 1388—1393.

Khaneghah, A. M., Chaves, R. D., & Akbarirad, H. (2017). Detoxification of aflatoxin M1 (AFM1) in dairy base beverages (Acidophilus milk) by using different types of lactic acid bacteria-mini review. Current Nutrition and Food Science, 13(2), 78—81.

Khaneghah, A. M., Martins, L. M., von Hertwig, A. M., Bertoldo, R., & Sant'Ana, A. S. (2018). Deoxynivalenol and its masked forms: Characteristics, incidence, control and fate during wheat and wheat based products processing — A review. Trends in Food Science and Technology, 71, 13—24.

Koubaa, M., Barba, F. J., Grimi, N., Mhemdi, H., Koubaa, W., Boussetta, N., & Vorobiev, E. (2016). Recovery of colorants from red prickly pear peels and pulps enhanced by pulsed electric field and ultrasound. Innovative Food Science & Emerging Technologies, 37(Part C), 336—344.

Lang, Y., & Jun, H. (2017). High intensity pulsed electric field as an innovative technique for extraction of bioactive compounds—A review. Critical Reviews in Food Science and Nutrition, 57(13), 2877—2888.

Majeed, M., Khaneghah, A. M., Kadmi, Y., Khan, M. U., & Shariati, M. A. (2018). Assessment of ochratoxin a in commercial corn and wheat products. Current Nutrition and Food Science, 14(2), 116—120.

Marin, S., Ramos, A. J., Cano-Sancho, G., & Sanchis, V. (2013). Mycotoxins:Occurrence, toxicology, and exposure assessment. Food and Chemical Toxicology, 60, 218—237.

Misra, N. N., Koubaa, M., Roohinejad, S., Juliano, P., Alpas, H., Ina`cio, R. S., &Barba, F. J. (2017). Landmarks in the historical development of twenty first century food processing technologies. Food Research International, 97, 318—339.

Mousavi Khaneghah, A., Fakhri, Y., Raeisi, S., Armoon, B., & Sant'Ana, A. S. (2018). Prevalence and concentration of ochratoxin A, zearalenone, deoxynivalenol and total aflatoxin in cereal-based products: A systematic review and meta-analysis. Food and Chemical Toxicology, 118, 830—848.

Mousavi Khaneghah, A., Fakhri, Y., & Sant'Ana, A. S. (2018). Impact of unit operations during processing of cereal-based products on the levels of deoxynivalenol, total aflatoxin, ochratoxin A, and zearalenone: A systematic review and meta-analysis. Food Chemistry, 268, 611—624.

Mousavi Khaneghah, A., Ismail, E. S¸., Raeisi, S., & Fakhri, Y. (2018). Aflatoxins in cereals: State of the art. Journal of Food Safety, 38(6), e12532. Available from https://doi.org/10.1111/jfs.12532.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., & Hens, L. (2016). Chemical pesticides and human health: The urgent need for a new concept in agriculture. Frontiers in Public Health, 4, 148. Available from https://doi.org/10.3389/fpubh.2016.00148.

Pue´rtolas, E., & Barba, F. J. (2016). Electro technologies applied to valorization of by-products from food industry: Main findings, energy and economic cost of their industrialization. Food and Bioproducts Processing, 100, 172—184.

Pue´rtolas, E., Koubaa, M., & Barba, F. J. (2016). An overview of the impact of electrotechnologies for the recovery of oil and high-value compounds

from vegetable oil industry: Energy and economic cost implications. Food Research International, 80, 19—26.

Puligundla, P., Pyun, Y. R., & Mok, C. (2018). Pulsed electric field (PEF) technology for microbial inactivation in low-alcohol red wine. Food Science and Biotechnology, 27(6), 1691—1696.

Rastegar, H., Shoeibi, S., Yazdanpanah, H., Amirahmadi, M., Khaneghah, A. M., Campagnollo, F. B., & Sant'Ana, A. S. (2017). Removal of aflatoxin B1 by roasting with lemon juice and/or citric acid in contaminated pistachio nuts. Food Control, 71, 279—284.

Razzaghi, N., Ziarati, P., Rastegar, H., Shoeibi, S., Amirahmadi, M., Conti, G. O., Mousavi Khaneghah, A. (2018). The concentration and probabilistic health risk assessment of pesticide residues in commercially available olive oils in Iran. Food and Chemical Toxicology, 120, 32—40.

Subramanian, V., Shanmugam, N., Ranganathan, K., Kumar, S., & Reddy, R. (2017). Effect of combination processing on aflatoxin reduction: Process optimization by response surface methodology. Journal of Food Processing and Preservation, 41(6), e13230. Available from https://doi.org/10.1111/jfpp.13230.

U¨ nu¨ san, N. (2019). Systematic review of mycotoxins in food and feeds in Turkey. Food Control, 97, 1—14.

Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., & Sunny Kumar, S. (2018). Effect of pH and pulsed electric field process parameters on the aflatoxin reduction in model system using response surface methodology: Effect of pH and PEF on aflatoxin reduction. Journal of Food Science and Technology, 55 (3), 868—878.

Wei, K., Li, W., Gao, S., Ji, B., Zang, Y., Su, B., .. . Wang, J. (2016). Inactivation of ricin toxin by nanosecond pulsed electric fields including evidences from cell and animal toxicity. Scientific Reports, 6. Available from https://doi.org/ 10.1038/srep18781.

Yadolahi, M., Babri, M., Sharif, A. A. M., & Khaneghah, A. M. (2012). Pesticide residue determination in Shahr-E-Rey tomatoes using QuEChERS method. Advances in Environmental Biology, 6(8), 2434—2438.

Yang, N., Huang, K., Lyu, C., & Wang, J. (2016). Pulsed electric field technology in the manufacturing processes of wine, beer, and rice wine: A review. Food Control, 61, 28—38.

Zhang, Y., Hou, Y., Chen, J., Chen, F., Liao, X., & Hu, X. (2012). Reduction of diazinon and dimethoate in apple juice by pulsed electric field treatment. Journal of the Science of Food and Agriculture, 92(4), 743—750.

Zhu, Z., He, J., Liu, G., Barba, F. J., Koubaa, M., Ding, L., Vorobiev, E. (2016). Recent insights for the green recovery of inulin from plant food materials using non-conventional extraction technologies: A review. Innovative Food Science and Emerging Technologies, 33, 1—9.

Zulueta, A., Barba, F. J., Esteve, M. J., & Fr´ıgola, A. (2010). Effects on the carotenoid pattern and vitamin A of a pulsed electric field-treated orange juice-milk beverage and behavior during storage. European Food Research and Technology, 231(4), 525—534.

3.4. Mycotoxins occurrence in herbal medicines dietary supplements and exposure assessment

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Mycotoxins occurrence in herbal medicines dietary supplements and exposure assessment

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ABSTRACT

Herbal medicine is one of the most ancient and traditional practice expanded over the world. The risk of herbal medicines contamination by mycotoxigenic fungi and subsequently with mycotoxins, increase with poor practices.

In this context, the multimycotoxin analysis of aflatoxins (AFs), zearalenone (ZEA), ochratoxin A (OTA), enniatins (ENNs) and beauvericin (BEA) was performed in 85 samples of herbal dietary supplements classified in 64 tablet samples based on one herbal ingredient and 21 mix tablet samples.
The extraction was performed by QuEChERS method and the determination by LC-MS/MS-IT. Then the risk characterization to mycotoxins through the consumption of herbal dietary supplements was assessed.

The results showed that ZEA, OTA, ENNs and BEA were present in the samples with incidences between 1 and 34% and mean of positives ranging from 65.53 to 1340.11 μg/kg. The Estimated Daily Intakes (EDIs) values obtained were in general far below the Tolerable Daily Intakes (TDIs) established.

Keywords: multimycotoxin; botanicals; QuEChERS; LC-MS/MS-IT; dietary exposure.

1. Introduction

The use of herbal medicine to improve quality of life is a practice expanded all over the world, not only in developing countries where high percentage of population depend upon herbal medicine as a primary health care source, but also in highly developed countries, due to the self-medicate tendency and the growing preference of the population for natural products (Abdel-Tawab, 2018). These botanicals are available in the corresponding markets in several forms: plant food supplements, homeopathic products, foods (teas and juices), and cosmetic products. They are commonly used to treat some acute and chronic neck pain, colds, anxiety, or depression (Abdel-Tawab, 2018; Hudson et al., 2018).

Some adverse effects related to herbal medicine drugs are hepatotoxicity, cardiovascular toxicity, and central nervous system alterations and can be caused by toxic effects of active plant compounds or due to other factors such as the contamination of supplements by adulterants during manufacturing process, negative interaction of some herbal ingredients with other drugs and the improper use (Hudson et al., 2018).

The increased popularity of herbal medicines has forced the introduction of several regulations all over the world to guarantee public health by assuring their quality, efficacy and safety (Qu et al., 2018).

In the European Union, from the legal point of view, food supplements are considered as food. There is no special category for these products that are not subject to any safety assessment, prior to their placement in the market. Furthermore, the frame legal of supplements is not totally harmonized, and a

substance or product that is considered food supplement in a European country may not be considered in another European country, so several unresolved problems like efficiency, safety and lack of legal harmonization are related to these products (Troncoso, 2019).

For herbal dietary supplements, the European Union (EU) monograph provides a system for the regulation together with the European Pharmacopeia which defined basic quality requirements for herbal medicinal products. The EU monograph is established by the Committee on Herbal Medicinal Products (HMPC), that was constituted as one of the scientific committees of the European Medicines Agency under the regulation 2004/24/EC, which amends Directive 2001/83/EC.

According to 2004/24/EC, herbal medicinal products must provide all information for market authorization, with exception of preclinical and clinical data, the efficacy and safety can be demonstrated by HMPC monographs (Abdel-Tawab, 2018).

Major contaminants of herbal medicines and products vary from microbiological to chemical contaminants such as heavy metals, pesticide residues and mycotoxins (Kosalec et al., 2009). Regarding mycotoxins, medicinal plants can be contaminated by various toxigenic fungi during harvesting, handling, storage and distribution, that may be responsible of mycotoxin production. The risk of contamination by mycotoxigenic fungi and subsequently with mycotoxins, increase with poor agricultural and harvesting practices or inadequate conditions of storage, distribution or transportation (Ashiq et al., 2014). Mycotoxins are natural toxicants that are produced by a high number of species belonging to different fungal generas, mainly to *Fusarium*, *Claviceps*,

Alternaria, *Aspergillus* and *Penicillium*. These compounds are related to adverse carcinogenic, genotoxic, teratogenic, dermatotoxic, nephrotoxic and hepatotoxic effects in animals and humans (Marin et al., 2013).

As far as mycotoxins are concerned, the European Pharmacopoeia Commission has implemented stricter limits for the presence of Aflatoxins (AFs) in herbal drugs, limit set to 2 μg/kg for aflatoxin B1(AFB_1) and to 4 μg/kg for total aflatoxins (European Pharmacopoeia, 2016). However, other mycotoxins such as emerging mycotoxins have not been regulated yet. There is an urgent need for the creation or updated legislation to cover traditional mycotoxins as well as emerging mycotoxins such as enniatin and beauvericin and masked mycotoxins. Only in this way, botanical herbs will meet the precepts of food safety.

Information about mycotoxin contamination in various types of herbal dietary supplements is scarce (Veprikova et al., 2015), focusing in one ingredient, such as green tea supplements (Martínez-Domínguez et al., 2016), milk thistle supplements (Arroyo-Manzanares et al., 2013) or ginkgo biloba supplements (Di Mavungu et al., 2009; Martínez-Domínguez et al., 2015).

In this context, the aim of the present study was to perform a multimycotoxin analysis (AFs, Zearalenone (ZEA), Ochratoxin A (OTA), Enniatins (ENNs) and Beauvericin (BEA)) in 64 tablet samples of herbal dietary supplements containing one herbal ingredient, and 21 mix tablet samples. The extraction was performed by QuEChERS method and the determination by liquid chromatography coupled to ion-trap tandem mass spectrometry (LC-MS/MS-IT). An estimation of the population's risk to mycotoxins through the intake of herbal dietary supplements was also performed.

2. Material and methods

2.1. Reagents and chemicals

Solvents acetonitrile (ACN) and methanol (MeOH) HPLC grade were purchased from Merck (Darmstadt, Germany). Deionized water (resistivity >18 MΩ cm^{-1}) was obtained using a Milli-Q SP® Reagent Water System (Millipore Corporation Bedford, USA). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain) and formic acid (reagent grade ≥ 95%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). All solvents were filtered through a 0.45 μm cellulose filter supplied by Scharlau (Barcelona, Spain) before use. Salts for QuEChERS extraction: sodium chloride (NaCl) was obtained from VWR Chemicals (Leuven, Belgium), Magnesium sulfate ($MgSO_4$), anhydrous 99.5% min powder was supplied by Alfa Aesar (Karlsruhe, Germany) and Octadecyl C18 sorbent was acquired from Phenomenex (Madrid, Spain). Before injection, samples were filtered through a nylon filter (13 mm/0.22 μm) from Membrane Solutions (TX, USA). Mycotoxins standards (AFB_1, AFB_2, AFG_1, AFG_2, ZEA, OTA, ENNA, $ENNA_1$, ENNB, $ENNB_1$ and BEA) were supplied by Sigma (St. Louis, MO, USA). Individual stock solutions of each mycotoxin were prepared in MeOH at concentration of 100 mg/l. The working solutions were prepared from these individual stock solutions. All prepared solutions were stored in darkness at −20 °C until the analysis.

2.2. Sample collection

85 tablet samples of the most common herbal medicines dietary supplements used as natural remedies in Spain were acquired from different herbalists or pharmacies located in Valencia (Spain). These samples were 64

tablets based on one herbal ingredient (horsetail "*Equisetum arvense L.*", artichoke "*Cynara scolymus*", valerian root "*Valeriana officinalis*", dandelion plant "*Taraxacum officinale*", *cardus marianus* "*Silybum marianum*", fucus "*Fucus vesiculosus L.*", boldus leaves "*Peumus boldus"*, ginkgo "*Ginkgo biloba"*, ginger "*Zingiber officinale"*, passionflower "*Passiflora incarnata*", devil's clawroot "*Harpagophytum procumbens"*, whitethorn "*Crataegus monogyna"*, lemon balm leaves "*Melissa officinalis"*, red tea "Aspalathus linearis" and green tea *"Camellia sinensis"*), acquiring at least four samples for each herbal type, and 21 samples that are based on more than one herbal ingredient (Table S1 annex 1), which are used to treat insomnia or to lose weight. Samples were stored in their original packaging in a dark and dry place until the analysis. Table S1 (annex 1) describes the botanical contents of the analyzed tablets, the dosage recommended by the manufacturer and the main health effects associated.

2.3. QuEChERS procedure extraction

The analysis was performed with QuEChERS method previously validated. The tablets were crushed and 2 g of their content were weighted in a 50 ml falcon tube before adding 10 ml of acidified water with 2% formic acid and shacked for 30 min in a shaker IKA KS 260. Then 10 ml of ACN were added and the resulting mixture was shacked for other 30 min. Then, 4 g of $MgSO_4$ and 1 g of NaCl salts were added to the tube and the mixture was vortexed for 30 sec and centrifuged at 5000 rpm during 10 min. 2 ml of the supernatant were taken and placed into a 15 ml falcon tube with 0.3g of $MgSO_4$ and 0.1 g of Octadecyl C18 sorbent, shacked and centrifuged at 5000 rpm for 10 min. The obtained supernatant was filtered with a 13 mm/0.22 µm nylon filter

(Membrane Solutions, TX, USA), prior to injection of 20 µl into the LC-MS/MS-IT system.

2.4. LC-MS/MS-IT analysis

An Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with Turbo Ion Spray (ESI) electrospray ionization was used for the determination. The QTRAP analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. The column for the analyte separation was a Gemini-NX column C_{18} (Phenomenex, 150 mm x 4.6 mm, 5 particle size) preceded by a guard column. The flow rate was set at 0.25 ml/min, and the oven temperature at 40 ºC. The elution mobile phases consisted in acidified water with 5 mM ammonium formate and 0.1% formic acid (mobile phase A) and in acidified methanol with 5 mM ammonium formate and 0.1% formic acid (mobile phase B). For the elution, the gradient started with 0% of eluent B; in 10 min increased to 100%, decreased to 80% in 5 min, and to 70% in 2 min. In the next 6 min, the column was readjusted to initial conditions and equilibrated for 7 min.

The Turbo Ion Spray was used in positive ionization mode (ESI+). Nitrogen was served as nebulizer and collision gas. The conditions employed were Ion spray voltage 5500 V; curtain gas 20 arbitrary units; GS1 and GS2 with 50 and 50 psi, respectively and probe temperature (TEM) of 450 ºC.

The quantification and confirmation transitions of mycotoxin monitored fragments and the spectrometric parameters (declustering potential, collision energy and cell exit potential) are shown in Table 1.

Table 1. Spectrometric parameters of Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS).

Mycotoxin	Retention Time (min)	DP[a]	Precursor ion	Quantification ion Q			Confirmation ion q		
				CE[b]	Product ion	CXP[c]	CE	Product ion	CXP
AFB_1	9.13	46	313.1	39	284.9	4	41	241.0	4
AFB_2	9.03	81	315.1	33	286.9	6	39	259.0	6
AFG_1	8.86	76	329.0	39	243.1	6	29	311.1	6
AFG_2	9.37	61	331.1	27	313.1	6	39	245.1	4
ZEA	10.40	26	319.0	15	301.0	10	19	282.9	4
OTA	10.27	55	404.3	97	102.1	6	27	239.0	6
ENNA	12.62	76	699.4	35	210.1	14	59	228.2	16
$ENNA_1$	12.22	66	685.4	37	210.2	8	59	214.2	10
ENNB	11.60	51	657.3	39	196.1	8	59	214.0	10
$ENNB_1$	11.89	66	671.2	61	214.1	10	57	228.1	12
BEA	12.00	116	801.2	27	784.1	10	39	244.1	6

[a] DP: decluster potential (volts); [b] CE: collision energy (volts); [c] CXP: cell exit potential (volts)

2.5. Method optimization

The method was optimized for herbal medicines dietary tablets in terms of recoveries, repeatability (intra-day precision), reproducibility (inter-day precision), matrix effects (signal suppression-enhancer), linearity, and limits of detection (LODs) and quantification (LOQs) according to Commission Decision (2002/657/EC). The analytical parameters are shown in Table 2.

Recoveries were determined by spiking blank horsetail tablet samples with each studied mycotoxin at 100× LOQ concentration level, before and after the QuEChERS extraction in triplicate. To assess the intra-day precision, three determinations were performed on the same day and on nonconsecutive days to

assess the inter-day precision. Intra-day and inter-day recoveries obtained ranged from 73 to 117% and were within the relative standard deviation (<20%).

Matrix effects (MEs) to evaluate a possible suppression or enhancement of the signal (SSE) was obtained comparing the slope of the calibration curve prepared in blank horsetail tablet samples with the slope of the calibration curve prepared in methanol. SSE (%) were calculated as follows: SSE(%)= 100×slope with matrix/slope without matrix. Signal Suppression-Enhancer (SSE) for matrix effects were between 46 to 98%, observing suppression matrix effect for AFs. To minimize matrix effects, analytical parameters were determined using matrix matched calibration curves.

The LODs and LOQs were calculated by spiking a blank horsetail tablet samples with decreasing concentrations of the analyzed mycotoxins using the criterion of S/N ≥ 3 for calculating the LOD and S/N ≥ 10 for the LOQ. The LODs and LOQs ranged from 0.15 μg/kg to 3 μg/kg and from 0.5 μg/kg to 10 μg/kg, respectively.

Calibration curves in both pure solvent (methanol) and blank horsetail tablet samples were constructed at eight concentration levels (from LOQs to 1000 μg/kg). Linearity (r2) was in the range from 0.991 to 0.999 for all studied mycotoxins. Therefore, matrix-matched calibration curves constructed by spiking blank horsetail tablet samples were used for effective quantification of samples.

Table 2. Analytical parameters for method optimization.

Mycotoxin	Recoveries 100xLOQ ± RSD (%)		Signal Suppression -Enhancer (SSE%)	Limits of Detection (LOD) ppb (µg/kg)	Limits of Quantification (LOQ) ppb (µg/kg)
	Intra-day Precision	Inter-day Precision			
AFB_1	112± 18	108± 14	58	1.5	5
AFB_2	76± 20	112± 19	62	0.3	1
AFG_1	99± 9	108± 20	52	0.3	1
AFG_2	95± 18	112± 5	46	1.5	5
ZEA	109± 5	105± 1	82	3	10
OTA	119± 1	115± 5	79	1.5	5
ENNA	98± 1	117± 20	94	0.3	1
$ENNA_1$	87± 18	116± 3	74	0.15	0.5
ENNB	73± 20	111± 5	83	0.15	0.5
$ENNB_1$	109± 9	103± 1	89	0.3	1
BEA	103± 9	105± 12	98	0.3	1

3. Results and discussion

3.1. Mycotoxin occurrence in herbal medicines dietary tablets

All studied mycotoxins were detected in the analyzed samples except AFs. ENNB was the most detected mycotoxin (34%), followed by $ENNA_1$ (14%) and $ENNB_1$ (13%) (figure 1). Although ENNB presented the highest incidence, the corresponding mean concentration of positive samples quantified (88.7 µg/kg) located in the bottom of the detected mycotoxins. ZEA showed up at 8% of tablet samples but presented the higher mean concentration (1340.1 µg/kg). OTA was detected in one herbal mix tablet for insomnia but at a considerable concentration (799 µg/kg). BEA was detected in 11% of analyzed tablets, with mean concentration of 137.89 µg/kg (Table 3). Figure 2 shows a chromatogram of a whitethorn tablet naturally contaminated by ENNB.

As mentioned above, European Pharmacopoeia recommendation established a maximum level of AFB_1 as well as total AFs in herbal drugs,

however in the present work AFs were not detected in none of the analyzed samples.

Table 3. Minimum, maximum mycotoxin concentrations, mean of positive samples (µg/kg) and incidences of detected in herbal dietary tablets.

Mycotoxin	ZEA	OTA	ENNA	$ENNA_1$	ENNB	$ENNB_1$	BEA
Minimum Concentration (µg/kg)	116.9	799	3.8	<LOQ	<LOQ	<LOQ	<LOQ
Maximum Concentration (µg/kg)	3850.5	799	170.8	534.9	1378.2	1188.3	542.7
Mean of positive tablets (µg/kg)	1340.1	799	65.5	82.7	88.7	324.9	137.9
Incidence[a]	7/85	1/85	8/85	12/85	29/85	11/85	9/85

[a] number of positive samples/ number of total samples

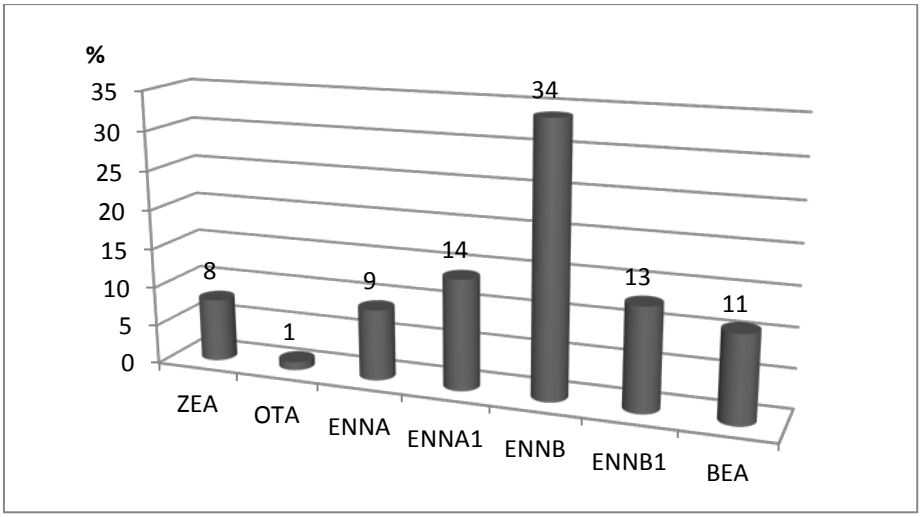

Figure 1. Incidence of mycotoxins (%) detected in analyzed tablets (n=85).

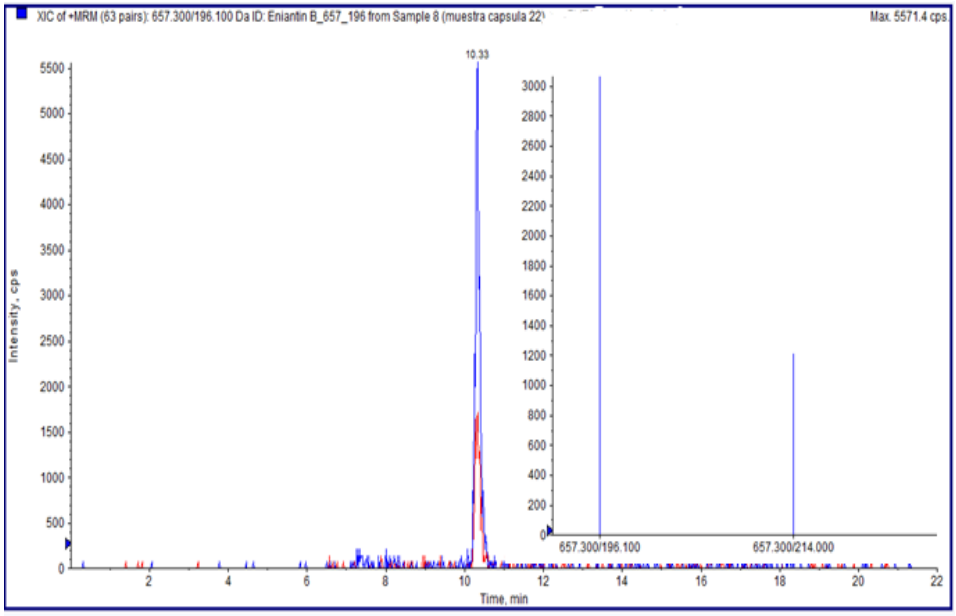

Figure 2. Chromatogram obtained from a sample of whitethorn naturally contaminated by ENNB (12.25 µg/kg) Retention Time= 10.33 min.

Artichoke, green tea, red tea, ginkgo tablets showed no mycotoxin contamination in any of the analyzed samples. The tablets for lose weight resulted also not contaminated, these tablets were made mainly with green tea and fucus, only one of the five samples of individual fucus tablets resulted contaminated by ZEA at 659.73 µg/kg. In a previous study, Pallarés et al. (2017) did not report either contamination of mycotoxins at levels above the limit of quantification in samples of green and red teas prepared as aqueous infusions. The major part of studies available in literature are focused on specific types of medicinal plants supplements, like ginkgo biloba, ginseng, *cardus marianus* or green tea. Di Mavungu et al. (2009) did not detect the presence of any mycotoxin in ginkgo. Contrary to these results, Martínez-Domínguez et al. (2015) observed

the presence of AFB_1, AFB_2, T-2 with incidences of 14, 29 and 29% respectively in seven samples of ginkgo biloba leaves extracts. In multimycotoxin analysis performed in green tea samples, Martínez-Domínguez et al. (2016) only detected AFB_1 in one of ten samples at 5.4 μg/Kg.

At least one of the analyzed tablets of valerian, dandelion, boldus, ginger, passionflower, horsetail, *cardus marianus*, devil's clawroot, whitethorn, lemon balm, fucus and herbal mix used to treat insomnia resulted contaminated by one mycotoxin. Per type of botanical contents, boldus, *cardus marianus*, horsetail and ginger tablets were the most contaminated tablets. These botanicals presented co-occurrence of mycotoxins at levels up to 1000 μg/kg (Table 4). In *cardus marianus*, ENNs were detected with high incidence (75%) and maximum concentrations between 109.19 and 1378.21 μg/Kg. ZEA was not detected. Arroyo-Manzanares et al. (2013) found no mycotoxin presence in a natural extract in vegetable glycerin of *cardus marianus*. Tournas et al. (2012) analyzed AFs presence in 2 samples of alcohol and in 8 samples of oil based *cardus marianus* liquid seed extracts, and observed the presence of AFs in 25% of oil based *cardus marianus* liquid seed extracts samples with mean concentration of positive samples of 0.06 μg/kg. Veprikova et al. (2015) reported also high incidence of trichothecens (13-78%), *Alternaria* toxins (22-97%), ZEA (78%) and ENNs (84-91%) in 32 tested samples of *cardus marianus*. ENNs were also detected at maximum concentrations ranging from 2340 to 9260 μg/Kg and ZEA at maximum concentration of 751 μg/Kg.

Table 4. Mycotoxins contents and incidence per type of botanical plant

Type of herbal medicine tablet (n[a])	Mycotoxin Concentration range μg/kg and Incidence[b]						
	ZEA	OTA	ENNA	$ENNA_1$	ENNB	$ENNB_1$	BEA
Artichoke (4)	nd	nd	nd	nd	nd	nd	nd
Boldus (4)	(1169-1995.9) (3/4)	nd	nd	nd	(1.8-5.4) (3/4)	(<LOQ) (1/4)	nd
Cardus Marianus (4)	nd	nd	(36.6-109.2) (2/4)	(57.6-534.9) (2/4)	(6.2-1378.2) (3/4)	(24.2-1165.9) (2/4)	(<LOQ-542.7) (2/4)
Dandelion (5)	nd	nd	(39.6) (1/5)	(26.6-28.3) (2/5)	(4.8-74.6) (4/5)	(<LOQ-71.5) (3/5)	nd
Devil's Clawroot (4)	(212.6) (1/4)	nd	nd	nd	(2.5-2.7) (2/4)	nd	nd
Fucus (5)	(659.7) (1/5)	nd	nd	nd	nd	nd	nd
Ginger (4)	(3850.5) (1/4)	nd	nd	nd	(3.3-15.1) (2/4)	nd	(95.7-136.8) (3/4)
Ginkgo (4)	nd	nd	nd	nd	nd	nd	nd
Green tea (5)	nd	nd	nd	nd	nd	nd	nd
Horsetail (5)	nd	nd	(12.6-170.8) (2/5)	(35.2-156.5) (2/5)	(7.1-588.6) (3/5)	(377-1188.3) (2/5)	(25.1-52.2) (2/5)
Lemon balm (3)	(117) (1/3)	nd	nd	nd	(6.6) (1/3)	nd	nd

Passionflower (4)	nd	nd	nd	nd	(3.1-9.1) (3/4)	nd	(70.2) (1/4)
Red tea (4)	nd	nd	nd	nd	nd	nd	nd
Valerian (5)	nd	nd	(63.1-88.7) (2/5)	(8.5-42.7) (2/5)	(0.8-27.8) (4/5)	(22.3) (1/5)	nd
Whitethorn (4)	nd	nd	nd	(6.6-12.3) (2/4)	(2.3-15) (2/4)	(10.7-22.2) (2/4)	(47.2) (1/4)
Herbal mix for treat insomnia (16)	nd	(799) (1/16)	(3.8) (1/16)	(<LOQ-1) (2/16)	(<LOQ-1.5) (2/16)	nd	nd
Herbal mix for lose weight (5)	nd	nd	nd	nd	nd	nd	nd

The experiment was not designed to evaluate differences between ecological and conventional tablets, for this reason the number of samples analyzed in the present study is different. The data obtained revealed that 58.3% of ecological samples front 41.1% of conventional samples resulted contaminated by at least one mycotoxin. In ecological samples co-occurrences of two and five mycotoxins were observed front co-occurrences of two, three, four, and five mycotoxins in conventional samples (Table 5). Regarding mycotoxins contents, significant differences ($p<0.05$) were observed for OTA, BEA, $ENNA_1$ and ENNB between ecological and conventional tablets samples after performing the *t* test, with higher contents observed in ecological samples. Not significant differences were observed for the rest of mycotoxins under this study. Very little information was available in bibliography comparing mycotoxins contents in ecological and non-ecological medicinal plant samples. However, for other food matrices like cereals, Pleadin et al. (2017) did not find significant differences in mycotoxin contents in 189 samples of unprocessed cereals and 61 samples of cereal-based products originated from conventional and organic production, except of ZEA and FBs.

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Table 5. Co-occurrence of mycotoxins in studied tablet samples

Co-occurrence of Mycotoxins	Non-ecological samples n=73		Ecological samples n=12	
	Number of samples	**Sum of concentrations (µg/kg)**	**Number of samples**	**Sum of concentrations (µg/kg)**
2 Mycotoxins	(8/73) 11% ZEA+ENNB (3/73) ENNB+BEA (3/73) $ENNA_1$+$ENNB_1$ (1/73) ENNB+$ENNB_1$ (1/73)	 215.25-1998.18 49.53-151.93 17.27 109.21	(3/12) 25% OTA+ENNB (1/12) ENNB+BEA (1/12) ENNB+$ENNB_1$ (1/12)	 799.01 76.64 5.41
3 Mycotoxins	(4/73) 5% $ENNA_1$+ENNB+$ENNB_1$ (2/73) ENNB+$ENNB_1$+ENNA (2/73)	 49.4-157.69 6.28- 128.39	(0/12) 0%	
4 Mycotoxins	(2/73) 3% ENNA+ $ENNA_1$+ ENNB+ $ENNB_1$	 77.48-154.24	(0/12) 0%	
5 Mycotoxins	(3/73) 4% ENNA+ $ENNA_1$+ ENNB+ $ENNB_1$+ BEA	 313.55-1345.02	(1/12) 8% ENNA+$ENNA_1$+ ENNB+$ENNB_1$+ BEA	 3739.86

3.2. Risk assessment

The risk assessment for adult population trough the consumption of herbal dietary supplements was evaluated. For this purpose, the Estimate Daily Intakes (EDIs) to mycotoxins were calculated and compared with their Tolerable Daily Intakes (TDIs).

EDI for each mycotoxin was calculated with the combination of supplement medium recommended dosage and mycotoxins mean concentration in each type of supplements, considering a medium corporal weight of 70 kg. EDI (μg/Kg bw/day)= recommended dosage daily consumption (g/kg bw/day)× medium concentration of each mycotoxin in each type of herbal dietary supplement (μg/g).

The medium concentration for detected mycotoxins has been calculated considering two scenarios, lower bound (LB) and upper bound (UB). In LB, value of zero was assigned to samples where mycotoxins were not detected or were detected at concentrations below LOQ. In UB, the values of LODs were assigned to samples where mycotoxins were not detected, and the values of LOQs were assigned to samples where mycotoxins were detected at concentrations below LOQ (EFSA, 2010).

The EDIs obtained for positive samples were compared with the TDIs established of 0.25 μg/kg bw/day for ZEA (EFSA, 2014) and Tolerable Weekly Intake (TWI) established of 0.12 μg/kg bw/week for OTA (EFSA, 2006) (Table 6). For ZEA, the EDIs obtained represented a percentage from 0.21 to 11.89% of TDI. Boldus tablets were the main contributor to ZEA dietary exposure. OTA, only was present in the group of herbal mix tablets to treat insomnia, and

the EDI obtained reached the 3.57% (LB) and 3.92% (UB) of the TWI established.

For emerging mycotoxins, TDI values have not yet been fixed, but the EDIs obtained in the present work can be compared with the lowest and highest TDI values fixed for other *Fusarium* mycotoxins, DON (1 µg/kg bw/day)(SCF, 2002) and the sum of the toxins HT2 and T2 (0.1 µg/kg bw/day) (EFSA, 2014) (Table 7).

The EDIs obtained for BEA at different scenarios ranged from 0.23 to 3.46% of the TDI established for HT-2 and T-2 and from 0.02 to 0.34% of the TDI established for DON. *Cardus marianus* tablets was the main contributor to BEA dietary exposure. The EDIs obtained for the sum of ENNs reached the 0.0048 to 22.2% of the cited TDI, representing a potential risk horsetail and *cardus marianus* tablets with EDIs that reached 15.6% and 22.2% of TDI, respectively. The percentages obtained decreased to unconcerned (from 0.00048 to 2.2%) when EDIs were compared with the TDI established for DON. In general, the consumption of herbal medicinal supplements at the recommended dosage doesn't suppose a considerable risk even in scarce cases considerable percentages of TDI were reached, highlighting that tablets may constitute an additional source of exposure to mycotoxins and their control is advisable considering that there is no specific regulation for emerging mycotoxins for food or for food supplements.

Table 6. Mycotoxin risk assessment through herbal dietary supplement tablets consumption.

Sample	Recommended dosage (g)	ZEA Concentration µg/g	EDI µg/kg bw/day	%TDI	OTA Concentration µg/g	EDI µg/kg bw/day	%TDI
Boldus	1.83			LB[a]			
		1.135	0.0297	11.87	nd	nd	nd
				UB[b]			
		1.138	0.0297	11.89	nd	nd	nd
Ginger	1.49			LB			
		0.962	0.0205	8.19	nd	nd	nd
				UB			
		0.97	0.0206	8.26	nd	nd	nd
Devil's clawroot	1.16			LB			
		0.053	0.0009	0.35	nd	nd	nd
				LB			
		0.061	0.001	0.4	nd	nd	nd
Lemon balm	0.93			LB			
		0.039	0.0005	0.21	nd	nd	nd
				UB			
		0.046	0.0006	0.24	nd	nd	nd
Fucus	1.3			LB			
		0.131	0.0024	0.98	nd	nd	nd
				UB			
		0.139	0.0026	1.04	nd	nd	nd
Herbal mixed for treat insomnia	0.85			LB			
		nd	nd	nd	0.05	0.0006	3.57
				UB			
		nd	nd	nd	0.05	0.0006	3.92

TDI ZEA (0.25 µg/kgbw/day) (EFSA, 2014); TWI OTA (0.12 µg/kg bw/week) (EFSA, 2006).

[a]LB (lower bound);[b]UB (upper bound).

Table 7. Mycotoxin risk assessment through herbal dietary supplement tablets consumption.

Sample	Recommended dosage (g)	ENN_1 Concentration µg/g	EDI µg/kg bw/ day	%TDI	BEA Concentration µg/g	EDI µg/kg bw/day	%TDI
Valerian	1.4				LB*		
		0.057	0.00114	1.15	nd	nd	nd
					UB*		
		0.059	0.00118	1.18	nd	nd	nd
Dandelion	1.84				LB		
		0.069	0.00183	1.83	nd	nd	nd
					UB		
		0.072	0.00188	1.88	nd	nd	nd
Boldus	1.83				LB		
		0.0024	6.19e-5	0.062	nd	nd	nd
					UB		
		0.0047	0.00012	0.124	nd	nd	nd
Ginger	1.49				LB		
		0.0046	9.79e-5	0.098	0.0914	0.0019	1.95
					UB		
		0.0067	0.00014	0.14	0.0916	0.002	1.95
Passionflower	1.72				LB		
		0.0046	0.00011	0.11	0.018	0.0004	0.43
					UB		
		0.0068	0.00017	0.17	0.018	0.0004	0.44
Horsetail	2.13				LB		
		0.5114	0.01556	15.56	0.015	0.0005	0.47
					UB		
		0.5128	0.0156	15.60	0.016	0.00049	0.49
Cardus Marianus	1.77				LB		
		0.877	0.022	22.18	0.136	0.0034	3.43
					UB		
		0.878	0.022	22.2	0.137	0.0034	3.46
Devil's clawroot	1.16				LB		
		0.0013	2.1555e5	0.022	nd	nd	nd
					UB		
		0.003	4.9727e5	0.05	nd	nd	nd
Whitethorn	1.36				LB		
		0.017	0.0003	0.34	0.012	0.0002	0.23
					UB		
		0.019	0.0004	0.37	0.013	0.0002	0.24

				UB			
		0.0044	5.7914e-5	0.058	nd	nd	nd
Herbal mixed for treat insomia	0.85			LB			
		0.0004	4.77e-6	0.0048			
				UB			
		0.0025	3.088e-5	0.031	nd	nd	nd

TDI WT2 and T2 (0.1 μg/kg bw/day) (EFSA, 2014).

*LB (lower bound);*UB (upper bound).

4. Conclusion

A simultaneous analysis of 11 mycotoxins (AFs, OTA, ZEA, ENNs and BEA) was performed by LC-MS/MS-IT to investigate herbal medicine dietary tablets contamination. 36 of 85 samples analyzed resulted positive for at least one mycotoxin. ZEA, OTA and emerging mycotoxins were present in samples with a mean ranging between 65.53 and 1340.11 μg/Kg and incidences from 1 to 34%. ENNB was the most detected mycotoxin. Co-occurrences from two to five mycotoxins were observed in 25% of the samples. Comparing ecological and conventional samples, no significant differences were observed for mycotoxins contents of ZEA, ENNA and $ENNB_1$. Data obtained showed that the consumption of this kind of herbal medicine at the recommended dosage did not increase mycotoxins exposure risk but vigilance should be kept for high consumers. It is important to highlight that there is an urgent need for an update of specific regulation for emerging mycotoxins in food and in botanical herbs.

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References

Abdel-Tawab, M. (2018). Do we need plant food supplements? A critical examination of quality, safety, efficacy, and necessity for a new regulatory framework. *Planta medica*, **84**, 372-393. https://doi.org/10.1055/s-0043-123764

Ashiq, S., Hussain, M., Ahmad, B. (2014). Natural occurrence of mycotoxins in medicinal plants: a review. *Fungal genetics and Biology*, **66**, 1-10. https://doi.org/10.1016/j.fgb.2014.02.005

Arroyo-Manzanares, N., García-Campaña, A. M., Gámiz-Gracia, L. (2013). Multiclass mycotoxin analysis in Silybum marianum by ultra high performance liquid chromatography–tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid–liquid microextraction. *Journal of Chromatography A,* **1282**, 11-19. https://doi.org/10.1016/j.chroma.2013.01.072

Di Mavungu, J. D., Monbaliu, S., Scippo, M. L., Maghuin-Rogister, G., Schneider, Y. J., Larondelle, Y., Callebaut, A., Robbens, J., Van Peteghem, C., De Saeger, S. (2009). LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. *Food additives and contaminants*, **26**(6), 885-895. https://doi.org/10.1080/02652030902774649

EFSA (European Food Safety Authority) (2006). Opinion of the Scientific panel on contaminants in the food chain on a request from the commission related to Ochratoxin A in food. *EFSA Journal*, **365**, 1 – 56.

EFSA (European Food Safety Authority) (2010). Management of left-censored data in dietary exposure assessment of chemical substances. *EFSA Journal*, **8**(3), 1557.

EFSA (European Food Safety Authority) (2014). Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA Journal*, **12**(12), 3916.

European Commission (2001). Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use. *Official Journal of the European*, L **311**, 67.

European Commission (2002). Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union*, L**221**, 8−36.

European Commission (2004). Directive 2004/24/EC of the European Parliament and of the Council of 31 March 2004 amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use. *Official Journal of the European Union*, L **136**, 85-90.

European Pharmacopoeia Commission. (2016). Determination of Aflatoxin B1 in herbal drugs. In European Pharmacopoeia 9th Edition 2.8.18. Council of Europe: Strasbourg, France, Volume 1, p. 289.

Hudson, A., Lopez, E., Almalki, A. J., Roe, A. L., Calderón, A. I. (2018). A review of the toxicity of compounds found in herbal dietary supplements. *Planta medica*, **84,** 613-626. https://doi.org/10.1055/a-0605-3786

Kosalec, I., Cvek, J., Tomić, S. (2009). Contaminants of medicinal herbs and herbal products. *Arhiv za higijenu rada i toksikologiju,* **60**(4), 485-500. https://doi.org/10.2478/10004-1254-60-2009-2005

Marin, S., Ramos, A. J., Cano-Sancho, G., Sanchis, V. (2013). Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food and Chemical Toxicology*, **60**, 218-237. https://doi.org/10.1016/j.fct.2013.07.047

Martínez-Domínguez, G., Romero-González, R., Frenich, A. G. (2015). Determination of toxic substances, pesticides and mycotoxins, in ginkgo biloba nutraceutical products by liquid chromatography Orbitrap-mass spectrometry. *Microchemical Journal*, **118**, 124-130. https://doi.org/10.1016/j.microc.2014.09.002

Martínez-Domínguez, G., Romero-González, R., Frenich, A. G. (2016). Multi-class methodology to determine pesticides and mycotoxins in green tea and royal jelly supplements by liquid chromatography coupled to Orbitrap high resolution mass spectrometry. *Food chemistry,* **197**, 907-915. https://doi.org/10.1016/j.foodchem.2015.11.070

Pallarés, N., Font, G., Mañes, J., Ferrer, E. (2017). Multimycotoxin LC–MS/MS analysis in tea beverages after dispersive liquid–liquid Microextraction (DLLME). *Journal of agricultural and food chemistry*, **65**, 10282-10289. https://doi.org/10.1021/acs.jafc.7b03507

Pleadin, J., Staver, M. M., Markov, K., Frece, J., Zadravec, M., Jaki, V., Krupić, I., Vahčić, N. (2017). Mycotoxins in organic and conventional cereals and cereal products grown and marketed in Croatia. *Mycotoxin research,* **33**(3), 219-227. https://doi.org/10.1007/s12550-017-0280-3

Qu, L., Zou, W., Wang, Y., Wang, M. (2018). European regulation model for herbal medicine: The assessment of the EU monograph and the safety and efficacy evaluation in marketing authorization or registration in Member States. *Phytomedicine,* **42**, 219-225. https://doi.org/10.1016/j.phymed.2018.03.048

Scientific 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 by the SCF on 26 February 2002). Report SCF/CS/ CNTM/MYC/27; European Commission Scientific Committee on Food: Brussels, Belgium.

Tournas, V. H., Sapp, C., Trucksess, M. W. (2012). Occurrence of aflatoxins in milk thistle herbal supplements. *Food Additives & Contaminants: Part A,* **29**(6), 994-999. https://doi.org/10.1080/19440049.2012.664788

Troncoso, A.M. (2019). La evaluación y regulación de los complementos alimenticios en Europa. *Revista de Toxicología,* **36** (1), 13.

Veprikova, Z., Zachariasova, M., Dzuman, Z., Zachariasova, A., Fenclova, M., Slavikova, P., Vaclavikova, M., Mastovska, K., Hengst, D., Hajslova, J. (2015). Mycotoxins in plant-based dietary supplements: Hidden health risk for consumers. *Journal of agricultural and food chemistry*, **63**(29), 6633-6643. https://doi.org/10.1021/acs.jafc.5b02105

3.5. Multimycotoxin LC-MS/MS analysis in tea beverages after dispersive liquid-liquid microextraction (DLLME)

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Multimycotoxin LC-MS/MS analysis in tea beverages after dispersive liquid-liquid microextraction (DLLME)

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ABSTRACT

The aim of the present study was to develop a multimycotoxin Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) method with a dispersive liquid-liquid microextraction procedure (DLLME) for the analysis of AFs, 3aDON, 15aDON, NIV, HT-2, T-2, ZEA, OTA, ENNs and BEA in tea beverages and to evaluate their mycotoxin contents. The proposed method was characterized in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision), reproducibility (inter-day precision), and matrix effects to check suitability. The results show LODs (range: 0.05-10 µg/l), LOQs (range: 0.2-33 µg/l) and recoveries (range: 65-127%, RSD <20%). The method developed in this study has been applied to 44 commercial samples of black tea, red tea, green tea and green mint tea. The results show that of the analyzed mycotoxins, AFB2, AFG2, 15aDON, AFG1 and ENB were detected in the samples. AFB2 (14.4-32.2 µg/l) and 15aDON (60.5-61 µg/l) presented the highest levels. Green mint tea contained the highest concentration of mycotoxins. The risk assessment study shows that population is not much exposed to mycotoxins through the tea beverages consumption.

KEYWORDS: mycotoxins; tea beverage; LC-MS/MS-IT; DLLME extraction, risk assessment.

INTRODUCTION

The term mycotoxins refers to a variety of highly toxic compounds produced from different substrates as a result of fungal secondary metabolism.[1] Mycotoxins appear in the food chain as a result of fungal infection of crops destined for consumption by humans or for use as livestock feed. The toxigenic fungal species most often found in foods belong to the genera *Aspergillus*, *Fusarium*, *Penicillium, Alternaria* and *Claviceps*. Aflatoxins (AFs) are produced by species of *Aspergillus*; ochratoxin A (OTA) is produced by both *Aspergillus* and *Penicillium*; *Fusarium* species produces trichothecenes (HT-2, T-2, *deoxynivalenol* (DON) as well as nivalenol (NIV)), zearalenone (ZEA), fumonisins (FB1 and FB2) and emerging mycotoxins (fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA) and enniatins (ENs)).[2] Mycotoxins commonly occur in food and can appear in the field before harvesting, after harvesting, or during processing, storage and feeding.[3] Mycotoxins cause adverse effects such as hepatotoxicity, nephrotoxicity, immunosuppressive activity, mutagenicity, teratogenicity, carcinogenicity, estrogenicity and diabetogenic action.[4] Specifically, AFs are known as genotoxic and carcinogenic compounds relationed with hepatocellular carcinoma, furthermore have effects on the reproductive and on the immune system. OTA is recognized as teratogenic, genotoxic, carcinogenic and immunotoxic compound and also as a potent nephrotoxic. FBs present carcinogenicity and cardiovascular toxic effects. ZEA is relationed with reproductive problems. Finally, T-2 inhibit protein synthesis and mitocondrial function and shows immunosupressive and cytotoxic effects.[2]

The consumption of natural products such as teas has increased in recent years due to cultural or psychological factors. Actually, tea is currently the second most popular drink after water.[5] Tea production plays a major role in the

economy of several countries in Asia and Africa.[6] The FAO prevision for the next 10 years indicates a production growth of 1.9% annually in the case of black tea and of 4.5% annually in the case of green tea, which is increasingly consumed in western countries.[7] Assurance of the safety, quality, and efficacy of teas has become a key issue in the global tea market.[8] Tea has many benefits for health including antioxidant, antiinflammatory, antiproliferative, antimutagenic, antibacterial and antiviral effects, and also affords protection against cardiovascular disease, hyperglycaemia, metabolic disorders and some cancers.[9] However, tea is susceptible to mycotoxin contamination, and the levels of toxigenic fungi can increase during tea manufacture and processing, especially if the conditions are not appropriate. Tropical climate and poor storage are suitable environmental conditions for toxigenic mould growing, drought or insect and mechanical damages during cultivation or storage can also contribute.[10] Different studies have identified toxigenic mold species capable of producing mycotoxins in teas.[11] Most studies on the presence of mycotoxins in raw tea material have investigated the presence of AFs, FBs, OTA or ZEA.[12,13,14] To the best of our knowledge, no data are available on emerging mycotoxins, which have been studied in medicinal plants.[15] The European Commission has established maximum limits (MLs) of mycotoxins such as AFs, OTA, FBs and trichothecenes in various foods, however no MLs have been established for teas.[16]

Food processing in the industrial or home setting can influence mycotoxin content. The effect of tea infusion process on the mycotoxins contents has been studied by some studies: Malir et al.[17] investigated the transfer of OTA into tea beverages from raw materials and observed a transfer of 34.8% for black tea and of 4.1% for fruit tea. Toman et al.[18] obtained a transfer of 41.5% of OTA in

black tea prepared according to the Turkish tradition method. To the best of our knowledge, only Monbaliu et al.[12] studied multimycotoxins contamination in tea or infusion beverages ready to drink, and found no mycotoxins in tea drinkable products prepared from raw tea and herbal infusion materials.

Most methods for the extraction of mycotoxins involve liquid-liquid extraction or solid phase extraction using C18 cartridges or immunoaffinity clean-up columns. However, in the recent years, a new liquid phase microextraction method known as dispersive liquid-liquid microextraction (DLLME) has been developed. This method offers some advantages with respect to the traditional techniques in that it is simple, fast and low cost.[19] The technique has been used for the analysis of different analytes in food aqueous matrixes such as OTA in wine [20] or ZEA in beer.[21] Furthermore DLLME technique has been developed for the multimycotoxin analysis, Tolosa et al.[22] assessed the presence of 15 mycotoxins in water and fish plasma, Rodríguez-Carrasco et al.[23] used this method for the analysis of *Alternaria* toxins (alternariol, alternariol methyl-ether, tentoxin) in tomato and tomato-based products and Serrano et al.[24] for study the migration of enniatins from pasta to pasta cooking water. However no information on the application of this method in analysing mycotoxins in tea beverages was found in the literature.

The aim of the present study was to develop and validate a mycotoxin Liquid Chromatography Tandem Mass Spectrometry Ion Trap (LC-MS/MS-IT) method for multimycotoxin (AFs, 3aDON, 15aDON, NIV, HT-2, T-2, ZEA, OTA, ENA, ENA1, ENB, ENB1 and BEA) using samples prepared following the DLLME extraction procedure. The validated method was applied to different samples of tea purchased from supermarkets in order to determine the mycotoxins content in tea beverages ready for human consumption. Then, the

risk exposure of the population through to these mycotoxins by the consumption of tea ready for consumption was determined.

MATERIAL AND METHODS

Reagents and chemicals

HPLC grade acetonitrile (ACN) and methanol (MeOH) were supplied by Merck (Darmstadt, Germany). Chloroform ($CHCl_3$) (99%) was obtained from Merck (Darmstadt, Germany). Ethyl acetate (EtOAc) (HPLC grade 99.5+ %) was purchased from Alfa Aesar (Karlsruhe, Germany). Carbon tetrachloride (CCl_4) (99%) was obtained from Panreac (Barcelona, Spain). Deionized water (resistivity > 18 MΩ cm-1) was prepared in the laboratory using a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, USA). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Formic acid (reagent grade ≥ 95%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). All solvents were filtered through a 0.45-μm cellulose filter supplied by Scharlau (Barcelona, Spain) prior to use. All samples were passed through a 13 mm/0.22 μm nylon filter from Membrane Solutions (TX, USA) before injection. The standards of AFB1 (≥98% purity), AFB2 (≥98%), AFG1 (≥98%), AFG2 (≥98%), 3aDON (≥99.3%), 15aDON (≥99.3%), NIV (≥98.2%), HT-2 (≥98%), T-2 (≥98%), ZEA (≥99%), OTA (≥98%), BEA (≥97%), ENA (≥95%), ENA1 (≥95%), ENB (≥95%) and ENB1 (≥95%) were purchased from Sigma (St. Louis, MO, USA). Individual stock solutions containing a concentration of 100 mg/l were prepared in methanol. The appropriate working solutions were prepared starting from individual stock solutions. All solutions were prepared and stored in the dark at −20°C until LC–MS/MS-IT analysis.

Sample collection

A total of 44 commercial samples of tea bags were purchased from different supermarkets in Valencia (Spain). The 44 samples were classified into 12 samples of black tea, 14 samples of red tea, 10 samples of green tea, and 8 samples containing a mixture of green tea and mint. The samples were stored in a dark and dry place until analysis.

Sample preparation

The preparation of tea beverages for consumption was performed according to the instructions of the manufacturer. Briefly the contents of a tea bag (approximately 1.5 g) were infused in 200 ml of boiling water for 5 minutes in a glass container. Next, the tea bag was removed and the tea was used in the analysis.

Extraction procedure

A 5-ml aliquot of the tea beverage sample was placed in a 10-ml conical tube and 1 g of NaCl was added. After vortexing the tube for one minute, the mixture of the dispersion solvent (950 µl of ACN) and extraction solvent (620 µl of EtOAc) was added, the mixture shaken for one minute forming a cloudy solution of the three components. The mixture was centrifuged 4000 rpm for 5 min, and the organic phase at the top of the tube was recovered and placed in to a second conical tube. Next, the mixture of dispersion solvent (950 µl of MeOH) and extraction solvent (620 µl of $CHCl_3$) was added to the remaining residue, and after agitation and centrifugation the separated organic phase was recovered and added to the first organic phase. The solvent in the conical tube containing the two recovered phases was evaporated to near dryness under a

nitrogen stream using a Turvovap LV Evaporator (Zymark, Hoptikinton, USA). The residue was reconstituted with 1 ml of 20 mM ammonium formate (MeOH/ACN) (50/50 v/v) and filtered through a 13 mm/0.22 μm nylon filter (Membrane Solutions, TX, USA). Next, 20μl of the filtrate was injected into the LC-MS/MS-IT system.

LC-MS/MS analysis

The LC-MS/MS analysis was performed using an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with Turbo Ion Spray (ESI) electrospray ionization. The QTRAP analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. The separation of analytes was performed in a Gemini-NX column C_{18} (Phenomenex, 150 mm x 4.6 mm, 5 particle size) preceded by a guard column. The flow rate was maintained at 0.25 ml/min, and the oven temperature at 40ºC. The two elution mobile phases were made up of water acidified with 5 mM ammonium formate, 0.1% formic acid (mobile phase A) and methanol acidified with 5 mM ammonium formate, 0.1% formic acid (mobile phase B). The elution gradient started with a proportion of 0% for eluent B; in 10 min was increased to 100%, then decreased to 80% in 5 min, and finally decreased to 70% in 2 min. In the next 6 min, the column was cleaned, readjusted to the initial conditions, and equilibrated for 7 min.

The analysis was performed using the Turbo Ion Spray in positive ionization mode (ESI+). Nitrogen served as nebulizer and collision gas. The following parameters were used during the analysis: ion spray voltage 5500 V; curtain gas

20 arbitrary units; GS1 and GS2 with 50 and 50 psi, respectively; probe temperature (TEM) 450ºC.

The fragments monitored (retention time, quantification ion and confirmation ion), and spectrometric parameters (declustering potential, collision energy and cell exit potential) are shown in Table 1.

Table 1. LC-MS/MS parameters.

Mycotoxin	RetentionTime (min)	DP[a]	Precursor ion	Quantification ion Q			Confirmation ion q		
				CE[b]	Product ion	CXP[c]	CE	Product ion	CXP
AFB1	9.13	46	313.1	39	284.9	4	41	241.0	4
AFB2	9.03	81	315.1	33	286.9	6	39	259.0	6
AFG1	8.86	76	329.0	39	243.1	6	29	311.1	6
AFG2	9.37	61	331.1	27	313.1	6	39	245.1	4
HT2	9.52	21	442.2	19	262.8	4	19	215.4	8
T2	9.82	21	484.3	29	215.1	4	22	185.1	4
3aDON	12.8	44	339.2	20	231.1	3	20	203.1	3
15aDON	12.8	50	339.2	20	137.0	3	20	261.1	3
NIV	9.09	50	313.4	80	115.1	3	27	175.1	3
ZEA	10.40	26	319.0	15	301.0	10	19	282.9	4
OTA	10.27	55	404.3	97	102.1	6	27	239.0	6
ENA	12.62	76	699.4	35	210.1	14	59	228.2	16
ENA1	12.22	66	685.4	37	210.2	8	59	214.2	10
ENB	11.60	51	657.3	39	196.1	8	59	214.0	10
ENB1	11.89	66	671.2	61	214.1	10	57	228.1	12
BEA	12.00	116	801.2	27	784.1	10	39	244.1	6

[a] DP: decluster potential (volts)[b]; CE: collision energy (volts)[c]; CXP: cell exit potential (volts)

RESULTS AND DISCUSSION

Optimization of the DLLME extraction procedure

Different parameters were tested to optimize the DLLME extraction, namely the nature of the dispersion and extraction solvent, and the volume of extraction solvent. For optimization studies, the quantity of NaCl was determined to be 1 g, based on the results reported by other authors.[24] The optimization of various solvents and volumes was carried out through recovery experiments performed in triplicate, comparing absolute peak areas of each analyte in blank sample spiked before the extraction and absolute peak areas of each analyte spiked after the extraction procedure. The blank sample used was a sample of green tea (in which none of the analysed mycotoxins were detected) and was spiked with a concentration approximately equivalent to 10 LOQ of each mycotoxin: 0.1 mg/l for HT2, T2, 3aDON, NIV, OTA and AFB2; 0.01 mg/l for AFB1, AFG1, AFG2, ENA, ENA1 and BEA; and 0.001 mg/l for ENB, ENB1, 15aDON and ZEA.

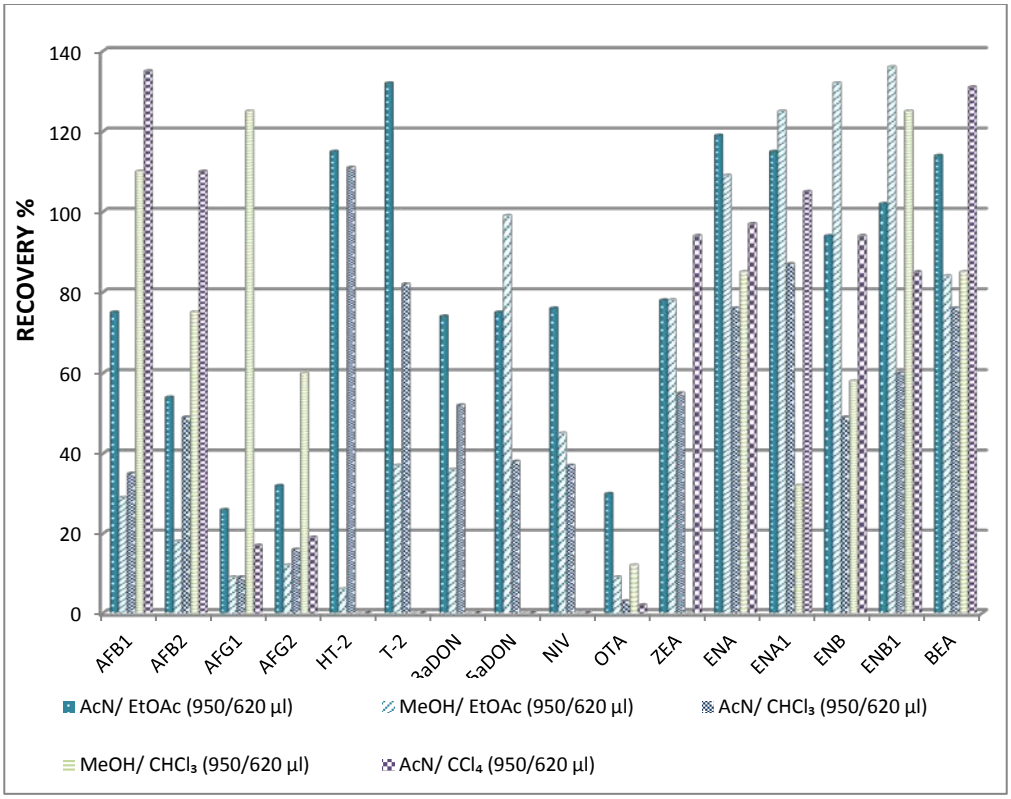
We first selected the dispersion and extraction solvents. Methanol (MeOH) and acetonitrile (ACN) were tested as dispersion solvents; and chloroform ($CHCl_3$), ethyl acetate (EtOAc) and carbon tetrachloride (CCl_4) were tested as extraction solvents. Figure 1 shows the effects of different combinations of dispersion and extraction solvents, and volume of extraction solvent, upon the recoveries of each studied mycotoxin.

The best recovery results for most of the mycotoxins studied (except AFs) were obtained using a combination of ACN and EtOAc (Figure 1a). For AFs, the best results were obtained with a combination of MeOH and $CHCl_3$. Therefore, extraction was carried out using two combinations of dispersion and

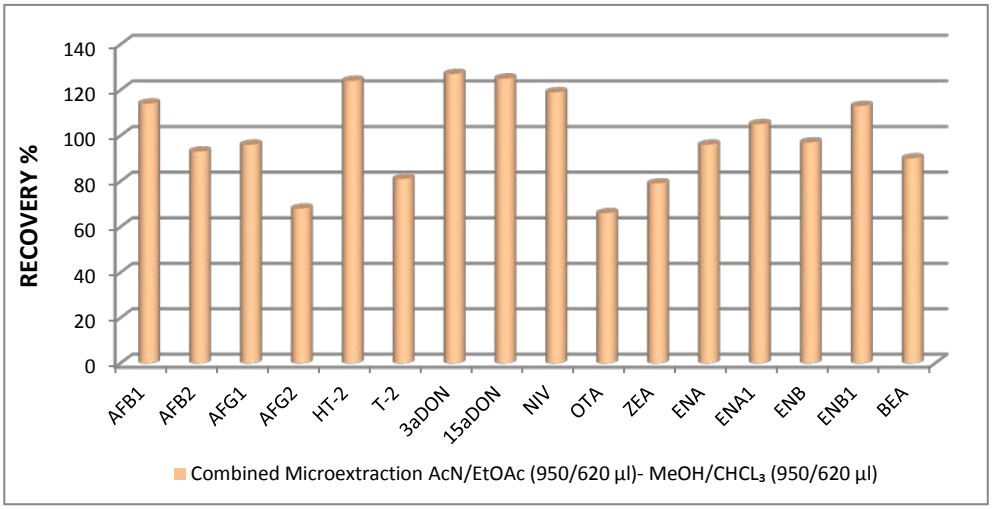
extraction solvents: ACN-EtOAc was used in the first step and MeOH-$CHCl_3$ in second step (Figure 1b).

Different volumes (500, 620 and 700 µl) of extraction solvent were then tested combined with 950 µl of dispersion solvent. The best recovery results, ranging from 66-127%, were obtained with the volume of 620 µl of extraction solvent (Figure 1c).

a)



b)



c)

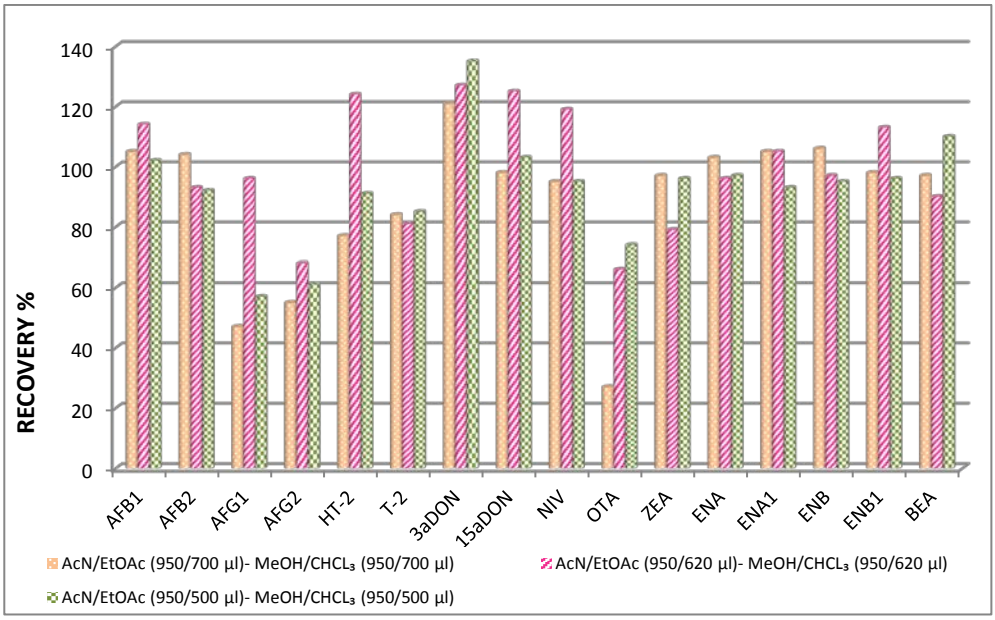


Figure 1. DLLME extraction procedure optimization performed by recoveries experiments in triplicate comparing absolute peaks area of analytes spiked in blank samples before and after the extraction. (a) Effect of different extraction and dispersion solvents on recoveries. (b) Recoveries obtained in combined microextraction with selected solvents ACN/EtOAc (950/620 µl)- MeOH/$CHCL_3$ (950/620 µl). (c) Effect of different volumes (500, 620 and 700 µl) of extraction solvents (EtOAc and $CHCL_3$) on recoveries.

Validation of the method

The proposed method was characterized in terms of linearity, limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision), reproducibility (inter-day precision), and matrix effects to check suitability. The analytical parameters characterized are shown in Table 2.

For the determination of linearity we constructed calibration curves for each studied mycotoxin standard dissolved in methanol and from standards in blank sample (a sample of green tea without mycotoxins) at concentration levels ranging from LOQ of each mycotoxin to 1000 μg/l. All studied mycotoxins presented good linearity, with correlation coefficients (r^2) between 0.992 and 0.999.

Matrix effects (MEs) occur because some substances present in the matrix modify the instrumental response of the analyte, resulting in enhancement or suppression of the analyte signal. For the evaluation of matrix effects, signal suppression/ enhancement (SSE) was compared based on the slope of calibration curves performed in methanol and in blank sample as follows:

SSE (%) = 100* slope with matrix / slope without matrix

SSE values higher than 100% indicate enhancement of the signal; SSE values lower than 100% indicate suppression of the signal; and SSE values near 100% indicate no significant matrix effect.

The results shown in Table 2 indicate that there was no SSE for most of the mycotoxins, though some of them, such as 3aDON, NIV and ZEA presented SSE - in all cases comprising suppression of the signal, except in the case of NIV, where enhancement was recorded. For this reason, the other

analytical parameters were determined using extracts of blank sample to minimize these matrix effects.

The LODs and LOQs were obtained spiking blank sample (green tea sample free of mycotoxins) with decreasing concentrations of studied analytes. The LODs and LOQs were calculated using the criterion of S/N ≥ 3 for LOD or S/N ≥ 10 for LOQ. The results obtained (LODs from 0.05 to 10 μg/l and LOQs from 0.2 to 33 μg/l) are lower than those obtained with Monbaliu et al.[12] that observed LOQs between 0.8 and 93 μg/l in the same analytical matrix.

Recoveries were evaluated by spiking blank sample with each analyte at two concentrations levels in triplicate. The low spiked level was 10xLOQ whilst the high spiked level was 100xLOQ for the determination of intra-day and inter-day precision. Intra-day precision was assessed based on three determinations on the same day, and inter-day precision was assessed based on three determinations on non-consecutive days. The experiments were performed by comparing absolute peak areas for each analyte in blank samples spiked before the extraction procedure and absolute peak areas in blank samples spiked after extraction. The results obtained were in accordance with the limits established by Commission Decision (2002/657/EC).[25] The recoveries ranged between 65 and 127%, and were within the relative standard deviation (<20%) (Table 2). Data from the 100xLOQ experiments are not shown in Table 2 because they were of little relevance.

Table 2. Analytical parameters for method validation: recoveries, intra-day and inter-day precisions, matrix effects and limits of detection and quantification.

Mycotoxin	Recovery[c] ± RSD[d] (%)		SSE (%)[b]	LOD[a]	LOQ[a]
	Intra-day precision	Inter-day precision			
AFB1	114± 7	111± 10	78	0.7	2.3
AFB2	93± 5	88± 6	102	2.4	8.0
AFG1	96± 6	91± 8	91	0.7	2.4
AFG2	68± 9	67± 11	82	0.5	1.6
HT2	124 ± 5	119 ±9	87	10	33
T2	81± 8	84± 11	81	9	30
3aDON	127±9	121± 12	47	8	27
15aDON	125 ± 5	117± 9	113	0.1	0.3
NIV	119± 11	115± 15	119	9.6	32
ZEA	79± 7	76± 9	60	0.05	0.2
OTA	66± 3	65± 5	69	5	17
ENA	96± 5	93± 8	75	0.4	1.4
ENA1	105± 5	104± 7	78	0.2	0.7
ENB	97± 4	94±5	107	0.05	0.2
ENB1	113± 6	110±9	70	0.1	0.3
BEA	90±8	87± 12	96	1	3.2

[a] LOD and LOQ are limits of detection and quantification, respectively (µg/l)
[b] SSE: Signal Suppression-Enhancer
[c] Recoveries: Analysis performed at concentrations of 10xLOQ
[d] RSD: Relative standard deviation (calculated injecting samples in triplicate)

Application to real samples

Occurrence and concentration levels of mycotoxins

The developed multimycotoxin method was applied to analyse 44 commercial tea samples.

Of the 16 analysed mycotoxins, AFB1, 3aDON, NIV, HT2, T2, ZEA, OTA, ENB1, ENA1, ENA and BEA were not detected in any of the samples. The mycotoxins that were most commonly found were: AFG2 (18% of positive samples) and AFB2 (14% of positive samples). AFG1 presented incidences of 9%. Mycotoxins showing the lowest incidence were 15aDON and ENB (5%). Figure 2 shows a chromatogram and the ion product spectrum corresponding

to a sample of green tea with mint that was contaminated with 15aDON (61µg/l).

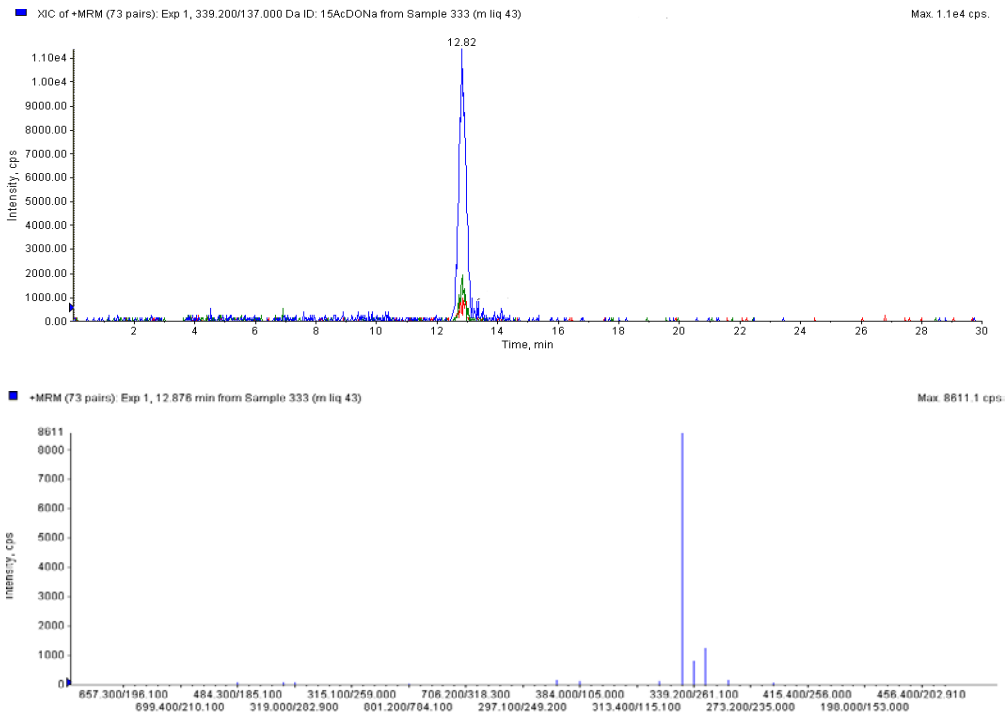


Figure 2. Chromatogram and mass spectrum of the ion product obtained from a sample of green mint tea naturally contaminated by 15aDON (61 µg/l). Retention time = 12.82 min.

Table 3 shows the incidence and the contents of mycotoxins per type of tea and in the global samples. Black tea and red tea were both positive for AFG2 but at concentrations below quantification limits. Green tea was positive for ENB at levels below quantification limit. Green tea with mint contained the highest amount of mycotoxins, with detected levels greater than > the LOQ for AFB2, AFG2 and 15aDON. AFB2 presented levels ranging from 14.4-32.2 µg/l, AFG2 was in the range of 1.9-2.6 µg/l and 15aDON presented levels from

60.5-61 µg/l. AFG1 was detected at levels below quantification limit. The levels obtained for AFB2 were higher than the limits set for AFs in spices, established as 10 µg/kg for the sum of AFs by Commission Regulation (2006),[16] however AFB2 was detected above the acceptable limits for spices in only a few of the samples of green tea containing mint. For other types of food like nuts, has been established the same limit for total AFs (10 µg/kg). Comparing our results with the results obtained in this type of matrix, for example in pistachios, the authors Cano-Sancho et al.,[26] obtained an incidence of 20% with a mean of 8.9 µg/kg for total AFs, slightly lower results than those obtained in our study, but similar in the sense that like in our case some samples exceeded the established limits.

As has been mentioned above, to the best of our knowledge the study of Monbaliu et al.[12] is the only study about the multimycotoxin presence in tea beverages. These authors investigated the presence of NIV, DON, neosolaniol (NEO), fusarenon-X, 3-acetyl-deoxynivalenol (3aDON), 15-acetyl-deoxynivalenol (15aDON), AFG2, AFG1, AFB2, AFB1, sterigmatocystin, OTA, FB1, alternariol, alternariol methyl ether, altenuene, HT-2, T-2, diacetoxyscirpenol, ZEA, mycophenolic acid, paxilline, fumigaclavin and citrinin in 91 samples of tea beverages collected in China and Belgium and unlike our study found no mycotoxins in any sample.

However, how not data about mycotoxins levels in tea beverages is available, we have compared our results with the results obtained by other authors who studied mycotoxins in raw tea, herbal infusion and medicinal plant materials.

Table 3. Incidence, concentration range and mean of mycotoxins in positive tea samples (µg/l).

Mycotoxin	Sample analyzed				
	Black tea	**Red tea**	**Green tea**	**Green mint tea**	**Total samples**
	I^a(n/N) Concentration range and mean in positive samples	I^a(n/N) Concentration range and mean in positive samples	I^a(n/N) Concentration range and mean in positive samples	I^a(n/N) Concentration range and mean in positive samples	I^a(n/N) Concentration range and mean in positive samples
AFB2	(0/12) nd	(0/14) nd	(0/10) nd	(6/8) 14.4-32.2 (26)	(6/44) 14.4-32.2 (26)
AFG1	(0/12) nd	(0/14) nd	(0/10) nd	(4/8) < LOQ	(4/44) <LOQ
AFG2	(2/12) <LOQ	(2/14) < LOQ	(0/10) nd	(4/8) 1.9-2.6 (2.3)	(8/44) <LOQ-2.6 (1.9)
15aDON	(0/12) nd	(0/14) nd	(0/10) nd	(2/8) 60.5-61 (60.7)	(2/44) 60.5-61 (60.7)
ENB	(0/12) nd	(0/14) nd	(2/10) < LOQ	(0/8) nd	(2/44) <LOQ

* nd, not detectable
I^a, Incidence
n, number of positive samples
N, total number of samples

The presence of AFs in raw tea has been studied by many authors. Haas et al.[14] studied the presence of AFs in 36 samples of raw Pu-erh tea (a type of post-fermented tea) and found that none of the samples exceeded the LOQ (1.7µg/kg). Pouretedal & Mazaheri [27] analyzed 40 samples of raw black tea including Iranian and imported tea and detected AFs with the follows incidences: AFB1 (27.5%), AFB2 (5%), AFG2 (7.5%). AFG1 was not detected. The mean detected for AFB1 was 10.0 µg/Kg and for total AFs 12.1 µg/Kg. In our study, the aflatoxin showing the highest levels in the tea beverages was AFB2 (14.4-32.2µg/l) (14% incidence), followed by AFG2 (< LOQ to 2.6 µg/l) (18%

incidence). AFG1 was detected with incidence of 9% at the LOQ, while AFB1 was not detected (table 3). Comparing our results with Haas et al.[14], in the present study AFB2 and AFG2 showed higher incidences and concentrations, higher incidences too than those obtained in the study performed by Pouretedal & Mazaheri [27] (5% and 7.5% respectively). For other part, in the case o black tea, the type of tea analyzed by Pouretedal & Mazaheri [27], as is mentioned above, in our study only has been detected AFG2 with incidence of 17% at levels <LOQ.

No data was available on the presence of trichothecenes in teas and only a small number of studies on medicinal herbs have been reported. Sharma et al.[28] did not find DON in any of the raw medicinal herb samples they analysed. In our study,15aDON was detected only in two tea samples at the concentrations of 60.5 µg/land 61µg/l, respectively. The other trichothecenes were not detected. In contrast, Arroyo-Manzanares et al.[29] found high concentrations of T2 and TH2 in seeds of Cardus marianus, at levels ranging from 363 µg/kg to 453.9 µg/kg in the case of T2 and from 826.9 µg/kg to 943.7 µg/kg in the case of HT2. However, T2 an TH2 were not found in tea samples.

In our study, ZEA was not detected in any tea beverage samples. In contrast, Kong et al.[30] found ZEA in 12% of raw medicinal plant materials they tested, and the levels were in concentrations ranging from 5.3µg/kg to 295.8µg/kg.

ENB was the only *Fusarium* emerging mycotoxin that was detected, at levels less that the LOQ. No reports were available on the presence of ENB mycotoxins in tea, but in medicinal plants Hu &Rychlik[15] recorded an incidence of 25%, with concentrations ranging between 2.5-751µg/kg. The previous results differ from ours, possibly because we analysed prepared ready-to-drink

tea beverages, and some authors have reported that these mycotoxins are sensitive to heat and likely to be destroyed during high heat food processing.[24]

Exposure assessment

The purpose of risk assessment is measuring the presence of contaminants to characterize the distribution of one or more substances in the food with the aim of estimating exposure to the substances in the population when consuming average or extremely high amounts of food. This kind of estimation requires knowledge of the entire range of contaminant concentrations.[31]

To calculate the dietary exposure to mycotoxins trough tea consumption, is necessary to combine information of levels of mycotoxins in tea beverages with the data of tea consumption. Data on mycotoxins concentrations in tea, was obtained analyzing 44 samples of tea beverages prepared for the consumption before the analysis. For calculate the estimated daily intake (EDI), the mean values of mycotoxins concentrations were obtained considering two different approaches: lower bound levels (LB) and upper bound levels (UB). In the LB approach the mean values were obtained assigning zero to samples where mycotoxins were no detected or were detected at levels below quantification limits, whereas in the UB approach limit of detection values were assigned to samples where mycotoxins were no detected and limit of quantification values were assigned to samples in which the levels of mycotoxins where below than quantification limits.

Data of annual consumption of tea was consulted in the data base of the Spanish Ministry of Agriculture, Food and Environment.[32] Annual consumption of tea raw material was 0.12 kg, equivalent to 16 l of tea ready for consumption. The Estimate Daily Intakes (EDIs) of each mycotoxin through tea consumption was calculated as EDI tea (μg/kg bw/day) = tea daily consumption (l/kg bw/

day) × mean value of mycotoxins concentration in teas (μg/l).To obtained the risk characterization for each mycotoxin, the EDIs were compared with the tolerable daily intakes (TDIs) μg/kg bw/day established by European Commission.

AFs are genotoxic carcinogens compounds, so is not possible to determine the threshold levels which aflatoxins have no effect, therefore no TDI can be recommended. It is recommended that the concentrations of aflatoxins in food be reduced to As Low As Reasonably Achievable (ALARA).

For 15aDON has been used the TDI value set for DON (1 μg/kg bw/day).[33] There is not available TDI value established for emerging mycotoxins, so the risk assessment is not possible. However, is possible to perform approximation to the estimation of the risk assessment. For this, we have compared the EDIs obtained for the emerging mycotoxin ENB with the safety guidelines established for other *Fusarium* mycotoxins, in this case we have used the lowest and highest TDI values established for the other Fusarium mycotoxins: DON (1 μg/kg bw/day)[33] and sum of T-2 and HT-2 toxins (0.1 μg/kg bw/day).[34]

The calculated EDIs values and the comparison with the TDIs for assess the risk characterization are shown in table 4. AFB2, AFG1, AFG2, 15aDON and ENB were the mycotoxins detected in tea samples, but for AFs, as is explained above, performer the risk characterization is not possible. The EDIs calculated for 15aDON were 1,74 ng/kg bw/day (LB approach) and 1.80 ng/kg bw/day (UB approach), the % TDI were 0.17 and 0.18 % in the LB and UB approach respectively.

The EDI calculated for ENB only has been performed in the UB approach, because has been detected in the samples at levels below of

quantification limits. For the risk characterization the EDI for ENB in the UB approach (0.038 ng/kg bw/day) has been compared with the lower and higher TDI value of *Fusarium* mycotoxins (0.1 µg/kg bw/day and 1 µg/kg bw/day respectively). The results were 0.0038 % and 0.038% of the TDI.

In both mycotoxins, 15aDON and ENB, the EDI values were far below than TDI values.

Table 4. Estimate daily Intakes and risk characterization of population to mycotoxins through the consumption of tea beverages.

Mycotoxin	**LB approach**				
	Mean concentration (µg/L)	**Daily consumption (l/kg bw/day)**	**Estimate Daily Intakes (ng/kg bw/day)**	**Tolerable Daily Intakes (ng/kg bw/day)**	**Risk Characterization (EDI/TDI)*100**
15aDON	2.76	0.00063	1.74	1000	0.17%
	UB approach				
15aDON	2.86	0.00063	1.80	1000	0.18%
ENB	0.06	0.00063	0.038	1000[a]	0.0038%
ENB	0.06	0.00063	0.038	100[b]	0.038%

[a] TDI DON (1 µg/kg bw/day) (SCF, 2002)
[b] TDI sum of T-2 and HT-2 toxins (0.1 µg/kg bw/day) (EFSA, 2014).

On the basis of the results obtained from the analysis of mycotoxins in tea beverages, we can conclude that the analysis of derived drinkable samples of commercial tea products show a low incidence of mycotoxins. Furthermore, the levels of mycotoxins detected in all types of tea analysed were low, exception for AFB2 and 15aDON in few samples of green tea with mint. The risk assessment shows that the intake of mycotoxins through tea beverages consumption not represent a risk for the population. Nevertheless, the limited presence of

mycotoxins in tea could increase exposure to mycotoxins in heavy tea consumers.

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REFERENCES

(1) Hathout, A. S.; Aly, S. E. Biological detoxification of mycotoxins: a review. *Ann Microbiol.* **2014**, 64(3), 905-919.

(2) Marin, S.; Ramos, A. J.; Cano-Sancho, G.; Sanchis, V. Mycotoxins: occurrence, toxicology, and exposure assessment. *Food Chem Toxicol.* **2013**, 60, 218-23.

(3) Serrano, A.B.; Font, G.; Ruiz M.J.; Ferrer, E. Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. *Food Chem.* **2012**, 135, 423-429.

(4) Da Rocha, M. E. B.; Freire, F. D. C. O.; Maia, F. E. F.; Guedes, M. I. F.; Rondina, D. Mycotoxins and their effects on human and animal health. *Food Control* **2014**, *36*(1), 159-165.

(5) McKinley, H.; Jamieson, M. Handbook of Green Tea and Health Research; Nova Publishers: New York, 2009.

(6) Hazarika, L. K.; Bhuyan, M.; Hazarika, B. N. Insect pests of tea and their management. *Annu. Rev. Entomol.* **2009**, 54, 267-284.

(7) FAO. Current situation and medium-term outlook. Eighteenth Session of Committee on Commodity Problems Intergovernmental of Group on Tea in Hangzhou, China, 14 – 16 May 2008. (ftp://ftp.fao.org/docrep/fao/meeting/013/k2054E.pdf).

(8) Ahmad, B.; Ashiq, S.; Hussain, A.; Bashir, S.; Hussain, M. Evaluation of mycotoxins, mycobiota, and toxigenic fungi in selected medicinal plants of Khyber Pakhtunkhwa, Pakistan. *Fungal biol.* **2014**, 118(9), 776-784.

(9) El-Aty, A. Residues and contaminants in tea and tea infusions: a review. *Food Addit.Contam., Part A* **2014**, 31(11), 1794-1804.

(10) Ashiq, S.; Hussain, M.; Ahmad, B. Natural occurrence of mycotoxins in medicinal plants: a review. *Fungal Genet. Biol.* **2014**, 66, 1-10.

(11) Khosravi, A. R.; Nosrati, A.; Shokri, H. Protein Profiles of Aspergillus Species Isolated From the Tea Gardens and Factories Air in Northern Iran. *Jundishapur J.Microbiol.* **2012**, *6*(1), 4-10.

(12) Monbaliu, S.; Wu, A.; Zhang, D.; Van Peteghem, C.; De Saeger, S. Multimycotoxin UPLC− MS/MS for tea, herbal infusions and the derived drinkable products. *J. Agric. Food Chem.* **2010**, 58(24), 12664-12671.

(13) Bokhari, F. M.; Aly, M. M. Unexpected hazard due to Fumonisins contaminating herbal teas used traditionally by Saudi people. *Afr. J. Microbiol. Res.* **2013**, *7*(1), 35-40.

(14) Haas, D.; Pfeifer, B.; Reiterich, C.; Partenheimer, R.; Reck, B.; Buzina, W. Identification and quantification of fungi and mycotoxins from Pu-erh tea. *Int. J. Food Microbiol.* **2013**, 166(2), 316-322.

(15) Hu, L.; Rychlik, M. Occurrence of enniatins and beauvericin in 60 Chinese medicinal herbs. *Food Addit. Contam., Part A* **2014**, 31(7), 1240-1245.

(16) European Commission. Commission Directive 2006/1881/EC of 19 December 2006, setting maximum levels for certain contaminants in food stuffs. Off. J. Eur. Union 2006, L364, 5-24.

(17) Malir, F.; Ostry, V.; Pfohl-Leszkowicz, A.; Toman, J.; Bazin, I.; Roubal, T. Transfer of ochratoxin A into tea and coffee beverages. *Toxins* **2014**, *6*(12), 3438-3453.

(18) Toman, J.; Malir, F.; Ostry, V.; Kilic, M. A.; Roubal, T.; Grosse, Y.; Pfohl-Leszkowicz, A. Transfer of ochratoxin A from raw black tea to tea infusions prepared according to the Turkish tradition. ***J. Sci. Food Agric.*** **2017**. http://dx.doi.org/10.1002/jsfa.8464

(19) Zgoła-Grześkowiak, A.; Grześkowiak, T. Dispersive liquid-liquid microextraction. *Trends Anal. Chem. TrAC* **2011,** 30(9), 1382-1399.

(20) Arroyo-Manzanares, N.; Gámiz-Gracia, L.; García-Campaña, A. M. Determination of ochratoxin A in wines by capillary liquid chromatography with laser induced fluorescence detection using dispersive liquid–liquid microextraction. *Food Chem.* **2012**, 135(2), 368- 372.

(21) Antep, H. M.; Merdivan, M. Development of new dispersive liquid–liquid microextraction technique for the identification of zearalenone in beer. *Anal. Methods* **2012**, 4(12), 4129- 4134.

(22) Tolosa, J.; Font, G.; Mañes, J.; Ferrer, E. Multimycotoxin analysis in water and fish plasma by liquid chromatography-tandem mass spectrometry. *Chemosphere* **2016**, 145, 402-408.

(23) Rodríguez-Carrasco, Y.; Mañes, J.; Berrada, H.; Juan, C. Development and Validation of a LC-ESI-MS/MS Method for the Determination of Alternaria Toxins Alternariol, Alternariol Methyl-Ether and Tentoxin in Tomato and Tomato-Based Products. *Toxins* **2016** , 8(11), 328.

(24) Serrano, A. B.; Font, G.; Mañes, J.; Ferrer, E. Development a mitigation strategy of enniatins in pasta under home-cooking conditions. *LWT-Food Sci. Technol.* ***2016***, 65, 1017-1024.

(25) European Commission. Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Off. J. Eur. Union* 2002, L221, 8−36.

(26) Cano-Sancho, G.; Sanchis, V.; Marín, S.; Ramos, A. J. Occurrence and exposure assessment of aflatoxins in Catalonia (Spain). *Food Chem Toxicol.* **2013**, 51, 188-193.

(27) Pouretedal, Z.; Mazaheri, M. Aflatoxins in black tea in Iran. *Food Addit. Contam., Part B* **2013**, 6(2), 127-129.

(28) Sharma, S.; Sumbali, G.; Sharma, V. Mycobial contamination and mycotoxinogenesis of Tinosporacordifolia: an important medicinal plant of India. *International Journal of Agricultural Research, Innovation and Technology* **2013**, *3*(2), 16- 21.

(29) Arroyo-Manzanares, N.; García-Campaña, A. M.; Gámiz-Gracia, L. Multiclass mycotoxin analysis in Silybummarianum by ultra high performance liquid chromatography–tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid–liquid microextraction. *J. Chromatogr. A* **2013**, 1282, 11- 19.

(30) Kong, W. J.; Shen, H. H.; Zhang, X. F.; Yang, X. L.; Qiu, F.; Ou-yang, Z.; Yang, M. H. Analysis of zearalenone and α-zearalenol in 100 foods and medicinal plants determined by HPLC-FLD and positive confirmation by LC-MS-MS. *J. Sci. Food Agric.* **2013**, 93(7), 1584- 1590.

(31) EFSA (European Food Safety Authority). Management of left-censored data in dietary exposure assessment of chemical substances. *EFSA Journal* 2010, 8(3), 96. doi:10.2903/j.efsa.2010.1557.

(32) MAGRAMA. Ministry of Agriculture, Food and Environment, 2013. Data on food consumption, Tea consumption (2013). Statistics database-home consumption. http://www.mapama.gob.es/

(33) SCF. 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. https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_fusarium_out123_en.pdf

(34) EFSA (European Food Safety Authority). Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA Journal* 2014*, 12(12)*, 3916

3.6. Occurrence of Mycotoxins in Botanical Dietary Supplement Infusion Beverages

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Occurrence of Mycotoxins in Botanical Dietary Supplement Infusion Beverages

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ABSTRACT

The aim of the present work was to study the ocurrence of mycotoxins [aflatoxins (**1-4**), 3-acetyldeoxyniavlenol (**5**), 15-acetyldeoxynivalenol (**6**), nivalenol (**7**), HT-2 (**8**), T-2 (**9**), ochratoxin A (**10**), zearalenone (**11**), enniatin A (**12**), enniatin A1 (**13**), enniatin B (**14**), enniatin B1 (**15**), and beauvericin (**16**)] present in potable products derived from herbal teas. Analysis was carried out by liquid chromatography coupled to ion-trap tandem mass spectrometry (LC-MS/MS-IT) after a dispersive liquid-liquid microextraction procedure (DLLME), was conducted. The DLLME method was applied to 52 commercial samples of chamomile, chamomile with anise, chamomile with honey, linden, pennyroyal mint, thyme, valerian and horsetail beverages. The results obtained showed that the following mycotoxins were detected in the samples: **2** (19.1 to 134.7 μg/L), **3** (below the limit of quantification) and **4** (2.2 to 13.5 μg/L). Also, **6** was detected in one sample at 112.5 μg/L and **14** was detected only in two samples, although at very low concentration levels. Pennyroyal mint and thyme showed the highest concentration levels of mycotoxins. A risk assessment, however, showed negative results regarding the consumption of herbal tea beverages and the presence of mycotoxins.

Herbal teas are infusions of dried plant species, such as leaves, flowers, seeds, fruits, and roots, and are prepared using warm or hot water.[1] Herbal teas have purported health- promoting properties based on the antioxidative potential of their extracts, due to the occurrence of commonly occurring compounds such as carotenoids, flavonoids, and other phenolic substances, and additional secondary metabolites with potential anti-inflammatory or cancer chemopreventive properties.[2] However, plants used as teas, can also be a substrate for the growth of toxigenic fungi, which may lead to the production of mycotoxins during production, processing, transport, and storage.

Mycotoxins are secondary metabolites produced by filamentous fungi, with no apparent function in the primary metabolism of the organism, and are normally produced when the fungus reaches maturity.[3] Molds responsible for the production of mycotoxins mainly belong to the genera *Fusarium*, *Aspergillus,* and *Penicillium*. It has been estimated that at least 300 of these fungal metabolites are potentially toxic to animals and humans.[4] Aflatoxins **1**-**4** are produced by species of *Aspergillus*, while ochratoxin A (**10**) is produced by the genera *Aspergillus* and *Penicillium*. The *Fusarium* species produces trichothecenes [HT-2 (**8**), T-2 (**9**), deoxynivalenol (DON) and nivalenol (**7**)], zearalenone (**11**), fumonisins FB1 and FB2, and emerging mycotoxins [fusaproliferin (FUS), moniliformin (MON), beauvericin (**16**) and enniatins (**12**-**15**)].[5] These mycotoxins can contribute to human health problems: in fact, aflatoxins **1**-**4** are among the most powerful mutagens and carcinogens known. The International Agency for Research on Cancer (IARC) has classified them as Group 1 human carcinogens,[6] and their toxic effects comprise genotoxicity, teratogenicity and immunosuppressive activity. Compound **10** induces nephrotoxic, teratogenic, genotoxic, neurotoxic, hepatotoxic and immunosuppressive effects. Fumonisins

are associated with human esophageal cancer, while other toxic effects include hepatotoxicity, nephrotoxicity, and immunosuppressive activity.[7] Mycotoxin **11** causes reproductive tract alterations that may result in severe reproductive disorders, infertility, and changes in serum progesterone levels.[8] Trichothecene **9** is a potent inhibitor of protein synthesis and mitochondrial function in vitro and in vivo, and exhibits immunosuppressive and cytotoxic effects. Deoxynivalenol inhibits DNA and protein synthesis and causes immunosuppression.[9] With respect to emerging mycotoxins of *Fusarium*, fusaproliferin is toxic for human B lymphocytes, while moniliformin inhibits several enzymes, and the emerging mycotoxins **12**-**16** are cytotoxic with the capacity to disrupt intracellular ionic homeostasis.[5] The European Commission has not established maximal concentration levels for mycotoxins in herbal infusions,[10] but in similar products, such as spices, the European Commission has set maximum limits for **10** and aflatoxins **1**-**4**. A maximum limit of 15 μg/kg has been legislated for **10** in *Piper* spp. (pepper), *Myristica fragrans* (nutmeg), *Zingiber officinale* (ginger), and *Curcuma longa* (turmeric), and of 20 μg/kg in *Capsicum* spp., and of 15 μg/kg in mixtures of these spices.[11] In regard to aflatoxins **1**-**4**, 5 μg/kg has been fixed as the maximum limit for aflatoxin **1** and 10 μg/kg for the sum of **1**-**4** in the abovementioned spices.[12]

Some authors have reported the presence of toxigenic fungi in medicinal herbs, aromatic herbs and herbal teas.[13,14] Most studies on herbal teas have analyzed the raw material before preparation of the derived potable products. Compounds **1**-**4**, **10** and fumonisins are the most well-studied mycotoxins in this type of matrix, and some authors have reported their presence in herbal raw materials.[15-17] Few information is available about the presence of mycotoxins in herbal teas beverages. In a prior study performed in these matrixes, mycotoxins

were not detected.[15] However, some samples were positive for mycotoxins in a previous study focused on *Camellia sinensis* beverages ready for human consumption.[18]

Therefore, the aim of the present work was to investigate the presence of mycotoxins (**1**-**16**) in potable preparations derived from different herbal tea possible samples available on the market. For this purpose, the samples were extracted by a dispersive liquid-liquid microextraction procedure (DLLME) and analyzed subsequently by liquid chromatography coupled to ion-trap tandem mass spectrometry (LC-IT-MS/MS). Then, with the results obtained, a risk assessment procedure was performed. Compounds **2**-**4**, **6** and **14** were detected in the analyzed samples. In contrast, compounds **1**, **5**, **7**-**13**, **15**, and **16** were not detected in any herbal tea beverage sample. The mycotoxins that were most frequently found were **2** (10%), **4** (10%), and **3** (8%). Mycotoxin **14** appeared in two samples and **6** appeared in one sample. Figure S1 (Supporting Information, annex 2) shows a chromatogram corresponding to a sample of thyme contaminated with **4**. In relation to the different types of herbal tea beverages investigated, Table 1 shows the incidence and the concentration means of the mycotoxins detected. Mycotoxins were not detected in any sample of conventional chamomile, chamomile with anise, and chamomile with honey and linden. In contrast, pennyroyal mint and thyme were positive for more than one aflatoxin (**1**-**4**) at levels above the limit of quantification. Pennyroyal mint samples were found to be contaminated with **2** (three out of ten samples), **3** (two out of ten samples), and **4** (one out of ten samples), with concentration means of 71.9 μg/L, less than the limit of quantification, and 3.8 μg/L, respectively. Thyme samples were also positive for **2** (two out of six samples), **3** (one out of six samples), and **4** (two out of six samples) with concentration means of 112.2

μg/L, less than the limit of quantification, and 4.7 μg/L, respectively. Horsetail samples were positive for **3** (one out of four) and **4** (one out of four) at levels below the limit of quantification, and 2.2 μg/L, respectively. Valerian samples were positive only for one of the aflatoxins (**1**-**4**), with one out of four samples containing **4** at a concentration of 13.5 μg/L.

Table 1. Mean Incidence and Concentration (μg/L) of Mycotoxins Detected in Types of Herbal Tea Beverages that Were Positive for Mycotoxins.

mycotoxin	sample analyzed			
	pennyroyal mint	thyme	valerian	horsetail
	I[b](n/t) concentration mean	I (n/t) concentration mean	I (n/t) concentration mean	I (n/t) concentration mean
AFB2 (**2**)	(3/10) 71.9	(2/6) 112.2	(0/4) nd[c]	(0/4) nd[c]
AFG1 (**3**)	(2/10) <LOQ	(1/6) <LOQ	(0/4) nd[c]	(1/4) <LOQ
AFG2 (**4**)	(1/10) 3.8	(2/6) 4.7	(1/4) 13.5	(1/4) 2.2
15aDON (**6**)	(0/10) nd[c]	(0/6) nd[c]	(1/4) 112.5	(0/4) nd[c]
ENB (**14**)	(0/10) nd[c]	(0/6) nd[c]	(0/4) nd[c]	(2/4) <LOQ

[a] n: number of positive samples, t: total number of samples.
[b] Incidence.
[c] nd: not detectable.

Regarding prior studies cited in the bibliography, in an investigation on *Camellia sinensis* tea beverages prepared for human consumption, the results obtained were similar to those obtained in the present study. Hence, **2**, **4**, **3**, **6**, and **14** were the mycotoxins detected in the samples, and **2** (14.4-32.2 μg/L) and **6** (60.5-61 μg/L) occurred at the highest levels.[18] Earlier, Monbaliu et al.[15] studied the presence of mycotoxins in herbal teas. These authors analyzed the presence of mycotoxins in beverages obtained from raw herbal and tea materials that resulted in contamination by mycotoxins. One raw herbal sample was

contaminated with fumonisin B1, and the other 15 raw herbal samples contained traces of alternariol, alternariol methyl ether, deoxynivalenol, **6**, sterigmatocystin, fumonisin B1 and **9**, but no mycotoxins were found in the beverages. In the present work, therefore, the results are compared with data obtained in prior works on raw herbal teas or medicinal plant materials before processing.

Thus, aflatoxin B2 (**2**) exhibited the highest levels (19.1 to 134.7 μg/L) in the herbal tea beverages investigated, while aflatoxin G2 (**4**) was found at levels ranging from 2.2 to 13.5 μg/L; aflatoxin G1 (**3**) was present at levels below the limit of quantification, and aflatoxin B1 (**1**) was not detected. These results are similar to those obtained by Ahmad et al.[19] who found 30% of 30 raw medicinal plants to be contaminated with aflatoxins **1**-**4** at levels between 2.3 and 37.4 μg/kg. In contrast to this study, Romagnoli et al.[20] found no aflatoxins (**1**-**4**) in any of the 48 analyzed samples of medicinal plants and herbal teas before processing. One valerian beverage sample was positive for **6** at a concentration of 112.5 μg/L. Contrary to the present results, Sharma et al.[21] did not find DON in any dried sample of *Tinospora cordifolia,* an Indian medicinal plant. With regard to the emerging mycotoxins, only **14** was detected in two out of four horsetail samples, but at levels below the limit of quantification. Only one study has been published concerning the presence of emerging mycotoxins in medicinal plants, Hu and Rychlik[22] analyzed 60 types of dried Chinese medicinal plant samples and found compounds **12**-**16** in 25% of these at concentration levels ranging from 2.5 to 751 μg/kg. The present results therefore differ from those obtained by these authors, perhaps because herbal tea beverages were analyzed, not raw materials prior to preparation of the infusions. Some authors have found these mycotoxins to be thermosensitive in the context of food processing.[23] Finally, mycotoxins **10** and **11** were not detected in any of the herbal infusion samples.

However, Zhang et al.[24] analyzed 107 samples of different traditional Chinese raw medicinal plants and only found **11** in one type ("coix seed") with levels between 18.7 and 211.4 μg/kg. In general, only a few samples were contaminated by some mycotoxins, and the incidence was low; thus, the majority of herbal infusions beverages were not contaminated by mycotoxins.

The risk assessment to mycotoxins through herbal teas beverages was assessed. The Estimate Daily Intake (EDI) of each mycotoxin was calculated as herbal tea daily consumption × mean value of mycotoxin concentration. Data on the annual consumption of herbal teas for the Spanish population were estimated as 0.12 kg.[25] Because the Spanish population is not a high consumer of herbal tea, we performed a risk assessment with the values of consumption from the British population, and were estimated at 1.84 kg.[26] These values of raw materials are equivalent to 16 and 245 liters, respectively, of herbal tea consumption per year/per person on average. The EDIs were calculated using two different approaches: upper bound levels (UB), limit of quantification values were assigned to samples where mycotoxins were detected at levels below quantification limits and limits of detection were assigned to samples where mycotoxins were not detected; and lower bound levels (LB), in this approach, samples where mycotoxins were not detected or were detected at levels below of quantification, were assigned as zero.

Compounds **2-4**, **6** and **14** were detected in the samples, and the EDIs were compared with tolerable daily intakes (TDIs) for assessment of the risk characterization (Table S2, Supporting Information annex 2).[27,28] In the case of aflatoxins (**1-4**), no TDI can be recommended, because these are genotoxic and carcinogenic, so it is not possible to perform a risk characterization for these

compounds. Compounds **6** and **14** had no established TDI value. However, the EDIs calculated can be compared with the lowest and highest TDIs established for other *Fusarium* mycotoxins: DON[27] and/or the sum of toxins **8** and **9**.[28] In all the cases, the EDI values were far below the TDIs established, so the risk assessment did not show a high risk for the Spanish and United Kingdom populations due to mycotoxins after herbal tea consumption, although the risk could increase in consumers, who ingest greater than normal quantities of herbal teas.

EXPERIMENTAL SECTION

General Experimental Procedures. HPLC grade acetonitrile (CH_3CN) and methanol (MeOH) were supplied by Merck (Darmstadt, Germany). Chloroform ($CHCl_3$) (99%) was obtained from Merck. Ethyl acetate (EtOAc) (HPLC grade 99.5%) was purchased from Alfa Aesar (Karlsruhe, Germany). Carbon tetrachloride (CCl_4) (99%) was obtained from PanReac (Barcelona, Spain). Deionized water (resistivity >18 MΩ cm^{-1}) was generated in the laboratory using a Milli-Q SP Reagent Water System (Millipore Corporation, Bedford, MA, USA). Ammonium formate (99%) was supplied by PanReac. Formic acid (reagent grade ≥ 95%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). All solvents were filtered through a 0.45 μm cellulose filter supplied by Scharlau (Barcelona, Spain). All samples were filtered through a 13 mm/0.22 μm nylon filter from Membrane Solutions (Dallas, TX, USA) before injection. Standards of mycotoxins **1-16** were purchased from Sigma. Individual stock solutions with a concentration of 100 mg/L were prepared in methanol. The appropriate working solutions were prepared from individual stock solutions.

All solutions were prepared and stored in darkness at −20 °C until LC–MS/MS-IT analysis.

Plant Material. Altogether, 52 samples of herbal teas corresponding to different commercial brands packaged in a single-dose container were collected from different supermarkets in Valencia (Spain) (Table S3, Supporting Information). The types of samples were: eight samples of chamomile (*Matricaria chamomilla* L.) (A), five samples of chamomile with honey (B), six samples of chamomile with anise (C), nine samples of linden (*Tilia europaea* L.) (D), ten samples of pennyroyal with mint (*Mentha pulegium* L.) (E), six samples of thyme (*Thymus vulgaris* L.) (F), four samples of valerian (*Valeriana officinalis* L.) (G), and four samples of horsetail (*Equisetum arvense* L.) (H). All samples were stored in their original packaging in a dark and dry place until analysis. For each botanical dietary supplement (A-H), an accession voucher number was provided (Table S3, Supporting Information annex 2). All the samples were located at the Laboratory of Toxicology, University of Valencia.

Sample Preparation. The preparation of products ready for consumption was carried out according to the instructions of the manufacturer: the contents of an herbal tea bag (approximately 2 g) were infused in 200 mL of boiling water for 5 min in a specific glass container. Then, the herbal tea bag was removed, and analysis of the resulting beverage was performed.

Dispersive Liquid-Liquid Microextraction (DLLME) Procedure. Most methods for the extraction of mycotoxins involved liquid-liquid extraction or, solid-phase extraction with C_{18} cartridges or with immunoaffinity clean-up

columns. However, in recent years, a new liquid-phase microextraction method has been developed, namely, dispersive liquid-liquid microextraction (DLLME). This method offers some advantages with respect to traditional techniques, including simplicity, rapidity, and low cost.[29] The DLLME technique usually has been used for the analysis of different analytes in matrices, such as wine or beer,[30,31] so it can be applied also to herbal infusion beverages.

For the DLLME extraction procedure, 5 mL of a previously prepared herbal tea were placed in a 10 mL conical tube, and 1 g of NaCl was added. After vortexing the tube for 1 min, a mixture of dispersant solvent (950 μL of CH_3CN) and extractant solvent (620 μL of EtOAc) was added, the solution was shaken for 1 min, and a cloudy solution of the components was formed. Then, centrifugation was performed at 4000 rpm for 5 min, and the organic phase at the top of the tube was recovered and placed in another conical tube. Next, a mixture of dispersant solvent (950 μL of MeOH) and extractant solvent (620 μL of $CHCL_3$) was added to the remaining residue, and after shaking and centrifugation, the sediment corresponding to the organic phase was recovered and added to the previously separated first organic phase. The conical tube with the two recovered phases was evaporated to near dryness under a nitrogen stream using a TurvoVap LV Evaporator (Zymark, Hoptikinton, MA, USA). The evaporated residue was reconstituted with 1 mL of 20 mM ammonium formate ($MeOH/CH_3CN$) (50/50 v/v) and filtered through a 13 mm/0.22 μm nylon filter (Membrane Solutions) prior to the injection of 20 μL into the LC-MS/MS-IT system.[18]

LC-MS/MS Analysis. The MS/MS analysis was performed using an Agilent 1200 chromatography system (Agilent Technologies, Palo Alto, CA, USA) equipped with a 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with Turbo Ion Spray electrospray ionization (ESI). The QTRAP analyzer combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. The separation of analytes was performed in a Gemini-NX column C_{18} (Phenomenex, 150 mm x 4.6 mm, 5 particle size) preceded by a guard column. The flow rate was maintained at 0.25 mL/min, and the oven temperature was 40 ºC. The mobile phases for elution were water acidified with 5 mM ammonium formate, 0.1% formic acid (mobile phase A) and methanol acidified with 5 mM ammonium formate, 0.1% formic acid (mobile phase B). The elution gradient started with a proportion of 0% eluent B; after 10 min, it increased to 100% eluent B, then decreased to 80% eluent B for 5 min, and finally decreased to 70% eluent B for 2 min. Then, during the subsequent 6 min, the column was cleaned, re-adjusted to the initial conditions, and equilibrated for 7 min. The injection volume was 20 µL.

The analysis was performed using the Turbo ion-spray in the positive-ionization mode (ESI+). Nitrogen served as the nebulizer and collision gas. The following parameters were used for the analysis: ion spray voltage 5500 V; curtain gas 20 arbitrary units; GS1 and GS2, 50 and 50 psi, respectively; and probe temperature (TEM), 450 ºC.

The fragments monitored (quantification ion and confirmation ion), and spectrometric parameters (declustering potential, collision energy and cell exit potential) are shown in Table S4 (Supporting Information annex 2).

Method Validation. The dispersive liquid-liquid microextraction method was characterized in terms of recoveries, repeatability (intra-day precision), reproducibility (inter-day precision), matrix effects, linearity and limits of detection (LODs) and limits of quantification (LOQs) in a previous study.[18] The intra-day and inter-day recoveries performed at a concentration of 10×LOQ ranged from 66±3 to 127±9% and from 65±5 to 121±12%, respectively. Signal Suppression/Enhancement for matrix effects characterization ranged from 47 to 119%, indicating that there was no SSE for most of the mycotoxins, except for **5** (47%), **7** (119%) and **11** (60%). The linearity registered was good, with correlation coefficients (r^2) between 0.993 and 0.999. The calculated LODs and LOQs values ranged from 0.05 μg/L (**14** and **11** mycotoxins) to 10 μg/L (**8**) and from 0.2 μg/L (**14** and **11**) to 33 μg/L (**8**), respectively. The results are shown in Table S5 (Supporting Information annex 2).

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REFERENCES

(1) Oh, J.; Jo, H.; Cho, A. R.; Kim, S. J.; Han, J. *Food Control* **2013**, *31*, 403-409.

(2) Farzaneh, V.; Carvalho, I. S. *Ind. Crop. Prod.* **2015**, *65*, 247-258.

(3) Da Rocha, M. E. B.; Freire, F. D. C. O.; Maia, F. E. F.; Guedes, M. I. F.; Rondina, D. *Food Control* **2014,** *36*, 159-165.

(4) Hathout, A. S.; Aly, S. E. *Ann. Microbiol.* **2014**, *64*, 905-919.

(5) Marin, S.; Ramos, A. J.; Cano-Sancho, G.; Sanchis, V. *Food. Chem. Toxicol.* **2013**, *60*, 218-237.

(6) IARC (International Agency for Research on Cancer). *Monographs on the Evaluation of Carcinogenic Risks to Humans: Chemical Agents and Related Occupations.* **2012**, 100F.

(7) Ashiq, S.; Hussain, M.; Ahmad, B. *Fungal Genet. Biol.* **2014**, *66*, 1-10.

(8) Tatay, E.; Meca, G.; Font, G.; Ruiz, M. J. *Rev. Toxicol.* **2014**, *31*, 187-195.

(9) Steinmetz, W. E.; Robustelli, P.; Edens, E.; Heineman, D. *J. Nat. Prod.* **2008**, *71*, 589-594.

(10) European Commission. *Commission Directive 2006/1881/EC of 19 December 2006, setting Maximum Levels for Certain Contaminants in Foodstuffs*. OJEU, **2006**, *L 364*, 5-24.

(11) European Commission. *Commission Regulation (EC) 2015/1137/EC of 13 July 2015, amending Regulation no. 1881/2006, as Regards the Maximum Level of Ochratoxin A in Capsicum spp. spices*. OJEU, **2015**, *L 185*, 11-12.

(12) European Commission. *Commission Regulation (EC) 165/2010 of 26 February 2010, amending Regulation no. 1881/2006, setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins*. OJEU, **2010**, *L 50*, 8-12.

(13) Rizzo, I.; Vedoya, G.; Maurutto, S.; Haidukowski, M.; Varsavsky, E. *Microbiol. Res.* **2004**, *159*, 113-120.

(14) Rodríguez Tito, J. C.; Puente, E.; Marín, D.; Pérez, N.; Ferrer, D.; Cedeño, N. *B. Latinoamer. Caribe Pl.* **2012**, *11*, 218-222.

(15) Monbaliu, S.; Wu, A.; Zhang, D.; Van Peteghem, C.; De Saeger, S. *J. Agric. Food Chem.* **2010**, *58*, 12664-12671.

(16) Waśkiewicz, A.; Beszterda, M.; Bocianowski, J.; Goliński, P. *Food Microbiol.* **2013**, *36*, 426-431.

(17) Tosun, H.; Arslan, R. *Sci. World J.* **2013**.

(18) Pallarés, N.; Font, G.; Mañes, J.; Ferrer, E. *J. Agric. Food Chem.* **2017**, *65*, 10282-10289.

(19) Ahmad, B.; Ashiq, S.; Hussain, A.; Bashir, S.; Hussain, M. *Fungal Biol.* **2014**, *118*, 776-784.

(20) Romagnoli, B.; Menna, V.; Gruppioni, N.; Bergamini, C. *Food Control* **2007**, *18*, 697-701.

(21) Sharma, S.; Sumbali, G.; Sharma, V. *Int. J. Agric. Res. Innov. Technol.* **2013**, *3*, 16- 21.

(22) Hu, L.; Rychlik, M. *Food. Addit. Contam. A* **2014**, *31*, 1240-1245.

(23) Serrano, A. B.; Font, G.; Mañes, J.; Ferrer, E. *LWT-Food Sci. Technol.* **2016**, *65*, 1017-1024.

(24) Zhang, X.; Liu, W.; Logrieco, A. F.; Yang, M.; Ou-yang, Z.; Wang, X.; Guo, Q. *Food. Addit. Contam. A* **2011**, *28*, 885-893.

(25) Spanish Ministry of Agriculture, Food and Environment. *Data on Food Consumption*, **2018**. http://www. mapama.gob.es/ (accessed July 15, 2018).

(26) Food and Agriculture Organization. *Food Balance Sheets*, **2013**. http://www.fao.org/faostat/en/#data/FBS (accessed July 15, 2018).

(27) Scientific Committee on Food. *Opinion on Fusarium toxins Part 6: Group of T-2 Toxin, HT-2 Toxin, Nivalenol and Deoxynivalenol (adopted by the SCF on 26 February 2002)*.https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_fusarium_out123_en.pdf (accessed December 10, 2017).

(28) EFSA (European Food Safety Authority). *Scientific Opinion on the Risks for Human and Animal Health Related to the Presence of Modified Forms of Certain Mycotoxins in Food and Feed. EFSA J*, **2014**, *12*, 3916-4023. https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2014.3916(accessed January 23, 2018).

(29) Zgoła-Grześkowiak, A.; Grześkowiak, T. *Trends. Anal. Chem.* **2011**, *30*, 1382-1399.

(30) Arroyo-Manzanares, N.; Gámiz-Gracia, L.; García-Campaña, A. M. *Food Chem.* **2012**, *135*, 368- 372.

(31) Antep, H. M.; Merdivan, M. *Anal. Methods* **2012**, *4*, 4129- 4134.

3. 7. Mycotoxin Dietary Exposure Assessment through Fruit Juices Consumption in Children and Adult Population

Toxins (2019)

Mycotoxin Dietary Exposure Assessment through Fruit Juices Consumption in Children and Adult Population

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Abstract: Consumption of fruit juice is becoming trendy for consumers seeking freshness and high vitamin and low caloric intake. Mycotoxigenic moulds may infect fruits during crop growth, harvest, and storage leading to mycotoxin production. Many mycotoxins are resistant to food processing, which make their presence in the final juice product very likely expected. In this way, the presence of 30 mycotoxins including aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2), alternariol (AOH), alternariol monomethyl ether (AME), Ochratoxin A (OTA), fumonisin B1 (FB1), fumonisin B2 (FB2), enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1), beauvericin (BEA), *sterigmatocystin (*STG), zearalenone (ZEA), α-zearalanol (α-ZAL), β-zearalanol (β-ZAL), α-zearalenol (α-ZOL), β-zearalenol (β-ZOL), *deoxynivalenol* (DON), *3*-acetyl-*deoxynivalenol* (3-ADON), *15*-acetyl-*deoxynivalenol* (15-ADON), diacetoxyscirpenol (DAS), nivalenol (NIV), fusarenon-X (FUS-X), *neosolaniol* (NEO), patulin (PAT), T-2 toxin and HT-2 toxin was evaluated in 80 juice samples collected from Valencia retail Market. An efficient Dispersive Liquid-Liquid Microextraction method (DLLME) was carried out before their trace level determination by chromatographic techniques coupled to tandem mass spectrometry. The results obtained revealed the presence of nine mycotoxins namely AOH, AME, PAT, OTA, AFB1, AFB2, AFG2, β-ZAL, and HT2 in the analyzed samples, with incidences ranging from 3 to 29% and mean contents between 0.14 and 59.52 μg/L. Considerable percentages of TDIs were reached by children when 200 mL was considered as daily fruit juice intake.

Keywords: mycotoxins; fruit juice; DLLME; risk assessment

1. Introduction

Fruit juices are excellent source of antioxidants, vitamins, and minerals, which play an important role in the prevention of heart diseases, cancer, and diabetes. The consumption of fruit juices is trendy nowadays, to meet the goals of five daily serving of fruits and vegetables. Fruit juices are mainly consumed by children and seekers of higher-quality diets due to their freshness, high vitamin content, and low caloric intake [1].

Moulds can infect agricultural crops during growth, harvest, storage, and processing. The fungal growth and the further mycotoxin production in fruits depend on various environmental factors such as moisture content, temperature and pH. Water activity of fruits boost fungal growth as many of them still tolerate the acidic pH of natural acids as citric, malic and tartaric acids [2].

Furthermore, some environmental conditions such as insect infestation, drought, cultivar susceptibility, mechanical damage, rainfall or humidity can promote the mycotoxins production in crops. *Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., and *Penicillum* spp. are the most common postharvest and storage fungi of fruits [1].

Aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT) and the *Alternaria* toxins such as alternariol (AOH), alternariol methyl ether (AME) and altenuene (ALT) are the most common mycotoxins reported in fruits and their processed products [3–9].

Mycotoxins, secondary fungi metabolites are related to different health adverse effects, such as cancer induction, mutagenicity, estrogenicity as well as gastrointestinal, urogenital, vascular, kidney and nervous disorders [10]. Mycotoxins showed stability against heat processes, which make its occurrence in processed juice highly likely expected [11]. A careful selection of fruits, a

proper storage, physical, chemical and biological detoxification methods are some strategies that are adopted to reduce the presence of mycotoxins in fruits [1,2].

The European Union Commission has set permissible maximum limits to control the contents of mycotoxins in fruit juices [12]. For OTA, 2 μg/kg have been established as maximum limit in grape juice, grape nectar, and its concentrated. In the case of PAT, 50 μg/kg have been set in fruit juices, its concentrated and fruits nectars while 10 μg/kg is the maximum established for apple juice and solid apple products, including apple compote and apple puree used by infants and young children. In the case of AFs, maximum limits have been established in dried fruits, but no AFs limits were set in fruit juices. *Alternaria* mycotoxins have been not regulated yet, despite some authors reported the toxicological implications of their presence in fruit juices [13].

Although patulin is the most studied mycotoxin in fruits and their processed products, the presence of *Alternaria* toxins, aflatoxins and ochratoxin A has also been reported [1]. These compounds can be of concern for human health due to their toxicity and the high consumption of juices by the young population, so multimycotoxin occurrence study in different types of juice available in the market will contribute to more realistic risk estimation.

Most methods for mycotoxins extraction involved liquid-liquid extraction, solid phase extraction using C18 cartridges and immunoaffinity clean-up columns to pre-concentrate and purify mycotoxins in food matrices. A dispersive liquid-liquid microextraction (DLLME) has also been developed for the purpose of miniaturizing, simplifying and automating the analytical procedure with good results [14]. DLLME is a ternary component system formed by an aqueous solution, an organic extraction solvent (frequent solvent

with high density) and a dispersive solvent (miscible in both of extractant and aqueous phases). DLLME has already proved some advantages such as high recoveries and low-cost applications [9,15,16].

The aim of the present study was to evaluate the presence of 30 mycotoxins (AFB1, AFB2, AFG1, AFG2, AOH, AME, OTA, FB1, FB2, ENNA, ENNA1, ENNB, ENNB1, BEA, STG, ZEA, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, PAT,T-2 and HT-2 toxins) in 80 fruit juice samples by Dispersive Liquid-Liquid Microextraction method (DLLME) and gas and liquid chromatography-tandem mass spectrometry determination. Liquid chromatography coupled to tandem mass spectrometry was used for the analysis of AFB1, AFB2, AFG1, AFG2, AOH, AME, OTA, FB1, FB2, ENNA, ENNA1, ENNB, ENNB1, BEA, STG while gas chromatography coupled to tandem mass spectrometry was used for the analysis of ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, PAT,T-2 and HT-2. Furthermore, the exposure to mycotoxins in adults and children population through the consumption of fruit juices was evaluated.

2. Results and Discussion

2.1. Method Validation

The method was validated in terms of recoveries, repeatability (intra-day precision), intermediate precision (inter-day precision), matrix effects, limits of detection (LODs), limits of quantification (LOQs) and linearity according to European Commission [17]. The results are shown in Table 1.

The acquisition by the triple quadrupole detector of two SRM transitions per compound accomplished the requirements set by the Commission Decision

2002/657/EC as regards ion transition ratio criteria and procedures for the validation of analytical methods satisfied by MS/MS.

Blank apple juice samples were tested for interference and selectivity. Matrix effects (SSE) that evaluate a possible suppression or enhancement of the signal was obtained comparing the slope of mycotoxins calibration curves prepared by spiking blank apple juice samples extract with the slope of calibration curves prepared in methanol. SSE (%) were calculated as follows: SSE (%) = 100× slope of curve in extracted matrix/slope of curve in methanol.

Matrix matched standards were used to compensate the signal suppression/enhancement (SSE) of matrix effects. Evidence for relative matrix effects have been registered for mycotoxins in apple juice as signal suppression (from 41–64%) was observed for α-ZOL, AFB1, AFG1, AFG2, OTA, ENNA1, ENNB1, and BEA. Therefore, matrix-matched calibration curves were used for effective quantification of samples. Matrix-matched calibration curves were prepared by spiking blank juice samples at concentrations between LOQs and 1000 μg/L.

The recoveries were obtained by spiking blank apple juice samples before and after the extraction procedure with the studied mycotoxins at three concentration levels (50, 100 and 200 μg/L) in three replicates. The values obtained ranged from 61 to 115%. To obtain Intra-day precision, three determinations were performed in the same day and in three of non-consecutive days for Inter-day precision. The intra-day and inter-day precision were lower than 14% and 19%, respectively, for all studied mycotoxins.

The LODs and LOQs were determined spiking blank apple juice samples, at decreasing concentrations of analyzed mycotoxins using the criterion for both transitions predetermined per each mycotoxin of S/N ≥ 3 for calculate LOD

and S/N ≥ 10 for LOQ. LODs obtained ranged from 0.15 to 2.34 μg/L and the LOQs from 0.5 to 7.81 μg/L. The relative error between prepared concentrations and the obtained ones ranged between 20% at LOQs and 16% at 1000 μg/L.

Linearity and matrix effects were studied using standard solutions in neat solvent and matrix-matched calibrations. Calibration curves in both pure solvent and matrix were constructed by plotting the analyte relative ion intensity against the concentration at eight concentration levels (from LOQs to 1000 μg/L). Regression coefficients obtained were higher than 0.990 in all cases. In everyday practice the calibration curve was constructed from a single lot of apple matrix. The degree of how SSE might vary in orange juice and pineapple matrixes was also evaluated. The relative errors between the obtained SSEs in the studied matrixes were lower than 18%. Due to the significant values obtained of SSEs, this parameter has been taken account and matrix matched curves were used for quantification.

The here proposed methodology is presented as a sensitive and robust analytical tool for the simultaneous determination of thirty mycotoxins in juice samples.

Table 1. Quantification and confirmation transitions of mycotoxins monitored fragments, retention time (Rt)) and analytical parameters obtained.

Mycotoxin	Quantification Transition	Confirmation Transition	Rt	SSE (%)[c]	LOD	LOQ	Recovery (%)		
							50	100	200
	m/z	m/z	min		µg/L	µg/L	µg/L	µg/L	µg/L
ZEA[a]	462 > 151	462 > 333	15.95	115	2.34	7.81	98	113	103
α-ZAL[a]	433 > 309	433 > 295	15.45	88	0.58	1.95	101	97	99
β-ZAL[a]	433 > 295	307 > 73	15.68	91	2.34	7.81	97	105	104
α-ZOL[a]	305 > 73	305 > 289	16.45	62	0.58	1.95	80	113	95
β-ZOL[a]	536 > 333	536 > 446	16.82	69	1.17	3.90	98	102	69
DON[a]	392 > 259	407 > 197	8.4	103	0.58	1.95	68	72	63
3-ADON[a]	392 > 287	467 > 147	9.68	88	1.17	3.90	103	103	105
15-ADON[a]	392 > 217	392 > 184	9.65	114	0.58	1.95	95	98	96
DAS[a]	350 > 229	378 > 124	9.85	96	1.17	3.90	103	91	84
NIV[a]	289 > 73	379 > 73	10.15	87	1.17	3.90	61	88	97
FUS-X[a]	450 > 260	450 > 245	9.73	106	2.34	7.81	84	98	106
NEO[a]	252 > 195	252 > 167	11.68	95	0.58	1.95	83	71	69
PAT[a]	226 > 73	183 > 75	4.3	78	2.34	7.81	61	96	92
T-2[a]	350 > 259	350 > 229	14.8	71	2.34	7.81	98	96	71
HT-2[a]	347 > 157	347 > 185	14.39	78	0.58	1.95	106	107	78
AFB1[b]	313 > 285	313 > 241	7.41	48	0.3	1.0	111	64	115
AFB2[b]	315 > 287	315 > 259	7.36	80	0.3	1.0	83	71	69
AFG1[b]	329 > 243	329 > 311	7.23	60	0.3	1.0	63	76	83
AFG2[b]	331 > 313	331 > 245	7.13	41	0.3	1.0	79	70	92
AOH[b]	259 > 128	258 > 184	8.01	67	0.3	1.0	105	114	90
AME[b]	273 > 128	273 > 228	9.10	120	0.3	1.0	78	89	71
OTA[b]	404 > 239	404 > 102	8.68	64	0.3	1.0	90	73	71
FB_1[b]	722 > 334	722 > 352	7.7	75	1.5	5.0	65	86	82
FB_2[b]	706 > 336	706 > 318	7.85	67	1.5	5.0	75	94	79
ENNA[b]	699 > 210	699 > 228	11.74	73	0.3	1.0	96	67	78
ENNA1[b]	685 > 210	685 > 214	11.3	60	0.3	1.0	109	66	87
ENNB[b]	657 > 196	657 > 214	10.73	66	0.15	0.5	104	66	85
ENNB1[b]	671 > 214	671 > 228	10.68	54	0.3	1.0	110	67	81
BEA[b]	801 > 784	801 > 244	10.84	52	1.5	5.0	112	65	82
STG[b]	325 > 281	325 > 310	9.08	106	1.5	5.0	62	66	71

[a] GC-MS/MS determination. [b]LC-MS/MS determination. [c] SSE: Signal Suppression-Enhancer.

2.2. Mycotoxin Contents in Juices

Only 9 of the 30 analyzed mycotoxins were detected in quantifiable amounts in 49% of the analyzed juices, mainly AOH, AME, PAT, OTA, AFB1, AFB2, AFG2, β-ZAL and HT-2. AOH and PAT, were the most detected mycotoxins with incidences of 29% and 18%, respectively, while β-ZAL and HT-2 were detected in only 3% of the samples (Table 2). The results obtained are consistent with the information available in bibliography, where PAT, AOH and AME are the most common mycotoxins found in fruit juices [2].

Chromatograms of real samples contaminated by PAT at 28.37 μg/L and AOH at 441.5 μg/L, respectively are shown in Figure 1.

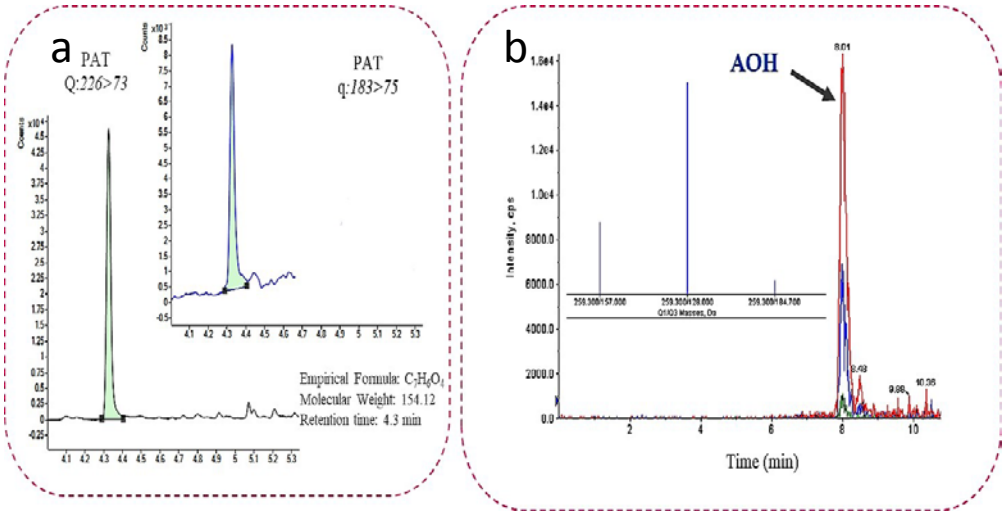

Figure 1. Chromatograms of contaminated samples with (**a**) patulin (PAT) (28.37 μg/L) and (**b**) AOH (441.5 μg/L) determined by GC-MS/MS and LC-MS/MS, respectively.

Table 2. Mycotoxins ranges and means found in the analyzed samples (μg/L).

MYCOTOXIN	Mean of Positive Samples	Minimum Concentration	Maximum Concentration	Incidence (%)	Lower Bound Scenario[a]	Upper Bound Scenario[b]
AFB1	9.57	4.68	18.1	8	0.71	0.99
AFB2	4.49	1.38	12.49	6	0.28	0.56
AFG2	3.75	1.24	7.60	4	0.14	0.43
AME	8.54	2.47	15.18	10	0.85	1.124
AOH	207	< LOQ	1213	29	59.50	59.73
βZAL	23.22	22.59	23.85	3	0.58	2.86
HT2	22.76	21.38	24.15	3	0.57	1.13
OTA	5.43	2.93	10.81	9	0.48	0.75
PAT	28.18	< LOQ	50.95	18	4.93	6.9

[a]Lower bound scenario: Mean assigning zero to not detected mycotoxins or mycotoxins detected below their corresponding LOQ. [b]Upper bound scenario: Mean assigning the LOD to not detected mycotoxins, and the corresponding LOQ to mycotoxins detected at levels below LOQ.

Regarding the contents determined, the mean of positive samples ranged from 3.75 to 207.01 μg/L. AOH and PAT were also the mycotoxins detected with the higher contents (Table 2).

The co-occurrence of mycotoxins was found in 9 analyzed samples (12%). Two apple juices showed co-occurrence of two mycotoxins (AOH + β-ZAL and BZAL + PAT). The most prevalent co-occurrence was AOH, AME and AFB1, detected simultaneously in 3 samples (2 samples of pineapple juices and one of peach juice) and coexistence of 7 mycotoxins was observed in two samples of fresh orange juice contaminated by AOH, AME, AFB1, AFB2, AFG2, OTA, PAT. Sum of mycotoxins concentrations quantified in the same sample are listed in Table 3. Although the sum of mycotoxins amounts in multi-contaminated samples cannot yet be used to obtain significant interpretations of the risk assessment, the results obtained provide data on the combined and simultaneous exposure to mycotoxins through juice intake. The natural co-occurrence of multiple mycotoxins in food products is an increasing health concern due to the exposure to multiple fungal growths. Very scarce data related to in vivo toxicity evaluation of exposure to combined mycotoxins are available in literature but in line with the in vitro observations, possible additive or synergistic effect may be registered, but, the impact of this effects usually rely on the concentration and duration of exposure [18].

Table 3. Sum of mycotoxins concentrations in multi-contaminated samples (μg/L).

Mycotoxins Co-occurrence	Number of Samples	Sum of Concentrations
AOH + β-ZAL (apple juice)	1	24.86
BZAL + PAT (apple juice)	1	50.96
AOH + AME+AFB1 (pineapple juice, peach juice)	3	9.36–36.2
AME + AFB2 + AFG2 + OTA + PAT (fresh orange juice)	1	33.3
AME + AFB1 + AFB2 + OTA + PAT (fresh orange juice)	1	71.33
AOH + AME + AFB1 + AFB2 + AFG2 + OTA + PAT (fresh orange juice)	2	65.47–97.36

PAT was detected in juices with incidence of 18%, concentrations ranged between < LOQ to 50.95 μg/L and mean of positive samples was about 28.18 μg/L. Only one sample, exceeded slightly the maximum limit set for PAT by the European Commission in fruit juices (50 μg/L) [12]. In Malaysia, Lee et al. [4] found to be PAT positive 5% of 56 analyzed fruit juices in a range of concentration between 13.1 and 33.7 μg/L. In Iran, Rahimi et al. [19] revealed also PAT presence in 16% of 161 fruit juices samples with levels between 5 to 190.7 μg/kg and mean of positives of 34.5 μg/kg. Contrary to the present study, in Tunisia, Zouaoui et al. [20] reported PAT incidence of 50% in 214 samples of fruit juices, compote and jam samples and concentrations ranging from 2 to 889 μg/L and a mean of 89 μg/L. In Pakistan, Iqbal et al. [8] also reported PAT incidence of 57.4% in 237 samples of fruits, juices, and smoothies, with concentrations ranging from 0.04 to 1100 μg/kg.

OTA was determined in 9% of the studied samples at concentrations between 2.93 and 10.81 μg/L. The European Commission has set 2 μg/kg as a limit for OTA on grape juice. In Turkey, Akdeniz et al. [3] found to be positive 20% of 10 grape juice samples with concentrations ranging from 0.9 to 1.9 μg/kg. Asadi et al. [7] also reported lower contents of OTA in 15% of 20 apple

juices and 25% of 20 grape juices, ranging from 0.06 to 0.1 μg/L and 0.06 to 0.12 μg/L, respectively.

AME and AOH were detected in 10 and 29% of the analyzed samples at mean concentration of 8.5 and 207 μg/L, respectively. In the study performed by Zwickel et al. [6], AOH and AME were reported at similar incidence (27 and 5% of 78 juice samples) respectively from the German market, similar incidences to the present work, but at lower contents: AOH (from 0.81 to 8.16 μg/L) and AME (from 0.89 to 1.54 μg/L). Myresiotis et al. [5] did not report AOH and AME presence in any of the pomegranate fruit and juice samples purchased from Greek markets, neither Ruan et al. [16] did detect them in commercial fruit juices from the local market of Guangzhou (China).

AFB1, AFB2 and AFG2 were detected in 8, 6 and 4% of the analyzed samples, respectively at levels in the range of 1.24 and 18.1 μg/L. In a study performed in Egypt, Abdel-Sater et al. [21] reported that 100% of five apple beverages resulted positive for AFB1 and AFG1 in a range from 20 to 30 μg/L and two of five guava juices presented AFB1 at 12 μg/L.

Only 2 apple juice samples of 80 samples resulted contaminated by β-ZAL with concentrations of 22.59 and 23.85 μg/L, respectively. Scarce information is available in the literature about the presence of ZEA and its metabolites in fruit juices. In a prior study, Carballo et al. [9] did not find ZEA and β-ZAL presence in a multimycotoxin study performed in 42 samples of fruit juices. Abdel-Sater et al. [21] also found no ZEA presence in fruit juices and beverages. The same case was observed for HT-2, that only was present in 2 samples of mixed fruit juices with concentrations of 21.38 and 24.15 μg/L, respectively. HT-2 is also not commonly found in fruits and its derived products and little information is

available in bibliography. López et al. [22] detected HT-2 in apple juice composite but at levels below the LOQ.

Comparing the results per type of juice samples, mono-fruit juice samples presented contamination up to 8 different mycotoxins (AOH, AME, PAT, AFB1, AFB2, AFG2, OTA, β-ZAL) at levels below 50 μg/L, while blended beverages presented contamination by AOH, PAT and HT-2 (Table 4). However, the contents of AOH, the most detected mycotoxin and with the highest levels, were higher in blended beverages, maybe for the addition of other ingredients, like ginger, vegetables as cucumber and spinach or passion fruits. In the case of Ginger, even that its essential oils may inhibit fungal growth, some authors reported ginger contamination by mycotoxins specially OTA and AFs [23]. Vegetables like tomato or red fruits have also been reported contaminated by AOH [24,25]. Juan et al. [26] also reported AOH in strawberry samples up to 752 μg/kg, amounts similar to those reported in the present study. Fresh orange juice samples resulted the most contaminated juice mainly by PAT, AME and AFB2 even at trace levels. AME was previously detected in 11% of citrus juices samples at concentration levels between 0.11 and 0.20 μg/kg [27]. Juan et al. [28] reported AFs, OTA, AOH and AME contamination with mean concentration of 26.12, 0.68, 18.7 and 160.5 μg/L, respectively in berry juices.

Table 4. Mycotoxin incidence, concentration range and mean of positive samples (μg/L).

Mycotoxin	Mono-Fruit Juices (n = 40)						Blended Beverages (n = 40)								
	Fresh Orange Juices (n = 7)			Packed Juices (n = 33)			Ecological Label (n = 5)			Health Claims Label (n = 24)			Common Label (n = 11)		
	I[a]	Mean ± SD μg/L	Range μg/L	I	Mean ± SD μg/L	Range μg/L	I	Mean ± SD μg/L	Range μg/L	I	Mean ± SD μg/L	Range μg/L	I	Mean ± SD μg/L	Range μg/L
AFB1	3/7	9.37 ± 3.03	5.93–11.65	3/33	9.77 ± 7.3	4.68–18.1	nd	---	--	nd	--	--	nd	--	--
AFB2	5/7	4.49 ± 4.53	1.38–12.49	nd	---	--	nd	---	--	nd	---	--	nd	---	--
AFG2	3/7	3.76 ± 3.4	1.24–7.6	nd	---	--	nd	---	--	nd	---	--	nd	---	--
AME	5/7	9.53 ± 2.05	7.03–11.77	3/33	6.87 ± 7.2	2.47–15.18	nd	---	--	nd	---	--	nd	---	--
AOH	2/7	3.59 ± 3.14	1.37–5.81	11/33	3.71 ± 3.55	< LOQ–11.16	2/5	629,74 ± 266	441.5–817.98	8/24	528.01 ± 362.9	93.5–1213.87	nd	---	--
B-ZAL	nd[b]	---	--	2/33	23.22 ± 0.89	22.59–23.85	nd	---	--	nd	---	--	nd	---	--
HT2	nd	---	--	nd	---	--	nd	---	--	nd	---	--	2/11	22.77 ± 1.95	21.38–24.15
OTA	5/7	6.25 ± 3.93	2.93–10.81	2/33	3.4 ± 0.38	3.13–3.67	nd	---	--	nd	---	--	nd	---	--
PAT	7/7	34.59 ± 13.9	14.78–50.59	7/33	28.34 ± 14	8.05–47.82	nd	---	--	nd	---	--	2/11	10.89 ± 0	< LOQ–10.89

[a] Incidence (number positive samples/number total samples). [b] not detected

2.3. Dietary Exposure to Mycotoxins through Fruit Juices Consumption in Children and Adult Population.

With the purpose to evaluate the exposure of children and adult population to mycotoxins through the consumption of fruit juices, the Estimate Daily Intakes (EDIs) were calculated and compared with the Tolerable Daily Intakes (TDIs).

The European Commission has established TDIs for OTA, PAT, and HT2 and βZAL. A provisional maximum tolerable daily intake of 0.4 μg/kg bw/day has been fixed for PAT [29], a TDI of 0.25 μg/kg bw/day for ZEA and of 0.1 μg/kg bw/day for the sum of HT2 and T2 have been set [30]. Tolerable Weekly Intake (TWI) of 0.12 μg/kg bw/week has been also fixed for OTA [31].

The EDIs (μg/kg bw/day) for each mycotoxin were calculated by following formula = C × K/bw where C is the mean concentration of each mycotoxin in juice (μg/L), K is the daily average of juice consumption per person (L/day) and bw is the bodyweight used for the population group.

Left-censored results (data below LOQ) were processed according to EFSA recommendations considering two exposure scenarios [32]. In the lower bound scenario (LB) zero was assigned when mycotoxins were not detected or were detected below the limit of quantification. In the upper bound (UB) scenario, the limit of detection was assigned when mycotoxins were not detected, and the limit of quantification when mycotoxins were detected at levels below LOQ.

A bodyweight of 70 kg was considered for adult population and of 25 kg for children population. The daily consumption of juice extracted from the database of the Spanish Ministry of Agriculture, Fisheries and Food was on

average of 9.2 L per year/per person [33]. Children populations are higher consumers of juices and a daily consumption of 200 mL corresponding to single portion was considered for a more realistic approach.

Considering the mean of positive samples as mean concentration, the EDIs obtained represented a notable percentage of TDIs fixed (Table 5). For adult population, percentages of (2.5% TDI), (11.5% TDI), (3.3% TDI) and (8.2% TDI) were respectively obtained for PAT, OTA, βZAL and HT-2. However, when the mean of total samples was used, the EDIs values obtained for adult population were far below the TDIs fixed, representing less than 2% of TDIs, in both LB and UB approaches.

For children, exposure rate of 56.4% TDI for PAT and 74.3% TDI for βZAL, and EDIs over than the fixed TDI for OTA and HT-2 were obtained with mean of positive samples. This data lowered to 13.8% of PAT TDI, 35.3% of OTA TDI, 9.15% of βZAL TDI and 9% of HT-2 TDI in UB scenario when the mean used was of the whole samples. These results evidenced an increasing risk for children.

Comparing these results with those obtained by Torovic et al. [34] in a study of risk assessment of patulin intake through apple juices in infants and preschool children in Serbia, similar risk exposure was observed by these authors after considering the same daily juice consumption of 0.2 L and the mean of total samples. In this case, the obtained values of EDI for preschool children reached the 6% of the TDI established for PAT, considering different scenarios for left censored results.

Table 5. Mycotoxin risk assessment through fruit juices consumption for children and adult population.

Mycotoxin	TDI (ng/kg bw)	Positive Samples				Lower Bound Scenario				Upper Bound Scenario			
		EDI (ng/kg bw)		%TDI		EDI (ng/kg bw)		%TDI		EDI (ng/kg bw)		%TDI	
		Children	Adults	Children	Adults	Children	Adults	Children	Adults	Children	Adults	Children	Adults
PAT	400	225.44	10.15	56.36	2.54	39.20	1.76	9.80	0.44	55.20	2.48	13.80	0.62
OTA	17	43.44	1.96	255.53	11.50	3.84	0.17	22.59	1.02	6.00	0.27	35.29	1.59
B-ZAL	250	185.76	8.36	74.30	3.34	4.64	0.21	1.86	0.08	22.88	1.03	9.15	0.41
HT-2	100	182.08	8.20	182.08	8.20	4.56	0.21	4.56	0.21	9.04	0.41	9.04	0.41

3. Conclusions

The analytical procedure employed was suitable for the analysis of 30 mycotoxins in the studied juice samples. The mycotoxins determined in the present study such as AOH, AME, and PAT were the most commonly reported in the literature. The main mycotoxins incidences and means in positive samples were lower than 30% and 28.18 μg/L, respectively. AOH was related to the higher mean detected (207 μg/L). A total of 12% of analyzed samples presented coexistence from 2 mycotoxins to 7 mycotoxins. The EDIs calculated for OTA and HT-2 overlapped the established TDIs when the mean of positives samples was considered for risk assessment for children, decreasing to unconcerned levels when the LB and UB scenarios where employed.

4. Materials and Methods

4.1. Reagents and Chemicals

Solvents (acetonitrile, methanol, and chloroform) were supplied by Merck (Darmstadt, Germany). Ethyl acetate was supplied by Alfa Aesar (Karlsruhe, Germany). Deionized water (resistivity > 18 MΩ cm-1) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Formic acid (reagent grade ≥ 95%) was obtained from Sigma Aldrich (St. Louis, MO, USA). Nylon filters (0.45-μm pore size) were supplied by Scharlau (Barcelona, Spain). Syringe nylon filters (13 mm diameter and 0.22-μm pore size) were obtained from Membrane Solutions (Plano, TX, USA). The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl) acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-

trimethylsilylimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA, USA). Disodium phosphate and Sodium dihydrogen phosphate, used to prepare phosphate buffer, were obtained from Panreac.

The standards of AFB1, AFB2, AFG1, AFG2, AOH, AME, OTA, FB1, FB2, ENNA, ENNA1, ENNB, ENNB1, BEA, STG, ZEA, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, PAT, T-2 and HT-2 toxins were purchased from Sigma Aldrich. Individual stock solutions were prepared to obtain 100 mg/L in methanol and working solutions were obtained diluting the individual stock solutions. All solutions were stored in darkness and kept at −20 °C.

4.2. Sampling

A total of 80 commercial juices were collected from various supermarkets of Valencia (Spain) during 2018. The samples were classified into two groups. 40 were mono-fruit; 7 were filtered orange juices with nothing added, and 33 resulting from concentrated, sweetened drinks made from fruit pulpe, where 3 were from orange, 4 from pear, 12 from apple and 7 were from pineapple and peach respectively.

40 blended beverages, containing fruit juices and mashed vegetables and, sometimes incorporating dairy or functional ingredients (e.g., chia seeds). In this group, 5 samples held ecological label, 24 health claims label and 11 common label. The composition of each sample is detailed in table S1 (annex 3). The samples were stored in darkness and dry place until analysis.

4.3. Dispersive Liquid-Liquid Microextraction Procedure (DLLME)

The samples were extracted according to Carballo et al., [9]. 5 mL of each juice sample were placed with 1 g of NaCl in a 10 mL conical tube, and the tube was shaken in vortex for one minute. After vortexing, a mixture of dispersion solvent (950 µL of acetonitrile) and extraction solvent (620 µL of ethyl acetate) was added and the tube was shaken in vortex for one minute, resulting in a cloudy solution of the three components. Then the mixture was centrifuged at 4000 rpm for 5 min, and 600 µL of the organic phase located at the top of the tube was separated and placed into other conical tube. Next, in a second step the mixture of dispersion solvent (950 µL of methanol) and extraction solvent (620 µL of chloroform) was added to the remaining residue in the tube. After centrifugation, 600 µL of the organic phase, located in this case in the bottom of the tube was recovered and added to the first organic phase separated before. Then the two recovered phases were evaporated to near dryness under a nitrogen stream using a Turvovap LV Evaporator (Zymark, Hoptikinton, MA, USA).

For LC-MS/MS analysis, the residue was reconstituted with 1 mL of 20 mM ammonium formate (MeOH/ACN) (50/50 v/v) and filtered through a 13 mm/0.22 µm nylon filter in a vial while for GC-MS/MS analysis, a derivatization process was carried out by adding 50 µL of BSA + TMCS + TMSI (3:2:3) to the dry extract and left at room temperature 30 min. Then 200 µL of hexane was added, mixed in vortex for 30 s, washed with 1 mL of phosphate buffer (60 mM, pH7) and mixed until the upper layer was clear. Finally, the hexane layer (200 µL) was transferred to an auto sampler vial.

4.4. LC-MS/MS Determination

The determination was performed using an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with turbo ion spray electrospray ionization (ESI). The QTRAP analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. The chromatographic separation of analytes was performed in a Gemini-NX column C_{18} (Phenomenex, 150 mm × 4.6 mm, 5 particle size) preceded by a guard column. Mobile phases were 5 mM ammonium formate, 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). The elution gradient initiated with a proportion of 0% for eluent B; in 10 min increased to 100%, then decreased to 80% in 5 min, and finally decreased to 70% in 2 min. In the next 6 min, the column was cleaned, readjusted to initial conditions, and equilibrated for 7 min. The flow rate was established at 0.25 mL/min, and the oven temperature at 40 °C.

The analysis was performed using the Turbo-ion spray in the positive ionization mode (ESI+). Nitrogen was served as nebulizer and collision gas. During the analysis, the following parameters were fixed: probe temperature 450 °C; ion spray voltage 5500 V; curtain gas 20 arbitrary units; GS1 and GS2, 50 and 50 psi, respectively.

The transitions used for the quantification and confirmation of the monitored fragments are shown in Table 1.

4.5. GC-MS/MS Determination

An GC system Agilent 7890A coupled with an Agilent 7000A triple quadruple mass spectrometer with inter electron-impact ion source (EI, 70Ev)

and Agilent 7693 auto sampler (Agilent Technologies, Palo Alto, USA) was used for the determination. Quantitation data were acquired at selection reaction monitoring mode (SRM). The transfer line and source temperatures were 280° and 230°, respectively. Nitrogen was used as collision gas for MS/MS experiments, and helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). A capillary column HP-5MS 30m × 0.25mm × 0.25µm was used for the separation of analytes. One microliter of the final clean derivatized extract of mycotoxins was injected in splitless mode in the programmable temperature vaporization (PTV) inlet at 250 °C, employing helium as carried gas at fixed pressure of 20.3 psi. Oven temperature started at 80 °C, and increased to 245 °C at 60 °C/min, hold their time for 3 min and progressively increased to 260 °C by 3 °C/min and finally to 270 °C at 10 °C/min and then held for 10 min. Agilent Mass hunter version B.04.00 software was employed to acquire and process data.

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References

1. Mandappa, I.M.; Basavaraj, K.; Manonmani, H.K. Analysis of Mycotoxins in Fruit Juices. In *Fruit Juices*; Academic Press: Wageningen, The Netherlands, 2018; pp. 763–777.
2. Fernández-Cruz, M.L.; Mansilla, M.L.; Tadeo, J.L. Mycotoxins in fruits and their processed products: Analysis, occurrence and health implications. *J. Adv. Res.* **2010**, *1*, 113–122.

3. Akdeniz, A.S.; Ozden, S.; Alpertunga, B. Ochratoxin A in dried grapes and grape-derived products in Turkey. *Food Addit. Contam. B* **2013**, *6*, 265–269.
4. Lee, T.P.; Sakai, R.; Manaf, N.A.; Rodhi, A.M.; Saad, B. High performance liquid chromatography method for the determination of patulin and 5-hydroxymethylfurfural in fruit juices marketed in Malaysia. *Food Control* **2014**, *38*, 142–149.
5. Myresiotis, C.K.; Testempasis, S.; Vryzas, Z.; Karaoglanidis, G.S.; Papadopoulou-Mourkidou, E. Determination of mycotoxins in pomegranate fruits and juices using a QuEChERS-based method. *Food Chem.* **2015**, *182*, 81–88.
6. Zwickel, T.; Klaffke, H.; Richards, K.; Rychlik, M. Development of a high performance liquid chromatography tandem mass spectrometry based analysis for the simultaneous quantification of various Alternaria toxins in wine, vegetable juices and fruit juices. *J. Chromatogr. A* **2016**, *1455*, 74–85.
7. Asadi, M. Determination of ochratoxin A in fruit juice by high-performance liquid chromatography after vortex-assisted emulsification microextraction based on solidification of floating organic drop. *Mycotoxin Res.* **2018**, *34*, 15–20.
8. Iqbal, S.Z.; Malik, S.; Asi, M.R.; Selamat, J.; Malik, N. Natural occurrence of patulin in different fruits, juices and smoothies and evaluation of dietary intake in Punjab, Pakistan. *Food Control* **2018**, *84*, 370–374.
9. Carballo, D.; Pinheiro-Fernandes-Vieira, P.; Tolosa, J.; Font, G.; Berrada, H.; Ferrer, E. Dietary exposure to mycotoxins through fruits juice consumption. *Rev. Toxicol.* **2018**, *35*, 2–6.

10. Marin, S.; Ramos, A.J.; Cano-Sancho, G.; Sanchis, V. Mycotoxins: Occurrence, toxicology, and exposure assessment. *Food Chem. Toxicol.* **2013**, *60*, 218–237.
11. Drusch, S.; Ragab, W. Mycotoxins in fruits, fruit juices, and dried fruits. *J. Food Prot.* **2003**, *66*, 1514–1527.
12. European Commission. Commission Directive 2006/1881/EC of 19 December 2006, Setting Maximum Levels for Certain Contaminants in Foodstuffs. *OJEU* **2006**, *L364*, 5–24. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32006R1881 (accessed on 12 February 2019).
13. Escrivá, L.; Oueslati, S.; Font, G.; Manyes, L. Alternaria mycotoxins in food and feed: An overview. *J. Food Qual.* **2017**, *2017*, 1–20.
14. Zgoła-Grześkowiak, A.; Grześkowiak, T. Dispersive liquid-liquid microextraction. *TrAC Trends Anal. Chem.* **2011**, *30*, 1382–1399.
15. Víctor-Ortega, M.D.; Lara, F.J.; García-Campaña, A.M; del Olmo-Iruela, M. Evaluation of dispersive liquid–liquid microextraction for the determination of patulin in apple juices using micellar electrokinetic capillary chromatography. *Food Control* **2013**, *31*, 353–358.
16. Ruan, C.; Diao, X; Zhang, H.; Zhang, L.; Liu, C. Development of a dispersive liquid–liquid microextraction technique for the analysis of citrinin, alternariol and alternariol monomethyl ether in fruit juices. *Anal. Methods* **2016**, *8*, 7944–7950.
17. European Commission. Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *OJEU* **2002**, *L221*, 8−36. Available online: https://op.europa.eu/en/publication-detail/-

/publication/ed928116-a955-4a84-b10a-cf7a82bad858/language-en (accessed on 8 January 2019).

18. Mallebrera, B.; Prosperini, A.; Font, G.; Ruiz, M.J. In vitro mechanisms of Beauvericin toxicity: A review. *Food Chem. Toxicol.* **2018**, *111*, 537–545.
19. Rahimi, E.; Rezapoor Jeiran, M. Patulin and its dietary intake by fruit juice consumption in Iran. *Food Addit. Contam. B* **2015**, *8*, 40–43.
20. Zouaoui, N.; Sbaii, N.; Bacha, H.; Abid-Essefi, S. Occurrence of patulin in various fruit juice marketed in Tunisia. *Food Control* **2015**, *51*, 356–360.
21. Abdel-Sater, M.A.; Zohri, A.A.; Ismail, M.A. Natural contamination of some Egyptian fruit juices and beverages by mycoflora and mycotoxins. *J. Food Sci. Tech. MYS* **2001**, *38*, 407–411.
22. López, P.; De Rijk, T.; Sprong, R.C.; Mengelers, M.J.B.; Castenmiller, J.J.M.; Alewijn, M. A mycotoxin-dedicated total diet study in the Netherlands in 2013: Part II–occurrence. *World Mycotoxin J.* **2016**, *9*, 89–108.
23. Kabak, B.; Dobson, A.D. Mycotoxins in spices and herbs—An update. *Crit. Rev. Food Sci.* **2017**, *57*, 18–34.
24. Qiao, X.; Yin, J.; Yang, Y.; Zhang, J.; Shao, B.; Li, H.; Chen, H. Determination of Alternaria Mycotoxins in Fresh Sweet Cherries and Cherry-Based Products: Method Validation and Occurrence. *J. Agric. Food Chem.* **2018**, *66*, 11846–11853.
25. Rodríguez-Carrasco, Y.; Mañes, J.; Berrada, H.; Juan, C. Development and validation of a LC-ESI-MS/MS method for the determination of Alternaria toxins alternariol, alternariol methyl-ether and tentoxin in tomato and tomato-based products. *Toxins* **2016**, *8*, 328.

26. Juan, C.; Oueslati, S.; Mañes, J. Evaluation of Alternaria mycotoxins in strawberries: Quantification and storage condition. *Food Addit. Contam. A* **2016**, *33*, 861–868.
27. Zhao, K.; Shao, B.;Yang, D.; Li, F. Natural occurrence of four Alternaria mycotoxins in tomato-and citrus-based foods in China. *J. Agric. Food Chem.* **2014**, *63*, 343–348.
28. Juan, C., Mañes, J.; Font, G.; Juan-García, A. Determination of mycotoxins in fruit berry by-products using QuEChERS extraction method. *LWT* **2017**, *86*, 344–351.
29. European Food Safety Authority (EFSA). Report of Experts Participating in Task 3.2.8. Assessment of Dietary Intake of Patulin by the Population of EU Member States. 2002. Available online: https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_patulin_3.2.8_en.pdf (accessed on 15 February 2019).
30. European Food Safety Authority (EFSA). Scientific Opinion on the Risks for Human and Animal Health Related to the Presence of Modified Forms of Certain Mycotoxins in Food and Feed. *EFSA J.* **2014**, *12*, 3916–4023.
31. European Food Safety Authority (EFSA). Opinion of the scientific Panel on Contaminants in the food chain on a request from the commission related to Ochratoxin A in food (Adopted on 4 April 2006). *EFSA J.* **2006**, *365*, 1–56.
32. European Food Safety Authority (EFSA). Management of left-censored data in dietary exposure assessment of chemical substances. *EFSA J.* **2010**, *8*, 1557.
33. Ministry of Agriculture, Fisheries and Food (MAPAMA). *Data on Food Consumption, Fruit Juices. Statistics Database-Home Consumption*; Ministry of

Agriculture, Fisheries and Food (MAPAMA): Madrid, Spain, 2017. Available online: https://www.mapa.gob.es/app/consumo-en-hogares/consulta10.asp (accessed on 22 January 2019).

34. Torović, L.; Dimitrov, N.; Assunção, R.; Alvito, P. Risk assessment of patulin intake through apple-based food by infants and preschool children in Serbia. *Addit. Contam. A* **2017**, *34*, 2023–2032.

3.8. Risk assessment and mitigation of the mycotoxin content in medicinal plants by the infusion process

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Risk assessment and mitigation of the mycotoxin content in medicinal plants by the infusion process

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ABSTRACT

Medicinal plants are often consumed as infusions with boiled water. Scarce information is available in the literature about the migration of mycotoxins into the resulting beverage and/or the effects of the infusion procedure on the final mycotoxin contents. The aim of the present study was to investigate the impact of the infusion process on mycotoxin contents during medicinal plant preparation. For this purpose, the contents of aflatoxins (AFs) [aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), aflatoxin G2 (AFG2)], zearalenone (ZEA), enniatins (ENNs) [enniatin B (ENNB), enniatin B1 (ENNB1), enniatin A (ENNA), enniatin A1 (ENNA1)] and beauvericin (BEA) were analyzed in 224 samples of medicinal plants and in their resulting beverages. The Quick, Easy, Cheap, Effective, Rugged and Safe extraction method (QuEChERS) was applied to the medicinal plants while the dispersive liquid-liquid microextraction procedure (DLLME) was applied to their infusions, and the mycotoxins were determined by liquid chromatography coupled to ion trap tandem mass spectrometry (LC-MS/MS-IT).

The results revealed that ZEA, ENNB, ENNB1, AFB2, AFG1 and AFG2 were detected in the beverages with incidences of ≤6% and at concentrations from less than the limit of quantification (LOQ) to 82.2 μg/L. Mycotoxins reduction ranged from 74% to 100% after the infusion process. The risk assessment revealed that the estimated daily intakes (EDIs) obtained for ZEA, ENNB and ENNB1 were far below the tolerable daily intakes (TDIs) established.

KEYWORDS: mycotoxins; medicinal plants; LC-MS/MS-IT; risk assessment

INTRODUCTION

According to the World Health Organization (WHO), a medicinal plant is any wild or cultivated plant that is used as a therapeutic agent or an active ingredient in a medicinal formulation [1]. The demand for herbal medicine is increasing worldwide, and the international trade of medicinal plants has increased tremendously; consequently, the need to examine the issues of efficacy, safety and quality assurance of medicinal plants has grown through the establishment of evidence-based approaches [2]. During harvesting, handling, storage and distribution, medicinal plants are subjected to contamination by various toxigenic fungi originating from the soil. Poor production and harvesting practices and/or storage and transportation contribute to increased fungal contamination [3]. Toxigenic fungi mainly belonging to genera *Aspergillus*, *Penicillium* and *Fusarium* are responsible for mycotoxin production, which are secondary metabolites that cause a variety of toxic effects, such as neurotoxicity, carcinogenicity, teratogenicity and immunotoxicity [4]. The increase in the use of medicinal plants may lead to an increase in the intake of mycotoxins that contribute to adverse human health problems [5]. The European Commission (EC) has established a regulatory list in relation to the safety and efficacy of herbal substances, preparations and combinations for use in certain traditional herbal medicinal products [6-7]. The EC has established maximal concentration levels of mycotoxins in some plants used as medicinal plants or spices. A maximum limit of 5 μg/kg has been set for aflatoxin B1 (AFB1), of 10 μg/kg for the sum of all aflatoxins (AFB1+AFB2+AFG1+AFG2) and 20 μg/kg for ochratoxin (OTA) [8-9]. The European Pharmacopoeia Commission has also established maximum limits for AFs in herbal drugs; 2 μg/kg for AFB1 and 4 μg/kg for the sum of all AFs [10].

Medicinal plants are usually consumed as infusions with boiling water. Thermal processes (cooking, boiling, extrusion, etc.) can have an impact on mycotoxin levels, but the details of these effects remain unclear. Most mycotoxins [aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FBs), zearalenone (ZEA), deoxynivalenol (DON), moniliformin (MON) and patulin (PAT)] are moderately heat-resistant within the range of conventional food-processing temperatures (80–121°C), so some modifications in overall toxin levels occurs as a result of normal cooking conditions [11]. Regarding their solubility, in general mycotoxins are slightly soluble in water thanks to some free carboxyl and amine groups, for example AFs present value of solubility around 10-20 μg/mL [12].

Currently, studies available on mycotoxins in medicinal plants are focused on the analysis of the plant raw materials, where mycotoxins are sometimes detected with high incidences and concentrations [13-15]. Santos et al. [13] reported ZEA and AFs as some of the most detected mycotoxins in medicinal and aromatic herbs materials, with high incidences and concentrations up to 45 and 855 μg/Kg, respectively. Hu et al. [15] also observed important presence of enniatins (ENNs) and beauvericin (BEA) in Chinese medicinal plants samples with 25% of samples contaminated by ENNs and BEA at levels between 2.5 and 751 μg/Kg. Scarce information about the migration of mycotoxins towards the resulting beverage and/or the impact of the infusion procedure on their final contents is available in bibliography [16-18]. Recently, Reinholds et al. [19] observed reduction rate from 0 to 70% for DON and ZEA during tea infusion process. Higher reduction rates were reported for AFs (from 60 to 70%) during the preparation of ginger beverages under different conditions in a study performed by Iha & Trucksess [16].

The Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) methodology, originally developed by Anastassiades et al. [20] consists in a simple, fast, and inexpensive extraction procedure based in an initial portioning with acetonitrile, followed by dispersive solid phase extraction by mixed salts as primary secondary amine and anhydrous magnesium sulfate to remove residual water and cleanup. QuEChERS procedure has been applied to multiple mycotoxins extraction from matrices such as breakfast cereals, dried fruits, vegetables, fruits, grapes and wines providing high recovery rates and good analytical performance [21].

The aim of the present study was to investigate the contents of 10 mycotoxins, AFB1, AFB2, AFG1, AFG2, ZEA, enniatin A (ENNA), enniatin A1 (ENNA1), enniatin B (ENNB), enniatin B1 (ENNB1) and beauvericin (BEA), in 224 samples of medicinal plants and in their infusion beverages. The QuEChERS method was applied to the extraction of studied mycotoxins from the medicinal plants samples and the dispersive liquid-liquid microextraction method (DLLME) from their infusions. Then, mycotoxin contents were determined by liquid chromatography coupled to ion trap tandem mass spectrometry (LC-MS/MS-IT). The effect of the infusion process on mycotoxin contents was also explored.

MATERIALS AND METHODS

Reagents and chemicals employed are described in detail as Supplementary Material (annex 4).

Sample collection

A total of 224 medicinal plant samples from 56 different species of herbs were purchased from different herbal stores in Valencia during 2018. The samples were packed in plastic containers and were stored in their original packaging in a dry and dark place until analysis. Table S1 (annex 4) shows the different types of medicinal plants analyzed.

Monitoring mycotoxin level to confirm compliance with regulatory values is essential. However, mycotoxin analysis is very sensitive to the sampling stage and the great heterogeneity in the distribution of toxigenic fungi. The European Union established sampling protocols for the analysis of mycotoxins in some food products as species [22]. This followed regulation summarized the recommendations to reach trueness and representativeness of the results.

Sample preparation

For the analysis of plant materials, each sample was homogenized, and 2 g was weighed. In addition, for the preparation of the infusions, 1.5 g was weighed from the bulk container and infused into a glass recipient with 200 mL of boiling water at 90-100°C for 5 min. Then, the content of the glass was filtered through a strainer, and the resulting beverage was analyzed.

Mycotoxin extraction from plant raw materials: QuEChERS procedure

Two grams of each raw medicinal plant were weighed in a 50 mL conical tube, 10 mL of water acidified with 2% formic acid were added, and the tube was shaken for 30 min in an IKA KS 260 orbital shaker. After thirty minutes, 10 mL of acetronitrile (ACN) were added, and the tube was shaken for an additional 30 min. Then, 4 g of MgSO4 and 1 g of NaCl were added to the tube containing the mixture of plant material and solvents, the tube was vortexed for 30 s, and the resulting mixture was centrifuged at 5000 rpm for 10 min. Next, 2 mL of the supernatant were placed in a 15 mL tube, 0.3 g of MgSO4 and 0.1 g of Octadecyl C18 sorbent were added. After shaking and centrifuging again with the same conditions detailed above, the supernatant was filtered through a 13 mm/0.22 μm nylon filter (Membrane Solutions, TX, USA), and 20 μL were injected into the LC-MS/MS-IT system.

Mycotoxins extraction from drinkable products: DLLME extraction

Five milliliters of the prepared infusion were placed in a 15 mL conical tube, then 1 g of NaCl was added, and the tube was vortexed for one minute. A mixture of dispersant solvent (950 μL of ACN) and extractant solvent [620 μL of ethyl acetate (EtOAc)] was added, and the tube was shaken for one minute. A cloudy solution of the three components formed. The mixture was centrifuged at 4000 rpm for 5 min, and the organic phase located at the top of the tube was recovered and placed into a second conical tube. Next, a mixture of dispersant solvent [950 μL of methanol (MeOH)] and extractant solvent [620 μL of chloroform ($CHCl_3$)] were added to the remaining residue, and after agitation and centrifugation, the separated organic phase placed at the bottom of the tube was recovered and added to the first organic phase. The solvent in the conical

tube containing the two recovered phases was evaporated to near dryness under a nitrogen stream using a Turbovap LV Evaporator (Zymark, Hoptikinton, USA). The residue was reconstituted with 1 mL of 20 mM ammonium formate (MeOH/ACN) (50/50 v/v) and filtered through a 13 mm/0.22 μm nylon filter (Membrane Solutions, TX, USA) prior to injection of 20 μL of the filtrate into the LC-MS/MS-IT system.

The LC-MS/MS-IT analysis is detailed in Supplementary Material (Table S2 annex 4).

Method validation

QuEChERS method was characterized in terms of recovery, repeatability (intraday precision), reproducibility (interday precision), matrix effects, linearity, limit of detection (LOD) and limit of quantification (LOQ) according to the Commission Decision [23], while DLLME data were obtained in a previous work for tea beverages [24]. The results obtained are shown in Supplementary Material (Table S3 annex 4).

RESULTS AND DISCUSSION

Occurrence of mycotoxins in medicinal plants

The incidence of studied mycotoxins in medicinal plant materials ranged from 1% (ENNA and BEA) to 15% (ENNB) (Figure S1 annex 4) while mean contents of total samples ranged from <LOQ (BEA) to 520.74 μg/kg (ZEA) (Table 1). ZEA and AFG2 were the mycotoxins reported with highest contents.

ZEA was presented in 11% of samples, mainly sage, stevia, eucalyptus,

marjoram, pennyroyal and mursalski tea. Kong et al. [14] observed a similar incidence (12%) for medicinal plants widely consumed in China but at medium concentration of 11.096 μg/kg, slightly lower than in the present study. Veprikova et al. [25], observed a maximum concentration of 824 μg/kg for ZEA and a mean concentration of all samples <50 μg/kg in herbal-based dietary supplements. In milk thistle-based dietary supplements, these authors reported ZEA incidence of 78%, higher than in the present study (11%).

The medicinal plants positive for AFs were sage, lemon balm leaves, chamomile, eucalyptus, peppermint, thyme, oregano, mugwort, rosemary, lemon verbena, basil and summer savory. AFs were detected in plant materials at mean concentrations between 64.76 and 383.53 μg/Kg even in very low incidences (4-11%). In a study performed in Spain, Santos et al. [13] also observed high levels of AFs (between <1.4 to 855 μg/kg) and incidence higher than in the present study (96%). Eucalyptus, peppermint, lemon verbena, sage, chamomile, rosemary and sage were also reported to be positive for AFs by these authors. Liu et al. [26] observed similar incidences to the present study: AFB1 (16%), AFB2 (14%), AFG1 (6%) and AFG2 (2%) in 174 commercial samples of medicinal herbs popular in China, but in lower contents (medium content of 3.85 μg/kg for AFs).

For the emerging mycotoxins, sage, mursalki tea, nettle, dandelion, licorice, anise, lemon balm, eyebright, willow, plantain, lungwort, vervain, Algerian tea, horsetail, whitethorn and linden were the species of positive medicinal plants. The incidences observed ranged from 1 to 15%, and the mean contents of total samples were between <LOQ (table S3 anexo 4) and 42.43 μg/kg. These results were similar to those reported by Hu & Rychlik [15] in Chinese traditional medicinal herbs (from 2.5 to 751 μg/kg). BEA was the most frequently detected

emerging mycotoxin (20%), and ENNs ranged from 6.7 to 11.7%. The mean concentration of the total samples reported by these authors was 7.33 μg/kg for ENNA, 11.28 μg/kg for ENNA1, 8.1 μg/kg for ENNB, 1.096 μg/kg for ENNB1 and 8.23 μg/kg for BEA. The incidences and contents reported in the present study were similar, even BEA was only detected in two samples at a level <LOQ and ENNB was detected at higher contents.

Occurrence of mycotoxins in infusions

Mycotoxin incidences in infusions were mainly lower than in the raw plant materials (1-6%) (Figure S1 anexo 4). AFB1, ENNA, ENNA1 and BEA were not detected in the infusions. ZEA and ENNB were detected with incidences of 6%, while AFB2, AFG1 and AFG2 registered incidences of 2, 4 and 3%, respectively. ENNB1 was only detected in two samples at levels <LOQ (Figure S1 annex 4). The concentrations ranged from <LOQ to 82.2 μg/L, and the mean contents of the total samples (Table 1) were 0.24 μg/L for AFG2, 0.11 μg/L for AFG1, below LOQ for AFB2 and 1.02 μg/L for ZEA. Finally, ENNB, the only emerging mycotoxin detected in the infusions at levels >LOQ, presented a mean concentration of 0.005 μg/L.

Comparing the contents of mycotoxins in plant materials with the contents in the resulting infusions (Table 1), the mycotoxin contents are lower in the infusions, and AFB1, AFB2, ENNA, ENNA1, ENNB1 and BEA were not detected in the infusions at levels above the LOQ, as previously mentioned. Figure S2 (annex 4) shows a chromatogram of a sample positive for AFB2, comparing the content in plant material with the resulting infusion. As mentioned above, the literature regarding the presence of mycotoxins in infusions prepared from teas, herbal and medicinal plant materials is scarce. In

a previous study performed on tea infusions, Pallarés et al. [24] obtained similar results to those obtained in the present study: AFB1, ENNA, ENNA1 and BEA were not detected in any analyzed sample, and ENNB was detected at levels below the LOQ. AFB2 was detected in samples at concentrations between 14.4 and 32.2 μg/L, AFG1 below the LOQ and AFG2 from <LOQ to 2.6 μg/L. Monbaliu et al., [17] observed no mycotoxin presence in infusions prepared from herbal samples containing several mycotoxins at levels < 80 μg/Kg.

Table 1. Mean concentrations of mycotoxins in total medicinal plant samples (n=224) analyzed in triplicate (raw materials *vs* infusions).

Mycotoxin	Medicinal plant material	Infusion	
	Mean concentration (μg/kg)	Mean concentration (μg/L)	Mean concentration (μg/kg)
AFB1	64.76	Nd[a]	Nd
AFB2	107.80	<LOQ	<LOQ
AFG1	82.12	0.11	14.66
AFG2	383.53	0.24	32
ZEA	520.74	1.02	136
ENNA	0.40	Nd	Nd
ENNA1	1.01	Nd	Nd
ENNB	42.43	0.005	0.66
ENNB1	3.06	<LOQ	<LOQ
BEA	<LOQ	Nd	Nd

Nd[a] (not detected)

During the preparation of the infusions from the medicinal plants, an important reduction in mycotoxin contents was achieved. The percent reductions ranged from 74% to 100%. AFB1, ENNA, ENNA1 and BEA were not detected in the resulting infusions, and AFB2 and ENNB1 were detected at

levels <LOQ. The reduction rate ranged between 48 to 100% (mean of 82%) for AFG1, between 63-100% (mean of 93%) for AFG2, between 41 to 100% (mean of 74%) for ZEA, and between 25 to 100% (mean of 95%) for ENNB (Figure S3 annex 4). ZEA achieved a lower reduction rate, which could be explained to rather high amounts detected in raw samples. These high reductions achieved during the infusion process may not be due to the temperature, since most of the studied mycotoxins are resistant to temperatures of 100°C, such as AFs and ZEA, but may instead be due to the low solubility and tendency of these mycotoxins to migrate to the resulting beverages during the time of the infusion process. Iha & Trucksess [16] investigated the migration of AFs and OTA from naturally contaminated powdered ginger to the resulting beverages under different conditions of process preparation and observed rate reduction up to 70% for AFs and up to 80% for OTA. Toman et al. [18] obtained higher transfer of OTA to the resulting beverages (around 83%) in a naturally contaminated Chinese medicinal plant while, Ariño et al. [27] only observed 1% of OTA transfer from licorice root to licorice infusion. Reinholds et al. [19] observed that 32 to 100% of DON and ZEA present in dry teas were extracted into the resulting beverages during the infusion process. Ryu et al. [28] concluded through the stability study that they carried out at temperatures form 100 to 225ºC, that ZEA was relatively stable at boiling temperatures.

Infusion impact has not been studied for emerging mycotoxins; however, the impact of boiling pasta and cooking fish have been already investigated on these mycotoxins. Serrano et al. [29] reported a reduction of ENNs while cooking pasta, from 98 to 100% for ENNA, 94-95% for ENNA1, 14-49% for ENNB and 53-65% for ENNB1 and Tolosa et al. [30] also observed ENNs reduction from 62 to 100% after boiling fish. In the present study, a high

reduction of emerging mycotoxins was achieved (from 95 to 100%). Having low solubility in water, little tendency of these mycotoxins to migrate towards their resulting beverages after infusion was registered. This trend was also reported by Serrano et al. [29] and Hu et al. [31], who observed the low transfer of ENNs into the water during cooking pasta and during beer production, respectively.

Risk assessment of mycotoxins through infusion consumption

To assess the risk of exposure of the population to mycotoxins through the studied infusions, the estimated daily intakes (EDIs) were calculated as medicinal plant daily consumption (L/kg bw/day) × mean value of the mycotoxin level in medicinal plants beverages (μg/L) and compared with the tolerable daily intakes (TDIs). Left-censored results (data below the LOQ) were processed according to EFSA [32] recommendations considering two exposures scenarios, a lower bound (LB) scenario and an upper bound (UB) scenario. In the LB, zero was assigned to samples where mycotoxins were not detected or detected at concentrations below the limit of quantification. In the UB, limits of detection were assigned to samples where mycotoxins were not detected, and limits of quantification were assigned to samples where mycotoxins were detected at concentrations below the limit of quantification. Data from the database of the Spanish Ministry of Agriculture, Fisheries and Food on the annual consumption of the group of infusions (including the medicinal plants) were consulted, and estimated to be 0.12 kg equivalent to 16 liters per year, per person, on average [33]. This consumption would be very low, less than one cup per day, and for this reason, the greater consumption of one cup (200 mL/day) and three cups (600 mL/day) per day were also contemplated, allowing a more realistic risk assessment approach.

For AFs, as genotoxic and carcinogenic compounds, they do not have an established TDI value to perform the risk characterization and the recommendations are to be reduced to as low as reasonably achievable (ALARA). For emerging mycotoxins, TDI values have not been fixed net either, but the EDIs obtained in the present work were compared with the lowest and highest TDI values fixed for other *Fusarium* mycotoxins as DON (1 μg/kg bw/day) [34] and the sum of the toxins HT2 and T2 (0.1 μg/kg bw/day) [35]. The TDI established for ZEA was 0.25 μg/kg bw/day [35].

For ZEA, the EDIs estimated from the LB scenario were 0,64 ng/kg bw/day, considering less than one cup/day consumption. This result changed to 2.9 ng/kg bw/day considering 1 cup/day and 8.8 ng/kg bw/day considering 3 cups/day, and these EDIs corresponded to 0.26%, 1.17% and 3.5%, respectively, of the TDI established for ZEA. In the UB scenario, the EDIs were 0.67 ng/kg bw/day (less than one cup/day), 3.06 ng/kg bw/day (1 cup/day) and 9.1 ng/kg bw/day (3 cups/day), corresponding to 0.27%, 1.22% and 3.67% of the TDI, respectively (Table 2).

The EDI values calculated for ENNB in the LB scenario were 0.003 ng/kg bw/day (less than one cup/day), 0.014 ng/kg bw/day (1 cup/day) and 0.04 ng/kg bw/day (3 cups/day) corresponding to 0.003%, 0.014% and 0.04% of the lower TDI value set for *Fusarium* mycotoxins (0.1 μg/kg bw/day), respectively, and corresponding to 0.0003%, 0.0014% and 0.004% of the higher TDI value set for *Fusarium* mycotoxins (1 μg/kg bw/day). In the UB scenario, the EDI values calculated for ENNB were 0.04 ng/kg bw/day (less than one cup/day), 0.17 ng/kg bw/day (1 cup/day) and 0.51 ng/kg bw/day (3 cups/day), corresponding to 0.04%, 0.17% and 0.51%, respectively, of the lower TDI value

set for *Fusarium* mycotoxins and to 0.004%, 0.017% and 0.051% of the higher TDI value set for *Fusarium* mycotoxins (Table 2).

ENNB1, was not detected at levels higher than LOQ, so EDIs were only approached for UB scenario. The EDIs obtained for ENNs, resulting of sum of ENNB+ENNB1 were 0.103 ng/kg bw/day (less than 1 cup/day), 0.47 ng/kg bw/day (1 cup/day) and 1.42 ng/kg bw/day (3 cups/day), representing 0.103%, 0.47% and 1.42% of the lower value set for *Fusarium mycotoxins*, and 0.0103%, 0.047% and 0.142% of the higher TDI value fixed for *Fusarium* mycotoxins, respectively (Table 2).

The calculated EDI values were far below the established TDIs. The risk of exposure to mycotoxins through infusion consumption is not high, although the risk may increase in higher consumers (3 or more cups/day). As the amounts of mycotoxins are higher in medicinal plants than in the resulting infusions, its consumption as condiments may also increase the calculated risk.

Table 2. Risk characterization of mycotoxins through the consumption of medicinal plant infusions.

Mycotoxin	Mean concentration in samples (µg/L)	Estimate Daily Intakes (EDI) (ng/kg pc /día)			Tolerable Daily Intake (TDI) (ng/kg bw /day)	Risk Characterization (EDI/TDI)*100		
		MAPAMA (16 L/ year)	1 cup (200 mL/day)	3 cups (600 mL/day)		MAPAMA (16 L/ year)	1 cup (200 mL/day)	3 cups (600 mL/day)
Lower bound Approach								
ZEA	1.02	0.64	2.90	8.80	250[a]	0.26%	1.17%	3.50%
ENNB	0.01	3e-03	0.01	0.04	100[b]	3e-03%	0.01%	0.04%
ENNB	0.01	3e-03	0.01	0.04	1000[c]	3e-04%	1e-03%	4e-03%
Upper bound Approach								
ZEA	1.07	0.67	3.06	9.10	250[a]	0.27%	1.22%	3.67%
ENNB	0.06	0.04	0.17	0.51	100[b]	0.04%	0.17%	0.51%
ENNB	0.06	0.04	0.17	0.51	1000[c]	4e-03%	0.02%	0.05%
ENNB1	0.10	0.06	0.30	0.90	100[b]	0.06%	0.30%	0.90%
ENNB1	0.10	0.06	0.30	0.90	1000[c]	0.01%	0.03%	0.09%
ENNs	0.17	0.10	0.47	1.42	100[b]	0.10%	0.47%	1.42%
ENNs	0.17	0.10	0.47	1.42	1000[c]	0.01%	0.05%	0.14%

[a] TDI ZEA (0.25 µg/kg bw/day) [35];[b] TDI sum of T-2 and HT-2 toxins (0.1 µg/kg bw/day) [35]; [c] TDI DON (1 µg/kg bw/day) [34].

Conclusion

Although some medicinal plant materials presented high concentrations of some mycotoxins [aflatoxin G2 (AFG2) and zearalenone (ZEA)], the contents were reduced during the preparation of the infusions, achieving an important reduction for all assayed mycotoxins with percentages ranging from 74% to 100%; this means that during medicinal plant preparation, only a small fraction of the mycotoxins migrated from the plant materials to the resulting beverages. Furthermore, the risk assessment study of the population reveals that the levels of estimated daily intakes (EDIs) obtained are far below the established tolerable daily intakes (TDIs). A higher percent was noted for ZEA and increased in higher consumers of medicinal plant infusions (3 or more cups/day).

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REFERENCES

(1) WHO Expert Committee on Specifications for Pharmaceutical Preparations (2018) WHO guidelines on good herbal processing practices for herbal medicines. WHO Technical Report Series, 81-152. https://www.who.int/medicines/areas/quality_safety/quality_assurance/TRS1010annex1.pdf?ua=

(2) Poswal FS, Russell G, Mackonochie M, MacLennan E, Adukwu EC, Rolfe V (2019) Herbal teas and their health benefits: A scoping review. Plant Foods

Hum Nutr 74: 266-276. https://doi.org/10.1007/s11130-019-00750-w.

(3) Ahmad B, Ashiq S, Hussain A, Bashir S, Hussain M (2014) Evaluation of mycotoxins, mycobiota, and toxigenic fungi in selected medicinal plants of Khyber Pakhtunkhwa, Pakistan. Fungal biol 118: 776-784. https://doi.org/10.1016/j.funbio.2014.06.002.

(4) Marin S, Ramos AJ, Cano-Sancho G, Sanchis V (2013) Mycotoxins: Occurrence, toxicology, and exposure assessment. Food Chem Toxicol 60: 218-237. https://doi.org/10.1016/j.fct.2013.07.047.

(5) Ashiq S, Hussain M, Ahmad B (2014) Natural occurrence of mycotoxins in medicinal plants: a review. Fungal Genet Biol 66: 1-10. https://doi.org/10.1016/j.fgb.2014.02.005.

(6) European Commission (2004) Directive 2004/24/EC of the European Parliament and of the Council of 31 March 2004 amending, as regards traditional herbal medicinal products, Directive 2001/83/EC on the Community code relating to medicinal products for human use. OJEU L 136: 85-90.

(7) European Commission (2008) Commission Decision 2008/911/EC of 21 November 2008, Establishing of a list of herbal substances, preparations and combinations thereof for use in traditional herb medicinal products. OJEU L328: 42-48.

(8) European Commission (2015) Commission Regulation (EC) 2015/1137/EC of 13 July 2015, amending Regulation no. 1881/2006, as Regards the Maximum Level of Ochratoxin A in Capsicum spp. spices. OJEU L 185: 11-12.

(9) European Commission (2010) Commission Regulation (EC) 165/2010 of 26 February 2010, amending Regulation no. 1881/2006, setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins. OJEU L

50: 8-12.

(10) European Pharmacopoeia Commission. Determination of Aflatoxin B1 in herbal drugs. In European Pharmacopoeia 9th Edition 2.8.18. Council of Europe: Strasbourg, France, 2016, Volume 1, p. 289.

(11) Kabak B (2009) The fate of mycotoxins during thermal food processing. J Sci Food Agric 89: 549-554. https://doi.org/10.1002/jsfa.3491.

(12) IARC (2012). Chemical and Physical Characteristics of the Principal Mycotoxins. IARC Scientific Publications 2012, 31-38.

(13) Santos L, Marín S, Sanchis V, Ramos AJ (2009) Screening of mycotoxin multicontamination in medicinal and aromatic herbs sampled in Spain. J Sci Food Agric 89: 1802-1807. https://doi.org/10.1002/jsfa.3647.

(14) Kong WJ, Shen HH, Zhang XF, Yang XL, Qiu F, Ou-yang Z, Yang MH (2013) Analysis of zearalenone and α-zearalenol in 100 foods and medicinal plants determined by HPLC-FLD and positive confirmation by LC-MS-MS. J Sci Food Agric 93: 1584- 1590. https://doi.org/10.1002/jsfa.5926.

(15) Hu L, Rychlik M (2014) Occurrence of enniatins and beauvericin in 60 Chinese medicinal herbs. Food Addit Contam Part A 31: 1240-1245. https://doi.org/10.1080/19440049.2014.913813.

(16) Iha MH, Trucksess MW (2010) Aflatoxins and ochratoxin A in tea prepared from naturally contaminated powdered ginger. Food Addit Contam 27: 1142-1147. https://doi.org/10.1080/19440041003795319.

(17) Monbaliu S, Wu A, Zhang D, Van Peteghem C, De Saeger, S (2010) Multimycotoxin UPLC− MS/MS for tea, herbal infusions and the derived drinkable products. J Agric Food Chem 58: 12664-12671. https://doi.org/10.1021/jf1033043.

(18) Toman J, Ostry V, Grosse Y, Roubal T, Malir, F (2018) Occurrence of ochratoxin A in Astragalus propinquus root and its transfer to decoction. Mycotoxin research 34: 223-227. https://doi.org/10.1007/s12550-018-0317-2.

(19) Reinholds I, Bogdanova E, Pugajeva I, Bartkevics V (2019). Mycotoxins in herbal teas marketed in Latvia and dietary exposure assessment. Food Addit Contam Part B 12(3): 199-208. https://doi.org/10.1080/19393210.2019.1597927

(20) Anastassiades M, Lehotay S J, Štajnbaher D, Schenck F J (2003). Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "dispersive solid-phase extraction" for the determination of pesticide residues in produce. Journal of AOAC international 86(2): 412-431. https://doi.org/10.1093/jaoac/86.2.412

(21) Perestrelo R, Silva P, Porto-Figueira P, Pereira J A, Silva C, Medina S, Câmara, J. S. (2019). QuEChERS-Fundamentals, relevant improvements, applications and future trends. Anal Chim Acta 1070: 1-28. https://doi.org/10.1016/j.aca.2019.02.036

(22) European Commission (2006) Commission Regulation EC nº 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs OJEU L 70: 12-34.

(23) European Commission (2002) Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. OJEU L 221: 8−36.

(24) Pallarés N, Font G, Mañes J, Ferrer E (2017) Multimycotoxin LC–MS/MS Analysis in Tea Beverages after Dispersive Liquid–Liquid Microextraction (DLLME). J Agric Food Chem 65: 10282-10289.

https://doi.org/10.1021/acs.jafc.7b03507.

(25) Veprikova Z, Zachariasova M, Dzuman Z, Zachariasova A, Fenclova M, Slavikova P, Vaclavikova M, Mastovska K, Hengst D, Hajslova, J (2015) Mycotoxins in plant- based dietary supplements: Hidden health risk for consumers. J Agric Food Chem 63: 6633-6643. https://doi.org/10.1021/acs.jafc.5b02105.

(26) Liu L, Jin H, Sun L, Ma S, Lin R (2012) Determination of Aflatoxins in Medicinal Herbs by High-performance Liquid Chromatography–Tandem Mass Spectrometry. Phytochem Analysis 23: 469-476. https://doi.org/10.1002/pca.2343.

(27) Ariño A, Herrera M, Estopañan G, Juan T. (2007). High levels of ochratoxin A in licorice and derived products. Int J Food Microbiol 114(3): 366-369.

(28) Ryu D, Hanna MA, Eskridge KM, Bullerman LB (2003) Heat stability of zearalenone in an aqueous buffered model system. J Agric Food Chem 51: 1746-1748. https://doi.org/10.1021/jf0210021.

(29) Serrano AB, Font G, Mañes J, Ferrer E (2016) Development a mitigation strategy of enniatins in pasta under home-cooking conditions. LWT-Food Sci Technol 65: 1017- 1024. https://doi.org/10.1016/j.lwt.2015.09.036.

(30) Tolosa J, Font G, Mañes J, Ferrer E (2017) Mitigation of enniatins in edible fish tissues by thermal processes and identification of degradation products. Food Chem Toxicol 101: 67-74. https://doi.org/10.1016/j.fct.2016.12.039.

(31) Hu L, Gastl M, Linkmeyer A, Hess M, Rychlik M (2014) Fate of enniatins and beauvericin during the malting and brewing process determined by stable isotope dilution assays. LWT-Food Sci Technol 56: 469-477.

https://doi.org/10.1016/j.lwt.2013.11.004.

(32) European Food Safety Authority (EFSA) (2010) Management of left-censored data in dietary exposure assessment of chemical substances. EFSA J 8: 1557. https://doi.org/10.2903/j.efsa.2010.1557.

(33) Ministry of Agriculture, Fisheries and Food (MAPAMA) (2017) *Data on Food Consumption, Tea Consumption. Statistics Database-home Consumption*; Madrid, Spain. Available: http://www. mapama.gob.es/ Accessed 8 January 2019.

(34) Scientific 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 by the SCF on 26 February 2002). Report SCF/CS/ CNTM/MYC/27; European Commission Scientific Committee on Food: Brussels, Belgium.

(35) European Food Safety Authority (EFSA) (2014) Scientific Opinion on the Risks for Human and Animal Health Related to the Presence of Modified Forms of Certain Mycotoxins in Food and Feed. EFSA J 12: 3916-4023. https://doi.org/10.2903/j.efsa.2014.3916.

3.9. Pulsed electric fields (PEF) to mitigate emerging mycotoxins in juice and smoothie

Food Control (under review)

Pulsed electric fields (PEF) to mitigate emerging mycotoxins in juice and smoothie

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Abstract

The development of emerging technologies has increased to respond to growing demanding supply of fresh-like products. The aim of the present study is to investigate the effect of pulsed electric fields technology (PEF) on reducing the emerging mycotoxins [enniatins (ENs) and beauvericin (BEA)] contents in juice and smoothie samples. The products of degradation obtained after PEF treatment have been identified and its toxicological endpoints toxicity predicted by Pro Tox-II web. Mycotoxin reduction ranged from 43 to 70% in juices and smoothies but in water the expected effect was lower. The acidified pH increased BEA reduction in water. The degradation products originated resulted from the loss of aminoacidic fragments of the original molecules, such as HyLv, Val, Ile or Phe. Pro Tox-II server assigned a toxicity class I for ENB degradation products with predicted LD50 of 3 mg/kg. The other degradation products were classified in toxicity class III and IV.

Keywords: enniatins; beauvericin; PEF; degradation compounds; Pro Tox-II.

1.Introduction

Nowadays the population is concerned about the safety and the nutritional and sensorial properties of food. That is why has increased the demand of high quality fresh-like foods (Pinela, & Ferreira, 2017). With applying emerging technologies, like high hydrostatic pressure (HPP), pulsed electric fields (PEFs) and ultrasounds (US), sensory properties of food are less changed and nutritional compounds are better retained allowing to obtain safe, healthy and minimally processed food that satisfy the consumer's preferences in fresh food (Knorr, Froehling, Jaeger, Reineke, Schlueter, & Schoessler, 2011).

These technologies are based on physical constraints and offers some advantages front traditional thermal processing, like to be effective at mild temperatures and use short treatment times (Picart-Palmade, Cunault, Chevalier-Lucia, Belleville, & Marchesseau, 2019). Nowadays, can partially or completely replace the traditional well-established processes of preservation (Zhang, Wang, Zeng, Han, & Brennan, 2019). PEF technology involves the application of high-voltage pulses (1-40 kv/cm) for short periods of time to a product placed between two electrodes. PEF is reported as an effective tool for inactivating microorganisms at low temperatures and has been widely used to sterilize foods such as vegetables, fruit juices, milk and liquid eggs (Morales-de La Peña, Elez-Martínez, & Martín-Belloso, 2011; Cserhalmi, Sass-Kiss, Tóth-Markus, & Lechner, 2006). PEF has also been applied to inactivate enzymes in food industry, to extract nutritionally valuable compounds from plant tissue and food by-products, for drying and freezing in food processing and to promote some selected properties of food macromolecules and some chemical reactions (Barba et al., 2015). In the other hand, PEF treatment has been applied successfully in

the degradation of some organophosphorus pesticides (Zhang et al., 2012).

Mycotoxins are toxic substances naturally present in food and feed, produced by secondary metabolism of some filamentous fungi. They are related to some adverse effects as nephrotoxicity, hepatotoxicity, carcinogenicity, mutagenicity and immunosuppressive effects. Emerging mycotoxins are mycotoxins produced by Fusarium genus and cause cytotoxic effects due their inophoric properties, which evoke changes in the ion intracellular concentration that consequently affects the cell functions. (Mallebrera, Prosperini, Font, & Ruiz. 2018; Prosperini et al., 2017).

Food processing can reduce mycotoxin levels and transform it into less toxic products. Development of detoxification technologies should be a priority for research (Karlovsky et al., 2016). However, only two studies are available so far in literature dealing on the effect of PEFs on aflatoxins (AFs) contents (Vijayalakshmi, Nadanasabhapathi, Kumar, Kumar, & Reddy, 2017; Vijayalakshmi, Nadanasabhapathi, Kumar, & Kumar, 2018). The cited authors reported important reductions of AFs contents after PEFs treatment.

In addition, during food processing, some degradation or modified mycotoxins products may also be formed. A large majority of these compounds generated after food processing has not been tested for potential adverse effects on human health, which makes it difficult to carry out an adequate risk assessment (Cotterill, Chaudhry, Matthews, & Watkins, 2008). In this case, computational approaches can be used as a preliminary tool to identify potential toxic of these degraded or modified mycotoxins compounds generated after food processing by using their chemical structure and may also be employed as a screening method to select which compounds will be later assayed by in vitro

assays.

In silico methods are nowadays one of the promising approaches for toxicity assessment by employing expert systems and multiple algorithms that use computation (Kar & Leszczynski, 2019) and are an efficient tool especially in concrete situations, such as evaluating the toxicity of new degradation or reaction products which standards are not available. Furthermore, these in silico methods prevent cost, duration and animal harming, thus with the compliance on the replace, reuse and refinement of experimental animals (3Rs) (Gozalbes, de Julián-Ortiz, & Fito-López, 2014).

In this context, the aim of the present work is to study the effect of PEF technology on emerging mycotoxins contents [enniatin A (ENA), enniatin A1 (ENA1), enniatin B (ENB), enniatin B1 (ENB1) and beauvericin (BEA)] in grape juices and smoothies. After the PEFs treatment, ENs and BEA were extracted by dispersive liquid-liquid microextraction (DLLME) and determined by HPLC-MS/MS-IT. In addition, HPLC-Q-TOF-MS was used for tentative identification of ENs and BEA degradation products. Finally, different toxicological endpoints have been predicted by ProTox-II web server *in silico* method in order to evaluate and compare the toxicity of the identified degradation products with the precursor mycotoxin.

2. Materials and methods

2.1. Reagents and Chemicals

Acetonitrile (ACN), methanol (MeOH) (HPLC grade) and Chloroform (CHCl3) (99% grade) used for the extraction were purchased from Merck (Darmstadt, Germany). Ethyl acetate (EtOAc) (HPLC grade 99.5+ %) was

supplied by Alfa Aesar (Karlsruhe, Germany). The deionized water employed for mobile phase with resistivity > 18 MΩ cm-1 was prepared using a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, USA). All solvents employed to prepare mobile phases were filtered prior to use through a 0.45-µm cellulose filter supplied by Scharlau (Barcelona, Spain).

The salts, ammonium formate (99%) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). Formic acid (reagent grade ≥ 95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl) was supplied by VWR Chemicals (Leuven, Belgium). All samples were filtered by a 13 mm/0.22 µm nylon filter acquired from Membrane Solutions (TX, USA) prior to injection.

Standards of mycotoxins ENA, ENA1, ENB, ENB1, BEA were supplied by Sigma (St. Louis, MO, USA) and prepared in methanol at concentration of 1000 mg/L. The appropriate working solutions were prepared from the stock solutions. All solutions were placed at −20°C until the analysis.

2.2. Samples

For this study, grape juice samples and smoothie samples were employed. Smoothie samples were made from apple, banana, grape juices, strawberry, blackberry, orange purees and cereals.

10 bottles of juice and smoothie samples were purchased from different supermarkets in Valencia. After their homogenization, aliquots were taken, and samples were tested for the absence of mycotoxins. After this, a volume of 215 mL were spiked individually with ENA, ENA1, ENB, ENB1 and BEA, respectively, at concentration of 100 µg/L. The same experiments were

performed in parallel in water samples spiked with each studied mycotoxin at concentration of 100 μg/L. All experiments were performed in triplicate.

2.3. Pulsed electric field treatment

An Elea pulsed electric field cellcrack III equipment with a container of 10 cm of gap between electrodes was employed for the treatment. The voltage was set at 30 kv resulting in a field strength of 3 kv/cm and the specific energy applied was 500 KJ/Kg. To reach the 500 KJ/Kg an average of 238 pulses were applied in different cycles. During the treatment the temperature did not exceed 75ºC with conductivity of 2890 μs/cm for grape juice and 3160 μs/cm for smoothies.

2.4. Dispersive Liquid-Liquid Microextraction procedure (DLLME)

The samples were extracted according to the methodology proposed in a previous work (Pallarés, Carballo, Ferrer, Fernández-Franzón, & Berrada, 2019). 5 mL of sample were placed in 10 mL conical tub with 1g of NaCl and shaken one minute in vortex. Then, a mixture of dispersant and extractant solvents (950 μL of AcN) and (620 μL of EtOAc) was added, and shaken for one minute, resulting in a cloudy solution of the three components. The mixture was centrifuged at 4000 rpm for 5 min, allowing the separation of phases. The organic phase was separated and placed into other conical tube. The mixture of dispersant and extractant solvents (950 μL of MeOH) and (620 μL of CHCL3) was added to the remaining residue. After shaking and centrifugation, the organic phase was separated and placed with the first organic phase. The organic phases were evaporated to near dryness under a nitrogen stream using a

Turvovap LV Evaporator (Zymark, Hoptikinton, USA). The residue obtained was reconstituted in a vial with 1 mL of 20 mM ammonium formate (MeOH/ACN) (50/50 v/v) and filtered through a 13 mm/0.22 μm nylon filter prior to the injection in LC-MS/MS-IT.

2.5. LC-MS/MS-IT determination

To determine the mycotoxins contents an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with Turbo Ion Spray (ESI) electrospray ionization was used. The QTRAP analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. The chromatographic separation of analytes was performed using a Gemini-NX column C18 (Phenomenex, 150 mm x 4.6 mm, 5 particle size) preceded by a guard column. Mobile phases were, mobile phase A: 5 mM ammonium formate, 0.1% formic acid water and mobile phase B: 5 mM ammonium formate, 0.1% formic acid methanol. The gradient program initiated with a proportion of 0% for eluent B; in 10 min increased to 100%, then decreased to 80% in 5 min, and finally decreased to 70% in 2 min. In the next 6 min, the column was cleaned, readjusted to initial conditions and equilibrated for 7 min. The flow rate was fixed at 0.25 mL/min, the injection volume was 20 μL and the oven temperature was set at 40ºC.

The Turbo Ion Spray operated in positive ionization mode (ESI+) for the analysis. Nitrogen was served as nebulizer and collision gas. During the analysis, the following parameters were fixed: ion spray voltage 5500 V; curtain gas 20 arbitrary units; GS1 and GS2, 50 and 50 psi, respectively; probe temperature

(TEM) 450ºC.

2.6. LC-ESI-qTOF-MS analysis

For the identification of degradation products an Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) for the chromatographic determination coupled to a 6540 Agilent Ultra- High-Definition Accurate-Mass q-TOF-MS, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) for mass spectrometry analysis was employed. Chromatographic separation was developed in a Gemini-NX column C18 (Phenomenex, 150 mm x 4.6 mm, 5 particle size). The mobile phases consisted in water (A) and acetonitrile (B), both with 0.1% of formic acid. The gradient was as follows: 0–6 min, 50% B; 7–12 min, 100% B; 13–20 min, 50% B. The injection volume was 5 μL and the flow rate 0.2 mL/min. The following conditions were employed for mass spectrometry: interface in positive ionization mode; drying gas flow (N2) 12.0 L min−1; nebulizer pressure, 50 psi; gas drying temperature, 370ºC; capillary voltage, 3500 V; fragmentor voltage, 160 V. Analysis were carried out in MS mode and MS spectra were collected within the scan range 50–1500 m/z.

2.7. Method Validation

Method was previously validated in the laboratory for emerging mycotoxins analysis in juices (Pallarés et al., 2019). For validation, the method was characterized in terms of recovery, repeatability (intraday precision), reproducibility (interday precision), matrix effects, limit of detection (LOD) and limit of quantification (LOQ) according to the Commission Decision (EC, 2002). The recoveries performed at three levels of contamination (50, 100, 200

μg/L) ranged from 66 to 112%. Intra-day and inter-day precision were lower than 14% and 19%, respectively. LODs were between 0.15 and 1.5 μg/l and LOQs between 0.5 μg/l and 5 μg/l, respectively. Matrix effects experiments revealed signal suppression from 52 to 73%. Regarding linearity, regression coefficients were higher than 0.990 in all cases.

2.8. *In silico* prediction methods

The ProTox-II (http://tox.charite.de/protox_II/) is a free web server to predict diverse toxicological endpoints for several chemical compounds (Drwal, Banerjee, Dunkel, Wettig, & Preissner, 2014; Banerjee, Eckert, Schrey, & Preissner, 2018). This tool incorporates molecular similarity, pharmacophores, fragment propensities and machine-learning models for the prediction of some toxicity endpoints; such as acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, adverse outcomes pathways (Tox21) and toxicity targets, representing therefore a novel approach in toxicity prediction (Banerjee et al., 2018).

The ProTox-II platform is divided into a five different classification steps constructed by different computational models: (i) acute toxicity (oral toxicity model with six different toxicity classes); (2) organ toxicity model (1 model); (3) toxicological and genotoxicological endpoints, mainly immunotoxicity, cytotoxicity, mutagenicity and carcinogenicity (4 models); (4) toxicological pathways (12 models) and (5) toxicity targets (15 models).

Regarding the oral acute toxicity, toxic doses are expressed as LD50 values in mg/kg body weight. The prediction accuracy derived from cross-validation results is also given. Depending on the LD50 for each substance, they can be

classified into different toxicity classes, which are defined according to the Globally Harmonized System (GHS) of classification in labelling of chemicals:

- Class I: fatal if swallowed (LD50 ≤ 5 mg/kg)
- Class II: fatal if swallowed (5mg/kg< LD50 ≤ 50mg/kg)
- Class III: toxic if swallowed (50mg/kg< LD50 ≤ 300mg/kg)
- Class IV: harmful if swallowed (300mg/kg< LD50 ≤ 2000mg/kg)
- Class V: may be harmful if swallowed (2000mg/kg< LD50 ≤ 5000mg/kg)
- Class VI: non-toxic (LD50 > 5000mg/kg)

Regarding the toxicity endpoint and organ toxicity prediction, the predictive models are based on data from both *in vitro* (e.g. Tox21 assays, Ames bacterial mutation assays, hepG2 cytotoxicity assays, immunotoxicity assays, among others) and *in vivo* assays (e.g. carcinogenicity, hepatotoxicity).
In ProTox-II two types of target-pathway based models can be found. The two pathways have been namely defined as (i) Nuclear Receptor Signalling Pathways (7 pathway assays shown in Table 4) and (ii) Stress Response Pathways (5 pathway assays shown in Table 5).

This approach is based on the fact that a chemical compound can activate or inhibit a receptor or an enzyme when it interacts with their, resulting in a perturbation in diverse biological pathways and thereby disrupting the cellular process and causing cell death. The main purpose of the initiative is to prioritize substances for further in-depth toxicological evaluation as well as to identify some mechanisms for further investigation such as disease-associated pathways. This, by applying this computational prediction tool is possible to test quickly

and efficiently whether certain chemical compounds, have the potential to disrupt processes in the human body that may lead to adverse health effects (Banerjee et al. 2018).

3. Results and discussion

3.1. Reduction of ENs and BEA contents after PEF treatment

After PEF treatment, a significant reduction of ENA, ENA1, ENB, ENB1 and BEA were observed in all tested samples. For juice samples, the contents of emerging mycotoxins obtained were 46.81±0.8 μg/L (ENA), 52.23±4.4 μg/L (ENA1), 56.98±10.88 μg/L (ENB), 56.8±2.79 μg/L (ENB1) and 54.04±0.4 μg/L (BEA), that correspond with reduction percentages from 43 to 53% (figure 1).

For smoothie samples, the contents after PEF treatment were 44.35±0.21μg/L (ENA), 40.84±2.12 μg/L (ENA1), 42.94±14.56 μg/L (ENB), 40.12±6.58 μg/L (ENB1) and 29.8±9.8 μg/L (BEA), corresponding with higher reductions percentages from 56 to 70 % (figure 1). Figure 2 shows a chromatograms of juice sample spiked with ENB1: PEF treated vs not treated samples.

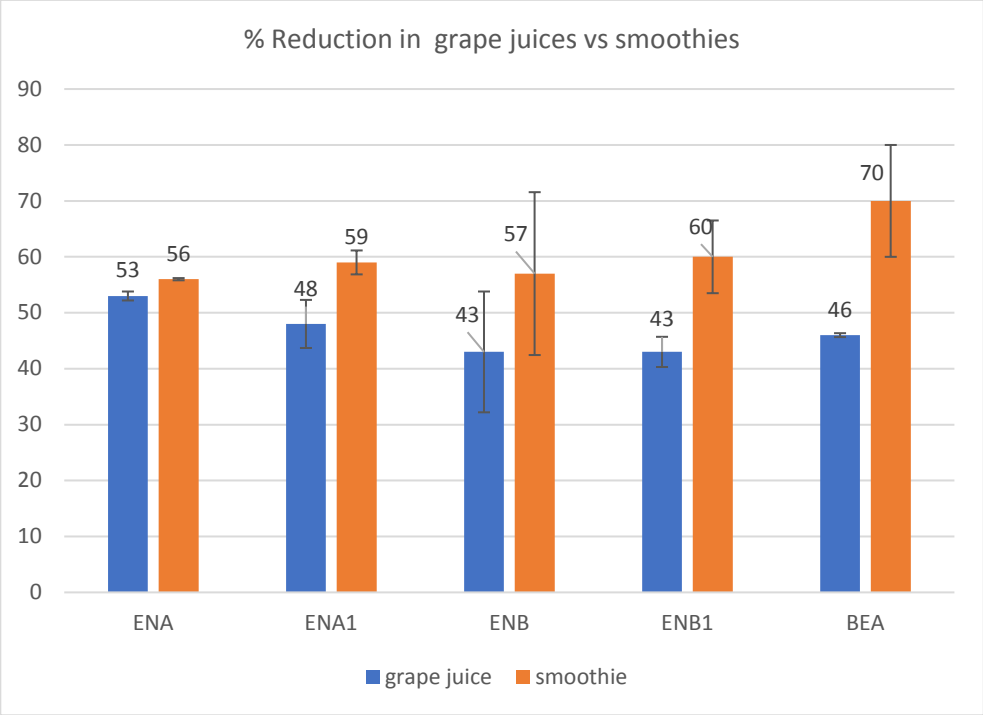


Figure 1. % Reduction of ENs and BEA in grape juice vs smoothie samples after PEF treatment.

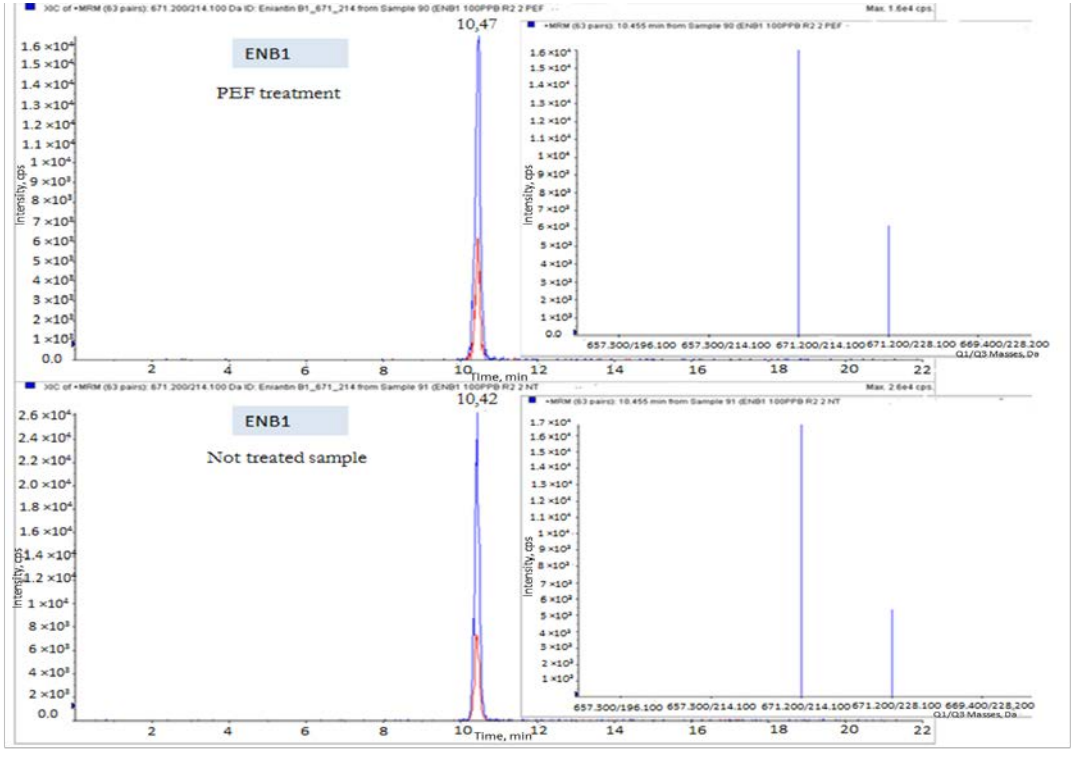


Figure 2. LC-MS/MS-IT chromatogram of juice sample contaminated by ENB1 treated by PEF vs not treated.

During PEF treatment temperatures around 70°C were reached. The emerging mycotoxins are relatively sensible to temperature compounds and experiments in H20 spiked with mycotoxins at the same conditions and warmed at 70°C during the same duration of PEF treatment were performed to check if the degradation observed was a consequence of the temperatures reached during the PEF treatment. The results obtained showed that temperature treatment caused degradations percentages until 26%.

In previous studies, Serrano, Font, Mañes, Ferrer, (2016) and Tolosa, Font, Mañes, & Ferrer, (2017) observed a similar ENB and ENB1 reductions after thermal treatments of cooking pasta and boiling fish at temperatures > 100°C, with reduction percentages from 14 to 49% for ENB and 53-65% for ENB1 after cooking pasta to near 60% (ENB and ENB1) after boiling fish.

As above mentioned, scarce information is available in the literature about the mycotoxin reduction after the PEF treatment. Vijayalakshmi et al., (2017) optimized the combination of thermal processing with PEF to reduce artificially spiked AFs in potato, dextrose and agar system, at different pH. The combination treatment was found to be more effective than individual processes, with reduction percentages from 92.3 to 96.9% for AFB1 in function of pH and from 82 to 95.7% for total AFs. These authors, (Vijayalakshmi et al., (2018) also observed a reduction of AFB1 and AFs contents from 77 to 97% after optimizing PEF methodology. These reductions were similar to the obtained in the present study for BEA in smoothie samples (70%).

The same treatment applied to H2O revealed lower percentages of reduction (from 31 to 45%) (figure 3). The lower percentages of reduction observed in water vs grape juices and smoothies (43 to 70%) may be because of

differences in matrix and/ or in Ph. The effect of pH 4 was assessed on ENs and BEA contents, using water model acidified with lemon juice. An additional degradation was observed when the H2O was acidified for BEA (56%) (figure 3) comparing with neutral H2O, but significant differences were not observed for ENs.

As it was reported (Avsaroglu, Bozoglu, Alpas, Largeteau, & Demazeau, 2015; Hao, Zhou, Koutchma, Wu, & Warriner, 2016), the degradation could be dependent of juice constituents. This fact may explain the different reductions observed in juices comparing with the reductions observed in water, and the higher reductions observed in smoothie samples (from 56 to 70 %) comparing with grape juice samples (from 43 to 53%). Smoothie was made with different fruit juices, purees and cereals, so constitute more complex matrix than grape juice.

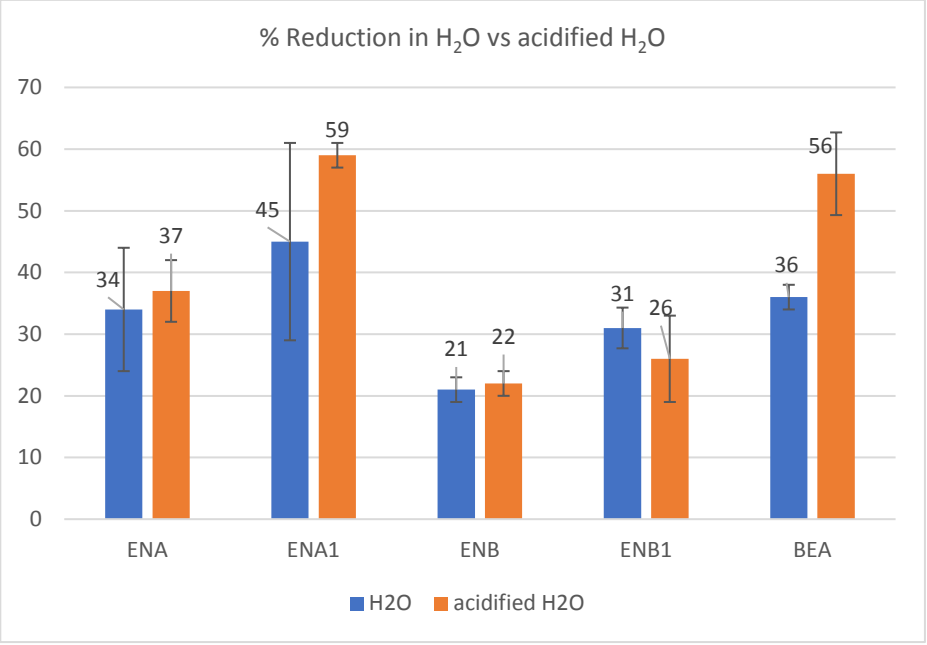


Figure 3. % Reduction of ENs and BEA in H_2O vs acidified H_2O at pH 4 after PEF treatment.

3.2. Identification of degradation products

Several degradation products of ENs and BEA were tentatively identified after PEFs treatment in juice and smoothie matrices (table 1). For this, the samples were injected in LC-ESI-qTOF-MS in the full scan modality. For BEA, the degradation product with m/z 517.3705, corresponds to BEA with the loss of one unit of phenylalanine (Phe) and hydroxyvaleric acid (HyLv). In a previous study Meca, Ritieni, & Mañes, (2012) also observed a degradation product from BEA with the loss of these two structural components (Phe+HyLv).

For ENA one degradation product was identified at m/z 475.3261, that corresponds to the loss of Isoleucine (Ile) and HyLv. This degradation product

was also previously identified by Serrano, Meca, Font, & Ferrer, (2013). For ENA1, the degradation product m/z 475.3244, corresponding to the loss of Valine (Val) and HyLv was identified. For ENB, two degradation products were observed. The degradation product 1 (m/z 437.1936), characterized as the sodium adduct of ENB, with the loss of Val and HyLv. The degradation product 2 with m/z 527.2000 corresponding to the loss of Val and previously reported by Serrano et al., (2013). Finally, for ENB1, the degradation product m/z 443.1674 was identified as the loss of Val and HyLv (figure 4).

The degradation products observed confirm the reduction of the effect of PEFs on emerging mycotoxins. The degradation products were originated from the loss of structural aminoacidic fragments of original molecules like HyLv, Val, Ile or Phe.

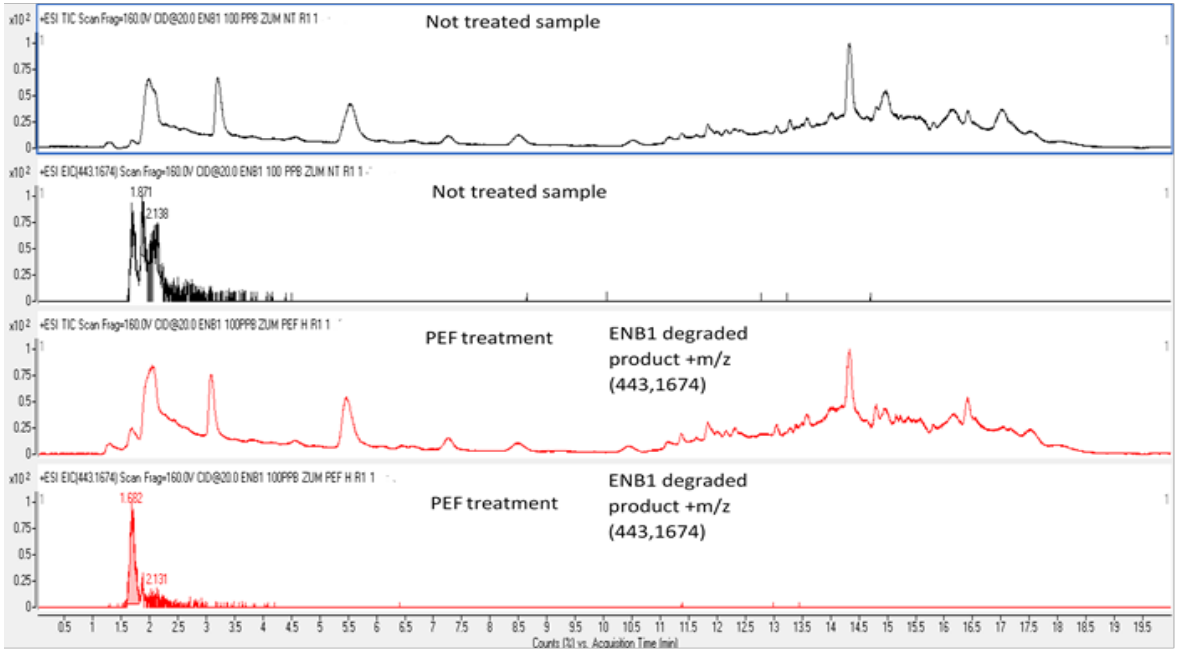
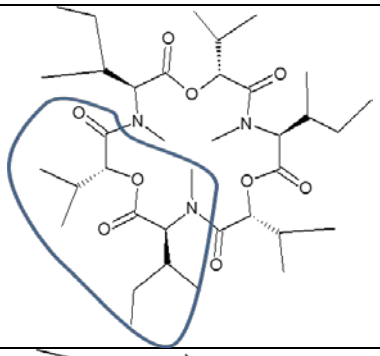
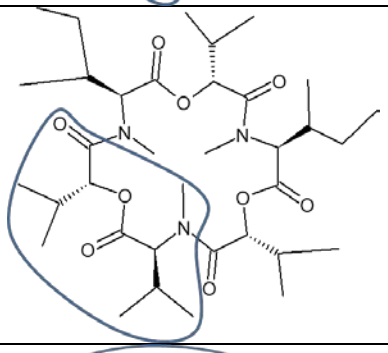
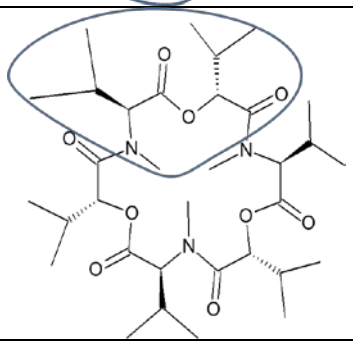
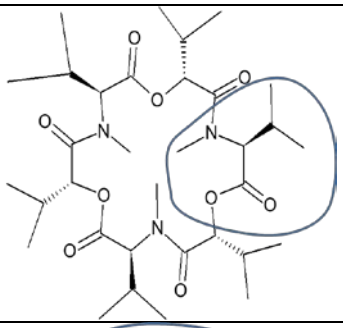
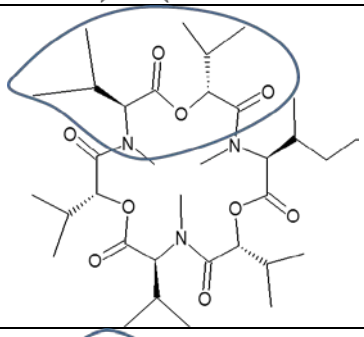
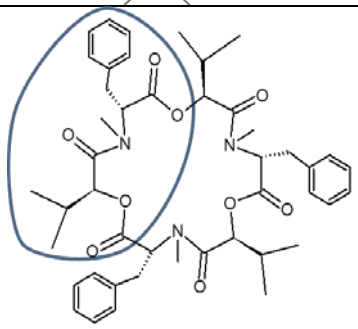


Figure 4. LC-ESI-qTOF-MS chromatogram of degraded product ENB1 +m/z 443.1674 obtained in matrix samples after PEF treatment.

Table 1. LC-ESI-qTOF-MS data (MS1) of the degradation products obtained in juice and smoothie samples after PEF treatment.

Mycotoxin	Degradation product	$[M+H]^+$ m/z	Lost fragment	Molecular Structure
ENA degradation product	$[ENA\text{-}HyLv\text{-}Ile+H_2O]^+$	475.3261	HyLV+Ile	
ENA1 degradation product	$[ENA1\text{-}HyLv\text{-}Val+H_2O]^+$	475.3244	HyLv+Val	
ENB degradation product 1	$[ENB\text{-}HyLv\text{-}Val+Na]^+$	437.1936	HyLv+Val	

ENB degradation product 2	[ENB-Val +H_2O]$^+$	527.2000	Val	
ENB1 degradation product	[ENB1-HyLv-Val]$^+$	443.1674	HyLv+Val	
BEA degradation product	[BEA-Phe-HyLv+2H_2O]$^+$	517.3705	Phe+HyLv	

3.3. *In silico* prediction methods

In silico prediction methods have been used in this survey to evaluate the toxicity of detected and identified degradation products by using the ProTox-II web server.

Results of oral acute toxicity expressed as LD50 (mg/kg) and the corresponding toxicity class for each identified compound are shown in table 2. It should be highlighted that, according to the obtained predictions, both ENB degradation products showed a predicted LD50 of 3 mg/kg, both with a 100% of average similarity and prediction accuracy. Thus, the assigned toxicity class was I. According to this result, special attention should be paid to those degradation products, as their predicted toxicity is comparable to that of ENB and also T-2 Toxin (table 2), which is known to be a toxic fungal metabolite with the lowest tolerable daily intake (TDI) within the *Fusarium* mycotoxins (EFSA, 2011).

Using the ProTox-II web server the organ toxicity can also be predicted, concretely the hepatotoxicity, which has been evaluated for different identified compounds, as liver is the organ where mycotoxins are metabolized. The results obtained regarding the organ toxicity and also the calculated predictions for diverse toxicological endpoints using the ProTox-II web server are reported in table 3. These results showed that identified degradation products were predicted as inactive compounds for hepatotoxicity. However, ENB1 degradation product was predicted as an active compound for immunotoxicity endpoint, although the percentage of prediction accuracy (probability score) was low (51%).

Table 2. Acute Oral Toxicity prediction obtained by using ProTox-II web server.

Mycotoxin	Oral toxicity prediction results			
	Predicted LD50 (mg/kg)	**Predicted Toxicity Class**	**Average similarity (%)**	**Prediction Accuracy (%)**
ENNA degration product	1600	IV	76.28	69.26
ENNA1 degration product	1600	IV	76.28	69.26
ENNB degradation product	3	I	100	100
ENNB degradation product (2)	3	I	100	100
ENNB1 degradation product	1600	IV	75.91	69.26
BEA degradation product	200	III	75.89	69.26

Class I: fatal if swallowed (LD50 ≤ 5 mg/kg); Class II: fatal if swallowed (5mg/kg< LD50 ≤ 50mg/kg); Class III: toxic if swallowed (50mg/kg< LD50 ≤ 300mg/kg); Class IV: harmful if swallowed (300mg/kg< LD50 ≤ 2000mg/kg); Class V: may be harmful if swallowed (2000mg/kg< LD50 ≤ 5000mg/kg); Class VI: non-toxic (LD50 > 5000mg/kg).

Table 3. Organ toxicity and toxicological endpoints predictions calculated using the ProTox-II web server.

Mycotoxin	Classification				
	Organ toxicity (% probability)	Toxicity endpoint (% probability)			
	Hepatotoxicity	Carcinogenicity	Immunotoxicity	Mutagenicity	Cytotoxicity
ENNA degradation product	Inactive (74)	Inactive (56)	Inactive (83)	Inactive (68)	Inactive (63)
ENNA1 degradation product	Inactive (74)	Inactive (56)	Inactive (83)	Inactive (68)	Inactive (63)
ENNB degradation product	Inactive (76)	Inactive (58)	Inactive (77)	Inactive (68)	Inactive (58)
ENNB degradation product (2)	Inactive (76)	Inactive (58)	Inactive (89)	Inactive (77)	Inactive (64)
ENNB1 degradation product	Inactive (75)	Inactive (56)	Active (51)	Inactive (76)	Inactive (66)
BEA degradation product	Inactive (85)	Inactive (53)	Inactive (91)	Inactive (76)	Inactive (70)

The prediction results obtained for the toxicological pathways, nuclear receptor signaling pathways and stress response pathways are reported in tables 4 and 5, respectively. According to the Tox21 Consortium, chemical compounds might have the potential to disrupt processes in the human body which may lead to negative health effects (Drwal et al, 2014). Regarding the nuclear receptor signaling pathway, 7 different pathways were assessed while for the stress response pathways, 5 diverse assays were evaluated. The computational

estimations revealed that degradation products identified in the present study were predicted as inactive for all the analyzed pathways.

The obtained probability scores in tables 4 and 5 showed an adequate accuracy in predicted values, ranging from 83% to 99%. However, for organ toxicity and toxicity endpoints (especially for carcinogenicity) (table 3), probability scores are low and with high variability (51%-91%). This fact could be explained by the quality or adequacy of the database employed for prediction model development. When performing the toxicity endpoint predictions, it could be expected to obtain the best results when using a database for model construction composed by chemical compounds or substances similar to those to be predicted. In our case, chemical compounds included in the databases used for model construction were not probably similar enough to degradation products evaluated. For this reason, the authors suggest revising those predicted results by developing more adequate prediction models.

Table 4. Toxicological pathways: Nuclear receptor signaling pathways predicted for detected mycotoxins.

Mycotoxin	Tox21 Nuclear receptor signaling pathways (% probability)						
	Aryl hydrocarbon Receptor (AhR)	**Androgen Receptor (AR)**	**Androgen Receptor LigandBinding Domain (AR-LBD)**	**Aromatase**	**Estrogen Receptor Alpha (ER)**	**Estrogen Receptor Ligand Binding Domain (ER-LBD)**	**Peroxisome Proliferator Activated Receptor Gamma (PPAR-Gamma)**
ENNA degradation product	Inactive (97)	Inactive (95)	Inactive (97)	Inactive (98)	Inactive (87)	Inactive (97)	Inactive (98)
ENNA1 degradation product	Inactive (97)	Inactive (95)	Inactive (97)	Inactive (98)	Inactive (87)	Inactive (97)	Inactive (98)
ENNB degradation product	Inactive (98)	Inactive (97)	Inactive (99)	Inactive (98)	Inactive (83)	Inactive (96)	Inactive (98)
ENNB degradation product (2)	Inactive (98)	Inactive (95)	Inactive (97)	Inactive (99)	Inactive (86)	Inactive (97)	Inactive (96)
ENNB1 degradation product	Inactive (97)	Inactive (96)	Inactive (99)	Inactive (99)	Inactive (90)	Inactive (97)	Inactive (93)
BEA degradation product	Inactive (93)	Inactive (95)	Inactive (98)	Inactive (95)	Inactive (89)	Inactive (97)	Inactive (95)

Table 5. Toxicological pathways: Stress response pathways predicted for detected mycotoxins.

Mycotoxin	Nuclear factor (erythroid-derived 2-like 2/antioxidant responsive element) (nrf2/ARE)	Heat shock factor response element (HSE)	Mitochondrial Membrane Potential (MMP)	Phosphoprotein (Tumor supressor) p53	ATPase family AAA domain containing protein 5 (ATAD5)
ENNA degradation product	Inactive (98)	Inactive (98)	Inactive (98)	Inactive (98)	Inactive (98)
ENNA1 degradation product	Inactive (98)	Inactive (98)	Inactive (98)	Inactive (98)	Inactive (98)
ENNB degradation product	Inactive (99)	Inactive (99)	Inactive (98)	Inactive (99)	Inactive (97)
ENNB degradation product (2)	Inactive (99)	Inactive (99)	Inactive (99)	Inactive (99)	Inactive (96)
ENNB1 degradation product	Inactive (98)	Inactive (98)	Inactive (97)	Inactive (97)	Inactive (99)
BEA degradation product	Inactive (97)	Inactive (97)	Inactive (91)	Inactive (96)	Inactive (95)

4. Conclusions

The application of PEF treatment to juice and smoothie samples produced mycotoxin reduction percentages from 43 to 70%. The same treatment applied to H_2O samples produced lower reductions showing that matrix constituents may affect PEFs results. After the treatment, degradation products originated by the loss of structural aminoacidic fragments of the original molecules, such as HyLv, Val, Ile or Phe were identified. Pro Tox-II server assigned a toxicity class I for ENB degradation products while the rest of degradation products were classified in toxicity class III and IV. PEFs is presented here as a good strategy to mitigate ENs and BEA contents in juice and smoothie samples, underlying the importance of the degradation products identification and toxicity assessment.

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References

(Avsaroglu et al., 2015). Avsaroglu, M. D., Bozoglu, F., Alpas, H., Largeteau, A., & Demazeau, G. (2015). Use of pulsed-high hydrostatic pressure treatment to decrease patulin in apple juice. *High pressure research*, *35*(2), 214-222. https://doi.org/10.1080/08957959.2015.1027700.

(Banerjee et al. 2018). Banerjee, P., Eckert, A. O., Schrey, A. K., & Preissner, R. (2018). ProTox-II: a webserver for the prediction of toxicity of chemicals. *Nucleic acids research*, *46*(W1), W257-W263. https://doi.org/10.1093/nar/gky318.

(Barba et al., 2015). Barba, F. J., Parniakov, O., Pereira, S. A., Wiktor, A., Grimi, N., Boussetta, N. Saraiva, J. A., Raso, J., Martin-Belloso, O., Witrowa-Rajchert, D., Lebovka, N., Vorobiev, E. (2015). Current applications and new opportunities for the use of pulsed electric fields in food science and industry. *Food Research International*, *77*, 773-798. https://doi.org/10.1016/j.foodres.2015.09.015.

(Cotterill et al., 2008). Cotterill, J. V., Chaudhry, M. Q., Matthews, W., & Watkins, R. W. (2008). *In silico* assessment of toxicity of heat-generated food contaminants. *Food and Chemical Toxicology*, 46(6), 1905-1918. https://doi.org/10.1016/j.fct.2008.01.030.

(Cserhalmi et al., 2006). Cserhalmi, Z., Sass-Kiss, A., Tóth-Markus, M., & Lechner, N. (2006). Study of pulsed electric field treated citrus juices. Innovative Food Science & Emerging Technologies, 7(1-2), 49-54. https://doi.org/10.1016/j.ifset.2005.07.001.

(Drwal et al., 2014). Drwal, M. N., Banerjee, P., Dunkel, M., Wettig, M. R., & Preissner, R. (2014). ProTox: a web server for the *in silico* prediction of rodent oral toxicity. *Nucleic acids research*, *42*(W1), W53-W58. https://doi.org/10.1093/nar/gku401.

EFSA Panel on Contaminants in the Food Chain (CONTAM). (2011). Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. *EFSA Journal*, *9*(12), 2481.

European Commission (2002) Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *OJEU L* 221, 8−36.

(Gozalbes et al., 2014). Gozalbes, R., de Julián-Ortiz, J. V., & Fito-López, C. (2014). Métodos computacionales en toxicología predictiva: aplicación a la reducción de ensayos con animales en el contexto de la legislación comunitaria REACH. *Revista de Toxicología*, *31*(2), 157-167.

(Hao et al., 2016). Hao, H., Zhou, T., Koutchma, T., Wu, F., & Warriner, K. (2016). High hydrostatic pressure assisted degradation of patulin in fruit and vegetable juice blends. *Food control*, *62*, 237-242. https://doi.org/10.1016/j.foodcont.2015.10.042.

(Kar & Leszczynski, 2019). Kar, S., & Leszczynski, J. (2019). Exploration of computational approaches to predict the toxicity of chemical mixtures. *Toxics*, *7*(1), 15. https://doi.org/10.3390/toxics7010015.

(Karlovsky et al., 2016). Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., Oswald, I. P., Speijers, G., Chiodini, A., Recker, T., & Dussort, P. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin research*, *32*(4), 179-205. https://doi.org/10.1007/s12550-016-0257-7

(Knorr et al., 2011). Knorr, D., Froehling, A., Jaeger, H., Reineke, K., Schlueter, O., & Schoessler, K. (2011). Emerging technologies in food processing. *Annual review of food science and technology*, *2*, 203-235. https://doi.org/10.1146/annurev.food.102308.124129.

(Mallebrera et al., 2018). Mallebrera, B., Prosperini, A., Font, G., & Ruiz, M. J. (2018). In vitro mechanisms of Beauvericin toxicity: A review. *Food and Chemical Toxicology*, *111*, 537-545. https://doi.org/10.1016/j.fct.2017.11.019.

(Meca et al., 2012). Meca, G., Ritieni, A., & Mañes, J. (2012). Influence of the heat treatment on the degradation of the minor Fusarium mycotoxin beauvericin. *Food Control*, *28*(1), 13-18. https://doi.org/10.1016/j.foodcont.2012.04.016.

(Morales-de la Peña et al., 2011). Morales-de La Peña, M., Elez-Martínez, P., & Martín-Belloso, O. (2011). Food preservation by pulsed electric fields: an engineering perspective. *Food Engineering Reviews*, *3*(2), 94-107. https://doi.org/10.1007/s12393-011-9035-7.

(Pallarés et al., 2019). Pallarés, N., Carballo, D., Ferrer, E., Fernández-Franzón, M., & Berrada, H. (2019). Mycotoxin Dietary Exposure Assessment through

Fruit Juices Consumption in Children and Adult Population. *Toxins*, *11*(12), 684.https://doi.org/10.3390/toxins11120684.

(Picart-Palmade et al., 2019). Picart-Palmade, L., Cunault, C., Chevalier-Lucia, D., Belleville, M. P., & Marchesseau, S. (2019). Potentialities and limits of some non-thermal technologies to improve sustainability of food processing. *Frontiers in nutrition*, *5*, 130. https://doi.org/10.3389/fnut.2018.00130.

(Pinela & Ferreira, 2017). Pinela, J., & Ferreira, I. C. (2017). Nonthermal physical technologies to decontaminate and extend the shelf-life of fruits and vegetables: Trends aiming at quality and safety. *Critical reviews in food science and nutrition*, *57*(10), 2095-2111. https://doi.org/10.1080/10408398.2015.1046547.

(Prosperini et al., 2017). Prosperini, A., Berrada, H., Ruiz, M. J., Caloni, F., Coccini, T., Spicer, L. J., Perego, M. C., & Lafranconi, A. (2017). A review of the mycotoxin enniatin B. *Frontiers in public health*, *5*, 304. https://doi.org/10.3389/fpubh.2017.00304.

(Serrano et al., 2013). Serrano, A. B., Meca, G., Font, G., & Ferrer, E. (2013). Degradation study of enniatins by liquid chromatography–triple quadrupole linear ion trap mass spectrometry. *Food chemistry*, *141*(4), 4215-4225. https://doi.org/10.1016/j.foodchem.2013.05.030.

(Serrano et al., 2016). Serrano, A. B.; Font, G.; Mañes, J., & Ferrer, E (2016). Development a mitigation strategy of enniatins in pasta under home-cooking conditions. *LWT-Food Science and Technology*, *65*, 1017-1024. https://doi.org/10.1016/j.lwt.2015.09.036.

(Tolosa et al., 2017) Tolosa, J., Font, G., Mañes, J., & Ferrer, E (2017). Mitigation of enniatins in edible fish tissues by thermal processes and identification of degradation products. *Food and chemical toxicology*, *101*, 67-74. https://doi.org/10.1016/j.fct.2016.12.039.

(Vijayalakshmi et al., 2017). Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., Kumar, S & Reddy, R. (2017). Effect of combination processing on aflatoxin reduction: process optimization by response surface methodology. *Journal of Food Processing and Preservation*, *41*(6), e13230. https://doi.org/10.1111/jfpp.13230.

(Vijayalakshmi et al., 2018). Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., & Kumar, S. S. (2018). Effect of pH and pulsed electric field process parameters on the aflatoxin reduction in model system using response surface methodology. *Journal of food science and technology*, *55*(3), 868-878. https://doi.org/10.1007/s13197-017-2939-3.

(Zhang et al., 2012). Zhang, Y., Hou, Y., Zhang, Y., Chen, J., Chen, F., Liao, X., & Hu, X. (2012). Reduction of diazinon and dimethoate in apple juice by pulsed electric field treatment. *Journal of the Science of Food and Agriculture*, *92*(4), 743-750. https://doi.org/10.1002/jsfa.4636.

(Zhang et al., 2019). Zhang, Z. H., Wang, L. H., Zeng, X. A., Han, Z., & Brennan, C. S. (2019). Non-thermal technologies and its current and future application in the food industry: a review. *International journal of food science & technology*, *54*(1), 1-13. https://doi.org/10.1111/ijfs.13903.

3.10. High hydrostatic pressure (HHP) and pulsed electric fields (PEF) technologies to mitigate aflatoxins contents in juice

Food and Chemical Toxicology (under review)

High hydrostatic pressure (HHP) and pulsed electric fields (PEF) technologies to mitigate aflatoxins contents in juice.

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ABSTRACT

The demand of fresh-like products to meet the five daily fruits and vegetables serving encouraged the implementation of non-thermal food processing techniques, such as high pressure processing (HPP) and pulsed electric fields (PEF), promoting low impact on nutritional components of juices fruit drinks, keeping their sensory properties unaltered and extending their shelf life. The aim of the present work is to study the application of HPP and PEF techniques as useful decontamination tool for aflatoxins (AFs) reduction in juices. Spiked juices samples with AFs were treated by PEF and HPP processes. After treatments, AFs contents were extracted using dispersive liquid-liquid microextraction (DLLME) and determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS-IT). Similar reductions percentages (14-29%) have been obtained for both treatments even higher reductions were obtained for AFB2 and AFG1 under PEF treatment. Results obtained in juice samples differed slightly from those obtained in water controls highlighting the matrix effect. Both HPP and PEF techniques showed impact on mycotxins levels. Furthermore, an AFB2 degradation product obtained after PEF has been identified by quadrupole time of flight mass spectrometry detector (qTOF-MS) and its toxicological endpoints predicted by Pro Tox-II web server.

Keywords: aflatoxins; HPP; PEF; LC-MS/MS-IT; LC-ESI-qTOF-MS; Pro Tox-II

1. Introduction

Mycotoxins are toxic compounds mainly produced by *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps* genera. Their presence is reported in a high variety of agricultural food products and its occurrence depends on several factors, such as, the product moisture content, the water activity, the relative air humidity temperature, the pH value, the composition of the food matrix and the presence of physical damages and mould spores (Pleadin et al., 2019; Shi et al., 2018). Aflatoxins (AFs) are toxic mycotoxins produced by *Aspergillus* genera related with carcinogenic, mutagenic and immuno-suppressive adverse effects. (Marín et al., 2013). The International Agency for Research on Cancer (IARC) classify the AFs [aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1), and aflatoxin G2 (AFG2)] as carcinogenic to humans (Group 1) (IARC, 2002).

AFs are largely reported in food commodities such as groundnuts, sesame seeds, millet, maize, rice, wheat, fig, spices and cocoa due to fungal infection during pre- and post-harvest stages (Mahato et al., 2019). Abdel-Sater et al. (2001) have observed their presence in apple and guava juices while Pallarés et al. (2019) have reported their occurrence in fresh orange juices and in pineapple and peach packed juices. The European Commission (EC) has set maximal concentrations of AFB1 and the sum of AFB1, AFB2, AFG1 and AFG2 in some foodstuffs, such as cereals, cereals-based foods, maize, some spices, nuts and dried fruits, but not maximum levels of AFs have been set in juices (EC, 2006). In addition, EFSA, (2020) assessed the human health risks related to the presence of AFs in food. To carry out the risk assessment, experts assessed the toxicity of AFs and estimated the dietary exposure of EU populations taking

into account new exposure data confirming that AFs are genotoxic and carcinogenic (EFSA, 2007).

Nowadays, further research is needed to implement methods for mycotoxin reduction or elimination. An efficient method for mycotoxins reduction should be able to remove or inactive mycotoxins without producing toxic residues or affecting the nutritive value, the palatability or the technological properties of products. Many physical, chemical and biological strategies have been suggested with acceptable results. Inside physical methods, different thermal processes such as extrusion, cooking, frying, baking, crumbling, pelleting, roasting, etc., can significantly reduce mycotoxins contents. However, the implementation does not result in the complete elimination of mycotoxins and high-temperature processes can cause losses of nutritional quality and/or organoleptic properties (Cˇolovic et al., 2019).

Currently, the industry is highly interested on the innovative nonthermal techniques, such as, high pressure processing (HPP) and pulsed electric fields (PEF), as a green alternative in food processing (Hassan and Zhou, 2018) for a better solid waste contribution to the biomass resources (Picart-Palmade et al., 2018). Furthermore, non-thermal processes aim to correctly meet the demand of the fresh population, keeping the nutritional components and sensory properties unchanged. Obtaining sterilized food changing the cell of microorganism rather than high temperatures makes these labelled "green techniques" very attractive to fresh fruits and vegetables industry (Pinela et al., 2017; Zhang et al., 2019).

Nowadays, fruit juice consumption is trendy and people are seeking freshness, high vitamin content, minerals and low-calorie diet to meet the recommendation of five daily pieces of fruits and vegetables (Mandappa et al.,

2018). In Europe, HPP technology is applied for industrial preservation of juices since 1994 leading fresh juices and smoothies supply in Spanish market (Sampedro et al., 2010; Kempkes et al., 2016).

HPP and PEF impact on the inactivation of spores and growth of mycotoxigenic fungi have been investigated even a few information is available about the effect of these technologies on mycotoxins levels (MacGregor et al., 2000; Evelyn et al., 2017; Kalagatur et al., 2018; Groot et al., 2019).

Regarding PEF, Vijayalakshmi et al. (2017 and 2018) observed AFs reduction percentages until 97% in potato dextrose agar model system for PEF while HPP treatment has evidenced citrinin reduction in black table olives (Tokusoğlu et al., 2010), Deoxynivalenol (DON) and Zearalenone (ZEA) decrease in maize (Kalagatur et al., 2018) and Patulin (PAT) cutback in juice blends (Hao et al., 2016; Avsaroglu et al., 2015). This reduction rates were up to 100% in some particular conditions.

In this context, the aim of the present study is to investigate the application of emerging technologies, HPP and PEF as useful tool for AFs reduction in juices. Spiked juice samples with AFB1, AFB2, AFG1 and AFG2 were treated under PEF and HPP technologies. Then mycotoxins were extracted using dispersive liquid-liquid microextraction (DLLME) and determined by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS-IT). Moreover, liquid chromatography coupled to electrospray ionisation and quadrupole time of flight mass spectrometry (HPLC-Q-TOF-MS) was used for tentative identification of AFs degradation products. Finally, Pro Tox-II web server *in silico* was employed to predict toxicological endpoints of degradation products obtained.

2. Materials and methods

2.1. Reagents and Chemicals

Ethyl acetate (EtOAc) (HPLC grade 99.5+ %) was supplied by Alfa Aesar (Karlsruhe, Germany). Acetonitrile (ACN) (HPLC grade), methanol (MeOH) (HPLC grade) and Chloroform (CHCl3) (99% grade) were purchased from Merck (Darmstadt, Germany). The deionized water with resistivity > 18 MΩ cm-1 was obtained from a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, USA). Mobile phases, water and MeOH, were filtered prior to use through a 0.45-μm cellulose filter supplied by Scharlau (Barcelona, Spain).

The salts, formic acid (reagent grade ≥ 95%) was purchased from Sigma-Aldrich (St. Louis, MO, USA), ammonium formate (99%) was obtained from Panreac Quimica S.A.U. (Barcelona, Spain) and sodium chloride (NaCl) was supplied by VWR Chemicals (Leuven, Belgium). All samples were filtered prior to injection using a 13 mm/0.22 μm nylon filter acquired from Membrane Solutions (TX, USA).

Standard of mycotoxins AFB1, AFB2, AFG1, AFG2 were purchased from Sigma (St. Louis, MO, USA). Standards were prepared at concentration of 1000 mg/l in methanol. The appropriate working solutions were prepared from the standrads solutions. All solutions were placed at −20°C until the analysis.

2.2. Samples

15 bottles of grape juice samples were purchased from a supermarket in Valencia. Samples were homogenized and aliquots were taken and tested for the absence of AFs. For PEFs treatment, a volume of 215 ml of grape juice spiked individually at concentration of 100 μg/L of AFB1, AFB2, AFG1 and AFG2

was employed. Prior to the treatment, aliquots of 5 ml were separated as not treated control.

For HPP treatment, a volume of 3 ml of grape juice was spiked with the same mycotoxins and concentration mentioned above. Prior to the treatment, aliquots of 1 ml were separated for controls. The experiments under PEFs and HPP treatments were also performed in water samples spiked of each AFs at concentration of 100 μg/L. All experiments were performed in triplicate. The pH of juice was measured in 3.9.

2.3. High hydrostatic pressure treatment (HPP)

A high-pressure equipment (EFSI NV) (Temse, Belgium) capable to produce nominal pressures up to 680 MPa equipped with 2.35 L pressure chamber filled with a mixture of water and anticorrosion additive was employed in the present study. In the pressure chamber, the temperature of the liquid can be adjusted at values between 15 and 90°C by a built-in heating system. Temperature increases in samples during pressurization process due to the adiabatic heat at approximately 3°C=100 MPa. Eppendorf containing samples were filled avoiding air bubbles and were enclosed in plastic bags. Plastic bags were filled with water and sealed prior to the treatment. Finally, samples were treated under pressure of 500 MPa during treatment time of 5 minutes. No temperature was applied during treatment.

2.4. Pulsed electric field treatment (PEF)

An Elea pulsed electric field cellcrack III equipment with 10 cm of gap between electrodes container was employed for the treatment. The treatment conditions consisted in voltage set at 30 kv, resulting in a field strength of 3

kv/cm, and in specific energy of 500 KJ/Kg. For reach the 500 KJ/Kg an average of 238 pulses were applied in different cycles. During PEF treatment temperature not exceeded 75ºC with conductivity of 2890 µs/cm for grape juice.

2.5. Dispersive Liquid-Liquid Microextraction procedure (DLLME)

Controls and PEFs and HPP samples were extracted according to Pallarés et al. (2019). For PEFs treated samples, 5 ml of samples were placed in 10 ml conical tub with 1g of NaCl and shaken one minute. Then, was added the combination of dispersant and extractant solvents AcN (950 µl) and EtOAc (620 µl), respectively. After shake for one minute, a cloudy solution of the three components was obtained. This mixture was centrifuged at 4000 rpm for 5 min, allowing the separation of the phases. The organic phase located at the top of the tube was separated and placed into other conical tube. Next, in a second step the mixture of MeOH (950 µl) (dispersant solvent) and CHCL3 (620 µl) (extractant solvent) was added to the remaining residue. After shake and centrifugation, the organic phase, located in the bottom of the tube was separated and placed with the first organic phase separated before. Two recovered organic phases were evaporated to near dryness employing a nitrogen stream using a Turvovap LV Evaporator (Zymark, Hoptikinton, USA). Finally, the dried residue was reconstituted with 1 ml of 20 mM ammonium formate (MeOH/AcN) (50/50 v/v) and filtered through a 13 mm/0.22 µm nylon filter prior to inject in LC-MS/MS-IT.

For HPP samples the extraction method was readjusted to the sample volume available, for this 1 ml of controls or samples was employed with 0.2 g of NaCl, and 523 µl of the mixture AcN and EtOAc and 523 µl of the mixture MeOH andCHCL3 (both prepared at the same proportion specified above, 9.5

ml of dispersant solvent and 6.2 ml of extractant solvent). The different steps were conducted as has been detailed above.

2.6. LC-MS/MS-IT determination

For mycotoxins determination an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with Turbo Ion Spray (ESI) electrospray ionization was employed. The QTRAP analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. The analysis was performed using a Gemini-NX column C18 (Phenomenex, 150 mm x 4.6 mm, 5 particle size) preceded by a guard column. The flow rate was set at 0.25 ml/min, the injection volume at 20 μL and the oven temperature at 40ºC.

Mobile phases employed consist in, mobile phase A: 5 mM ammonium formate, 0.1% formic acid water and mobile phase B: 5 mM ammonium formate, 0.1% formic acid methanol. The gradient was fixed as follows: started with a proportion of 0% for mobile phase B; in 10 min increased to 100%, then decreased to 80% in 5 min, and finally decreased to 70% in 2 min. In the next 6 min, the column was cleaned and readjusted to initial conditions, finally was equilibrated for 7 min.

The Turbo Ion Spray operated in positive ionization mode (ESI+). Nitrogen was served as nebulizer and collision gas. For the analysis, the following parameters were fixed: ion spray voltage 5500 V; curtain gas 20 arbitrary units; GS1 and GS2, 50 and 50 psi, respectively; probe temperature (TEM) 450°C.

2.7. LC-ESI-qTOF-MS analysis

Degradation products were identified by an Agilent 1200-LC chromatographic system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 6540 Agilent Ultra- High-Definition Accurate-Mass q-TOF-MS, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI). A Gemini-NX column C18 (Phenomenex, 150 mm x 4.6 mm, 5 particle size) was employed to chromatographic separation. Mobile phases consisted in water (A) and acetonitrile (B), both with 0.1% of formic acid. The gradient was fixed as follows: 0–6 min, 50% B; 7–12 min, 100% B; 13–20 min, 50% B. The injection volume was set in 5 μL and the flow rate in 0.2 mL/min. For mass spectrometry, the following parameters were fixed: interface in positive ionization mode, drying gas flow (N2) 12.0 L min−1; nebulizer pressure, 50 psi; gas drying temperature, 370ºC; capillary voltage, 3500 V; fragmentor voltage, 160 V. Analysis was performed in MS mode and MS spectra data were collected within the scan range 50–1500 m/z.

2.8. Method Validation

Method was characterized in terms of recovery, repeatability (intraday precision), reproducibility (interday precision), matrix effects, linearity, limit of detection (LOD) and limit of quantification (LOQ) according to the Commission Decision (EC, 2002). Recoveries obtained for AFs in juices were in the range from 63% to 115%. LODs and LOQs were 0.3 μg/l and 1 μg/l, respectively. Matrix effects experiments revealed signal suppression from 41 to 80%. To determine linearity, regression coefficients were higher than 0.990 in all cases (Pallarés et al., 2019).

2.9. *In silico* prediction methods

One identified degradation product from AFB2 was assessed in terms of toxicity by using the ProTox-II web server (http://tox.charite.de/protox_II/). This tool was employed to predict diverse toxicological endpoints such as acute toxicity, hepatotoxicity, cytotoxicity, carcinogenicity, mutagenicity, immunotoxicity, adverse outcomes pathways (Tox21) and toxicity targets for AFB2 degradation product (Drwal et al., 2014; Banerjee et al., 2018).

This tool incorporates molecular similarity, pharmacophores, fragment propensities and machine-learning models for the prediction of various toxicity endpoints (Banerjee et al., 2018). Moreover, all ProTox methods have been evaluated based on a diverse external validation set (sensitivity, specificity and precision of 76, 95 and 75%, respectively), indicating their possible applicability for other compound classes. The prediction method used by ProTox-II is based on the analysis of the two-dimensional (2D) similarity to compounds with known LD50 values and the identification of fragments over-represented in toxic compounds.

Regarding the toxicity endpoint and organ toxicity prediction, the predictive models are based on data from both *in vitro* (e.g. Tox21 assays, Ames bacterial mutation assays, hepG2 cytotoxicity assays, immunotoxicity assays, among others) and *in vivo* assays (e.g. carcinogenicity, hepatotoxicity).

To predict potential toxicities by using the ProTox-II web server, the chemical structure of identified degradation products has been drawn by using the chemical editor Chem.Doodle (https://www. chemdoodle.com/). This fact is essential when there is no standard available or the substances have not been synthesized, as for example reaction or degradation products formed after food treatment.

3. Results and Discussion

3.1. Effect of HPP treatment

After the HPP treatment, concentrations of 83.1±2μg/L (AFB1), 86.3±16 μg/L (AFB2), 80.8±6μg/L (AFG1) and 71±10μg/L (AFG2) were obtained corresponding to reduction percentages of 17% for AFB1, 14% forAFB2, 19% for AFG1 and 29% for AFG2. (figure 1). The same conditions treatment applied to water samples leaded to higher reductions of AFs, with observed concentrations of 26.8±5 μg/L (AFB1), 39.3±5 μg/L (AFB2), 12.9±5 μg/L (AFG1) and 14.5±5 μg/L (AFG2), equivalent to reduction percentages ranging from 61 to 87% (figure 1). These significative differences observed between juice and water samples may be attributed to matrix effects.

To the best of our knowledge, no studies are available about the effect of HPP on AFs contents in juice, since only PAT was studied. Hao et al. (2016) obtained similar results. A highest level of PAT reduction around 30% was observed in romaine, celery, cucumber, apple, spinach, kale parsley and lemon mixture juice spiked at concentration of 200 μg/L of PAT and treated under HPP at 600 MPa, during 300s, and temperature of 11°C (Hao et al., 2016). Avsaroglu et al. (2015) studied the reduction of PAT in apple juices spiked with 5, 50 and 100 μg/L, using two different pressure procedures, HPP and Pulsed-high hydrostatic pressure (p-HPP). The results showed higher percentages of reduction than in the present study, a decrease of PAT until to 51.16% for HPP (at 400 MPa and 30°C) and to 62.11% for p-HPP(6 pulse x 50 s, 300 MPa and 50°C).

Some authors studied the effect of HPP treatments in other food matrixes. Kalagatur et al. (2018) observed a complete reduction of DON and ZEA in maize after HPP treatment (550 MPa) at 45°C for 20 min of pressure holding

time, higher reductions than those obtained in the present work. Tokusoğlu et al. (2010), in olives sample spiked with CIT at concentration of 100 μg/Kg and treated by HPP at 250 MPa during 5 min, observed a reduction average of 1.3%. Higher reductions were obtained in the present study at the same concentration assayed, but under higher pressure (500 MPa).

Therefore, reduction percentages may depend on the food matrix, the studied mycotoxin and the treatment conditions applied.

3.2. Effect of PEF treatment

After PEF treatment, the concentrations observed were 28.5±5μg/L (AFB2), 16.4±6μg/L (AFG1), 74.4±10μg/L (AFB1), 76.7 ±5μg/L (AFG2). The reduction percentages reached 72% for AFB2 and 84% for AFG1, more than those observed for AFB1 and AFG2, 25% and 24% respectively (figure 1). In water samples, similar reduction percentages, 30% (AFB1), 75% (AFB2), 84% (AFG1) and 31% (AFG2) (figure 1), were obtained. So, no significative differences can be associated to the matrix. About AFs reduction for PEF treatment, only two studies were available in bibliography and were performed in potato dextrose agar model system.

Similar reduction percentages for AFB2 and AFG1 have been observed by Vijayalakshmi et al., (2017 and 2018). These authors applied PEF combined with thermal treatment in potato dextrose agar samples spiked with AFs and observed that the combination resulted more effective than individual processes. The basic pH (10) in model system resulted in additional AFs reduction, as they concluded after investigating the effect of several pHs (4,7 and 10).

Similar results were observed comparing the juices treated by PEF vs HPP for AFB1 and AFG2, with mean reductions in the range from 17 to 29%.

However, for AFB2 and AFG1, highest reductions were obtained for PEF treatment, 72 and 84%, respectively. On the other side, similar results were obtained by PEF treatment in juice and water controls, but differences were observed in the HPP treated juice and water, with higher reduction percentages observed in water (up to 61%). The differences obtained between two treatments may be attributed to their different physicochemical characteristics.

About thermal treatments, some studies are available on AFs contents. Gbashi et al. (2019) studied the thermal degradation of multiple mycotoxins as a function of temperature (120 to 200 °C) and time (6 to 60 min) in spiked maize flour. These authors observed an average degradation rates from 37% to 55% for AFs in maize, with practically complete reduction (90-100%) after 55 min at 200°C. Yazdanpanah et al. (2005) investigated the effect of roasting pistachio nuts on AFs reduction. The treatment at 150°C for 30 min in naturally contaminated pistachios with AFB1 and AFB2 showed degradation from 17% to 67%. The increase of time at 120 min degraded more than 95% of AFB1. Soliman et al. (2002) observed that AFs levels were reduced in green coffee beans naturally contaminated with AFs by approximately 56% during traditional roasting at 180 °C for 10 min. Hwang et al. (2006) investigate the effect of thermal treatment on the destruction of AFB1 in contaminated wheat samples at levels until 100 ppb heated in oven at various temperatures (50, 100, 150, 200 °C) and periods (30, 60, 90 min) and observed that AFB1 decreased from 50% to 90% depending to temperature and time. Less than reduction rate of 7% was observed by these authors at 50 °C during a treatment time of 30 min. Different authors suggested that, in general, AFs degradation was both, time and temperature dependent.

Reduction about 14-29% for AFs were achieved in juice after HPP treatment and about 25% for AFB1 and 24% for AFG2 after PEF treatment. However, higher reduction percentages were observed in the present study for AFB2 and AFG1 in juice treated by PEF, 72 and 84% respectively. Is important to highlight that for obtain higher reductions by thermal process are necessary temperatures higher than 150 °C and treatment times longer than 30 min. The non-thermal processing treatments proposed in the present study, allowed to reach significative AFs reductions rates at treatment times of 5 min, lower than those necessary to reach the same reductions employing conventional thermal processes.

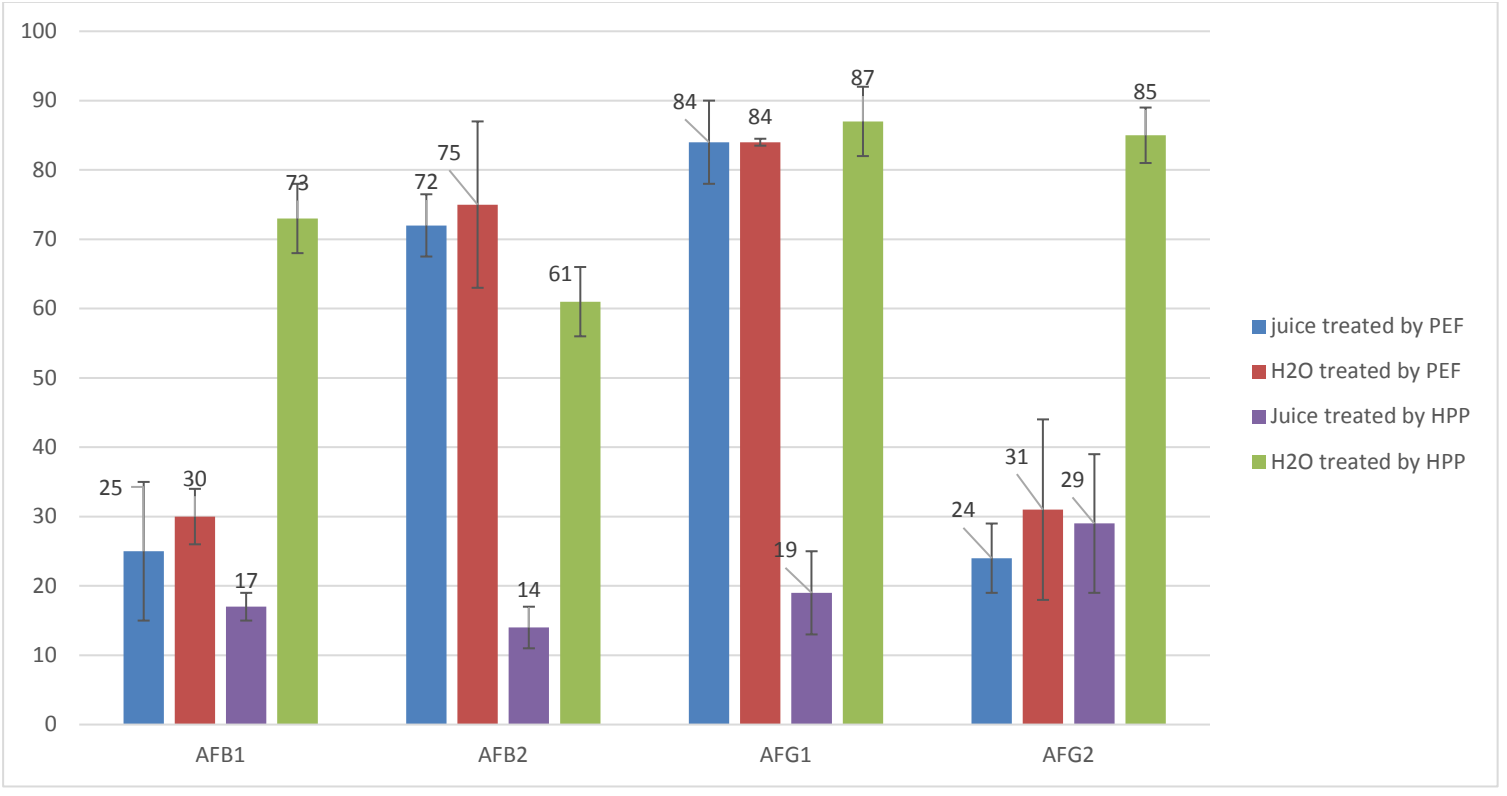

Figure 1. % AFs reduction in juice and H_2O samples after PEF treatment vs HPP treatment.

3.3. Degradation products identification

The juice samples with higher percentage of reduction were injected in LC-ESI-qTOF-MS in the full scan modality for tentatively identification of degradation products. An AFB2 degradation product was identified in juice after PEF with $[M+H]^+$ m/z 355.0711, corresponding with higher reduction achievement after treatment (approximately 72%). $[M+H]^+$ m/z may be attributed to the addition of OH^- and H^+ reaction groups to double bounds of AFB2 structure and the loss of methylene group (-CH2). Similar degradation product was observed previously for AFB1 by Wang et al. (2015). Figure 2 shows the LC-ESI-qTOF-MS chromatogram of AFB2 degraded product.

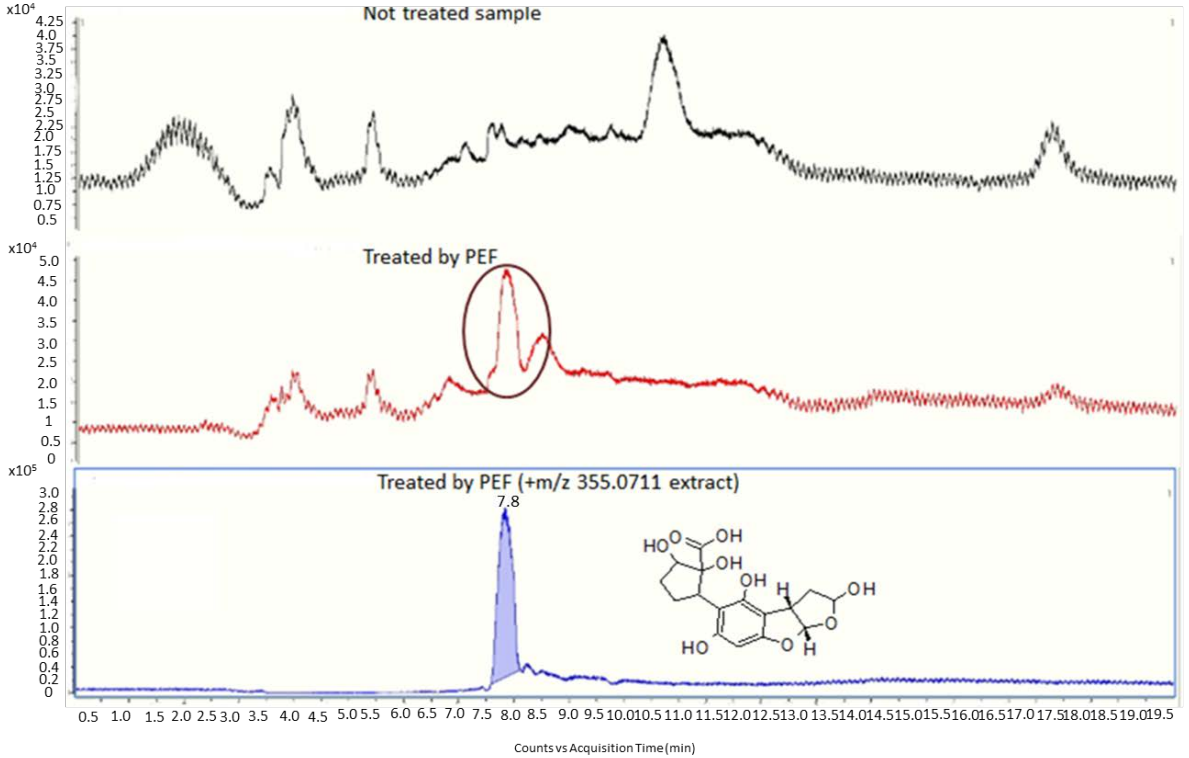

Figure 2. LC-ESI-qTOF-MS chromatogram of degraded product AFB2 +m/z 355.0711 obtained in juice after PEF treatment. A: not treated sample. B: treated by PEF sample. C: extracted chromatogram for m/z 355.0711from treated by PEF sample and possible structure of degraded product.

3.4. *In silico* prediction for degradation products

In silico prediction methods have been used in this survey to evaluate the toxicity of detected and identified products of mycotoxins degradation using the ProTox-II web server.

Results for oral acute toxicity expressed as LD50 (mg/kg) and the corresponding toxicity class for the identified compound showed that AFB2 degradation product was less toxic than its parent compound.

AFs are classified as carcinogenic compounds by the IARC; furthermore, they have shown to be hepatotoxic, mutagenic, immunotoxic, among other adverse effects. However, the identified AFB2 degradation product showed to be "inactive" for carcinogenicity, hepatotoxicity and mutagenicity, with 62%, 72% and 70% of probability, respectively. On the other hand, this identified degradation product was predicted to be immunotoxic with 99% of probability, may be due to its different chemical structure compared to AFB2 and other AFs. Although the probability of prediction is high, it is recommended to isolate the degradation product and to perform *in vitro* studies to confirm these results and to be able to compare them with those obtained in studies with AFB2.

4. Conclusions

HPP and PEF treatments leaded to significant reduction of AFs in treated juices. The reduction was similar for AFB1 and AFG2, however higher reductions were reached for AFG1 and AFB2 with PEF. Both treatments allowed in a shorter application time similar reductions to those obtained with thermal processing being more ecological friendly and keeping some organoleptic and nutritional advantages. Moreover, an AFB2 degradation

product under PEF treatment has been identified and toxicity predicted by the ProTox-II web server.

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References

Abdel-Sater, M. A., Zohri, A. A., Ismail, M. A., 2001. Natural contamination of some Egyptian fruit juices and beverages by mycoflora and mycotoxins. J Food Sci. Tech. MYS 38(4), 407-411.

Avsaroglu, M. D., Bozoglu, F., Alpas, H., Largeteau, A., Demazeau, G., 2015. Use of pulsed-high hydrostatic pressure treatment to decrease patulin in apple juice. High Pressure Res. 35(2), 214-222.https://doi.org/10.1080/08957959.2015.1027700.

Banerjee, P., Eckert, A. O., Schrey, A. K., Preissner, R., 2018. ProTox-II: a webserver for the prediction of toxicity of chemicals. Nucleic acids Res. 46(W1), W257-W263.

Čolović, R., Puvača, N., Cheli, F., Avantaggiato, G., Greco, D., Đuragić, O., Kos, J., Pinotti, L., 2019. Decontamination of Mycotoxin-Contaminated Feedstuffs and Compound Feed. Toxins 11(11), 617.https://doi.org/10.3390/toxins11110617.

Drwal, M. N., Banerjee, P., Dunkel, M., Wettig, M. R., Preissner, R. 2014. ProTox: a web server for the in silico prediction of rodent oral toxicity. Nucleic acids Res. 42(W1), W53-W58.

EFSA (European Food Safety Authority), 2007. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to the potential increase of consumer health risk by a possible increase of the existing maximum levels for aflatoxins in almonds, hazelnuts and pistachios and derived products. EFSA Journal 446, 1-127.

EFSA (European Food Safety Authority), 2020. Risk assessment of aflatoxins in food. EFSA Journal 18(3), 6040.

European Commission, 2002. Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. OJEU L 221, 8−36.

European Commission, 2006. Commission Directive 2006/1881/EC of 19 December 2006, setting Maximum Levels for Certain Contaminants in Foodstuffs. OJEU L 364, 5-24.

Evelyn, E., Silva, F. V. M., 2017. Resistance of Byssochlamys nivea and Neosartorya fischeri mould spores of different age to high pressure thermal processing and thermosonication. J. Food Eng. 201, 9-16.https://doi.org/10.1016/j.jfoodeng.2017.01.007.

Gbashi, S., Madala, N. E., De Saeger, S., De Boevre, M., Njobeh, P. B., 2019. Numerical optimization of temperature-time degradation of multiple mycotoxins. Food Chem. Toxicol. 125, 289-304.https://doi.org/10.1016/j.fct.2019.01.009.

Groot, M. N., Abee, T., van Bokhorst-van de Veen, H., 2019. Inactivation of conidia from three Penicillium spp. isolated from fruit juices by conventional and alternative mild preservation technologies and disinfection treatments. Food microbiol. 81, 108-114.https://doi.org/10.1016/j.fm.2018.06.004.

Hao, H., Zhou, T., Koutchma, T., Wu, F., & Warriner, K., 2016. High hydrostatic pressure assisted degradation of patulin in fruit and vegetable juice blends. Food control 62, 237-242.https://doi.org/10.1016/j.foodcont.2015.10.042.

Hassan, Y. I., Zhou, T., 2018. Promising Detoxification Strategies to Mitigate Mycotoxins in Food and Feed. Toxins 10, 116. https://doi.org/10.3390/toxins10030116.

Hwang, J. H., Lee, K. G., 2006. Reduction of aflatoxin B1 contamination in wheat by various cooking treatments. Food Chem. 98(1), 71-75.https://doi.org/10.1016/j.foodchem.2005.04.038.

IARC (International Agency for Research on Cancer), 2002. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 82. Lyon.

Kalagatur, N. K., Kamasani, J. R., Mudili, V., Krishna, K., Chauhan, O. P., Sreepathi, M. H., 2018. Effect of high pressure processing on growth and mycotoxin production of Fusarium graminearum in maize. Food Biosci. 21, 53-59.https://doi.org/10.1016/j.fbio.2017.11.005.

Kempkes, M., Simpson, R., Roth, I., 2016. Removing barriers to commercialization of PEF systems and processes. Proceedings of 3rd School on Pulsed Electric Field Processing of Food. Institute of Food and Health, University College Dublin, Dublin, 1-6.

MacGregor, S. J., Farish, O., Fouracre, R., Rowan, N. J., Anderson, J. G., 2000. Inactivation of pathogenic and spoilage microorganisms in a test liquid using pulsed electric fields. IEEE T Plasma Sci. 28(1), 144-149.

Mahato, D. K., Lee, K. E., Kamle, M., Devi, S., Dewangan, K., Kumar, P., Kang, S. G., 2019. Aflatoxins in food and feed: An overview on prevalence, detection and control strategies. Front. Microbiol. 10, 2266. https://doi.org/10.3389/fmicb.2019.02266.

Mandappa, I. M., Basavaraj, K., Manonmani, H.K., 2018. Analysis of Mycotoxins in Fruit Juices. In Fruit Juices; Academic Press: Wageningen, The Netherlands, pp. 763–777.

Marin, S., Ramos, A. J., Cano-Sancho, G., Sanchis, V., 2013. Mycotoxins: Occurrence, toxicology, and exposure assessment. Food Chem. Toxicol. 60, 218-237.https://doi.org/10.1016/j.fct.2013.07.047.

Pallarés, N., Carballo, D., Ferrer, E., Fernández-Franzón, M., Berrada, H., 2019. Mycotoxin Dietary Exposure Assessment through Fruit Juices Consumption in Children and Adult Population. Toxins 11(12), 684.https://doi.org/10.3390/toxins11120684.

Picart-Palmade, L., Cunault, C., Chevalier-Lucia, D., Belleville, M. P., Marchesseau, S., 2019. Potentialities and limits of some non-thermal technologies to improve sustainability of food processing. Front. Nutr. 5, 130.https://doi.org/10.3389/fnut.2018.00130.

Pinela, J., Ferreira, I. C., 2017. Nonthermal physical technologies to decontaminate and extend the shelf-life of fruits and vegetables: Trends aiming at quality and safety. Crit. Rev. Food Sci. Nutr. 57(10), 2095-2111.https://doi.org/10.1080/10408398.2015.1046547.

Pleadin, J., Frece, J., Markov, K., 2019. Mycotoxins in food and feed. Adv. Food Nutr. Res. 89, 297-345.https://doi.org/10.1016/bs.afnr.2019.02.007.

Sampedro, F., Fan, X., Rodrigo, D., 2010. High hydrostatic pressure processing of fruit juices and smoothies: research and commercial application. In Case studies in novel food processing technologies (pp. 34-72). Woodhead Publishing.

Shi, H., Li, S., Bai, Y., Prates, L. L., Lei, Y., Yu, P., 2018. Mycotoxin contamination of food and feed in China: Occurrence, detection techniques, toxicological effects and advances in mitigation technologies. Food Control 91, 202-215.https://doi.org/10.1016/j.foodcont.2018.03.036.

Soliman, K. M., 2002. Incidence, level, and behavior of aflatoxins during coffee bean roasting and decaffeination. J. Agr. Food Chem. 50(25), 7477-7481. https://doi.org/10.1021/jf011338v.

Tokuşoğlu, Ö., Alpas, H., Bozoğlu, F., 2010. High hydrostatic pressure effects on mold flora, citrinin mycotoxin, hydroxytyrosol, oleuropein phenolics and antioxidant activity of black table olives. Innov. Food Sci. Emerg. 11(2), 250-258. https://doi.org/10.1016/j.ifset.2009.11.005

Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., Kumar, S, Reddy, R., 2017. Effect of combination processing on aflatoxin reduction: process optimization by response surface methodology. J. Food Process Pres. 41(6), e13230. https://doi.org/10.1111/jfpp.13230.

Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., Kumar, S. S., 2018. Effect of pH and pulsed electric field process parameters on the aflatoxin reduction in model system using response surface methodology. J. Food Sci. Tech. 55(3), 868-878.https://doi.org/10.1007/s13197-017-2939-3.

Wang, S. Q., Huang, G. Q., Li, Y. P., Xiao, J. X., Zhang, Y., & Jiang, W. L., 2015. Degradation of aflatoxin B 1 by low-temperature radio frequency plasma and degradation product elucidation. Eur Food Res Technol. 241(1), 103-113. https://doi.org/10.1007/s00217-015-2439-5.

Yazdanpanah, H., Mohammadi, T., Abouhossain, G., Cheraghali, A. M., 2005. Effect of roasting on degradation of aflatoxins in contaminated pistachio nuts. Food Chem. Toxicol. 43(7), 1135-1139.https://doi.org/10.1016/j.fct.2005.03.004

Zhang, Z. H., Wang, L. H., Zeng, X. A., Han, Z., Brennan, C. S., 2019. Non-thermal technologies and its current and future application in the food industry: a review. Int. J. Food Sci. Tech. 54(1), 1-13.https://doi.org/10.1111/ijfs.13903

3.11. Investigating the in vitro catabolic fate of Enniatin B in a human gastrointestinal and colonic model

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Investigating the in *vitro* catabolic fate of Enniatin B in a human gastrointestinal and colonic model

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ABSTRACT

Enniatin B is an emerging mycotoxin known to present biological activity because of its ionophoric characteristics. This compound has demonstrated strong *in vitro* cytotoxicity against different cancer cells, also at low molecular concentrations. Its natural occurrence in food commodities and feed is highly reported world-wide, but few information is available about its stability in the human gastro-intestinal tract. The present work evaluates the catabolic fate of enniatin B upon *in vitro* simulated digestion and colonic fermentation. LC-MS target and untargeted analysis have been performed to quantify the extent of enniatin B degradation and the formation of catabolic products. The results obtained showed significant degradation of enniatin B (degradation rate 79 ± 5%) along the gastrointestinal tract and further degradation of residual enniatin B was observed during colonic fermentation after 24 h of incubation. Moreover, 5 catabolic metabolites of enniatin B were putatively identified after gastrointestinal digestion resulting from the oxidation and opening of the depsipeptide ring. As a final step, the pharmacokinetic properties of enniatin B degradation products were tested *in silico* revealing that some of them may be adsorbed at the gastrointestinal level more than the parent compound. Additionally, the smaller degradation products showed moderate blood-brain-barrier crossing.

KEYWORDS: Digestive model; Emerging mycotoxins; Grains; Gastrointestinal stability; Colonic fermentation; Degradation products

1. Introduction

Enniatins (ENNs) are a class of emerging mycotoxins deriving from secondary metabolism of different species of various fungal genera such as Alternaria, Fusarium, Halosarpheia and Verticillium. The chemical structure of these mycotoxins corresponds to that of a cyclic depsi- peptide and so far, more than 23 belonging to A, B and J types, have been identified (Feifel et al., 2007).

Among enniatins, Enniatin B (ENNB, see Fig. 1 for chemical structure) is known to present biological activity because of its ionophoric properties, and different studies have evidenced cytotoxic effects *in vitro* in both human and animal cell lines (Alonso-Garrido et al., 2018; Jonsson et al., 2016). This compound presented strong *in vitro* cytotoxicity against different cancer cells also at low molecular concentrations; in fact, it was demonstrated that ENNB had a higher cytotoxic potential than several other mycotoxins such as patulin, ochratoxin A, zearalenon and citrinin (Föllmann et al., 2009). The relevant ENNB cytotoxic activity was supposed to be due to the degradation of mitochondrial membrane and the consequent apoptosis in human tumor cell lines (Dornethuber et al., 2007). On the other side, possible genotoxicity was ruled out by Behm et al. (2009), using Comet assay tests. In addition, enniatin B1 was proven to exert adrenal endocrine toxicity (Prosperini et al., 2017).

The risk related to the exposure to enniatins and beauvericin in humans and animals was assessed by EFSA in 2014 (EFSA CONTAM Panel, 2014), however the Panel was unable to conclude in terms of chronic toxicity due to

lack of reliable *in vivo* data. The *in vivo* toxicity and genotoxicity of the same compounds were therefore investigated by EFSA in 2018 (EFSA Supporting Publication, 2018). The results supported a genotoxic effect in bone marrow and liver cells after acute treatment, but not after repeated exposure in mice. However, the toxicological role of enniatins in animals and humans is still unclear.

According to previous studies, ENNs were supposed to have a low bioavailability upon oral ingestion and to be almost stable in the gastrointestinal tract (Behm et al., 2009). However, a recent *in vivo* study showed that enniatin B1 presented high level of absolute oral bioavailability in pigs (91%) but low toxicity probably due to a rapid metabolism and elimination (Devreese et al., 2014). However, despite their supposed stability in human gastrointestinal tract, enniatins showed cytotoxic effects *in vitro* on human hepatocellular carcinoma cell line Hep-G2 as well as on two human colon cell lines such as epithelial colorectal adenocarcinoma cells (Caco-2) and colon carcinoma cells (HT-29) (Meca et al., 2011). In spite of their attested toxicity, to date no legal limits and tolerable daily intake for ENNs have been fixed. According to the recent reports, ENNs are frequent in Mediterranean grains, with concentration in the range of μg/Kg – mg/Kg (Meca et al., 2010; Juan et al., 2013, Fraeyman et al. 2016). According to Meca et al. (2010), who analysed cereal-based food from Spanish local market, enniatins were found in about 73% of the samples, being enniatin A1 the most occurring compound (33.38–814.42 mg/kg). An higher occurrence was reported for Scandinavian area, with a prevalence of ENNs in the range 96%–100% (Jestoi et al., 2009; Lindblad et al., 2013; Fredlund et al., 2013,

Fraeyman et al. 2016). According to the literature so far (vide INFRA), enniatins always co-occur as a structurally related group of mycotoxins, but co-occurrence with other Fusarium toxins, both regulated and emerging ones, is likely observed.

The formation of ENN metabolites in the gastrointestinal tract of mices have been reported, suggesting a possible result of enzyme degradation, gastrointestinal fermentation or intestinal metabolism (Rodríguez-Carrasco et al., 2016; Manyes et al., 2014). In particular, the authors tentatively identified three phase-I metabolites occurring in liver and colon tissues, annotated as dioxygenated-ENN B, mono- and di-demethylated-ENN B, with dioxygenated-ENN B being most prominent (Rodríguez-Carrasco et al., 2016). As far as the liver metabolism is concerned, main phase I ENNB biotransformation was investigated *in vitro* using rat, dog and human liver microsomes (Faeste et al., 2011; Ivanova et al., 2011). The authors identified 12 ENNB metabolites as the result of hydroxylation, carboxylation and N-demethylation processes, with specie-specific differences. Three of these compounds were further confirmed *in vivo* by Rodríguez-Carrasco et al. (2018), who observed in a biomonitoring study the occurrence of ENNB mono- oxygenated, deoxygenated and N-demethylated derivatives in 87.7%, 6.7% and 96.3% of human plasma, respectively.

The discrepancy between ENNs high cytotoxic effects *in vitro* and the reported low toxicity *in vivo*, could be therefore ascribed to the fast and extensive metabolism followed by a rapid excretion. However, Ivanova et al. (2017) performed a comparative study of the metabolism of ENN B1 in pigs, and

noticed that the exposure to the main metabolites appeared to be higher after oral than after intravenous administration likely due to the pre-systemic metabolism contribute. For this reason, information about the degradation/transformation of ENNs during the digestion process and the identification of ENNs products is so far very limited. On the basis of the occurrence data available so far and on the possible toxicological concern, a careful evaluation of ENNs bioaccessability and stability in the human gastro-intestinal tract is needed.

Bioaccessibility *in vitro* of contaminants, among them mycotoxins, has been assessed so far using consensus protocols such as those proposed by Versantvoort et al. (2005), Minekus et al. (2014) and Brodkorb et al. (2019). In particular, the standardised model proposed by Minekus et al. (2014) and followed up by Brodkorb et al. (2019), is based on an international consensus developed by the COST INFOGEST network.

Compared to the one developed by Versantvoort et al. (2005), the INFOGEST consensus method shows several differences. On one side, the digestive solutions are simpler in terms of organic and inorganic salts composition, with a lower ionic strength and a decreased buffering and emulsifying capacity. On the other side, the method provides a more efficient control of the pH along the digestion process, thus keeping constant the enzymatic effectiveness over time. Although in terms of protein digestion, the INFOGEST consensus method is reported to be more efficient, the performance is usually similar when bioactive/ toxic compounds are considered.

These protocols offer a quick and reproducible tool for studying the effect of the gastrointestinal digestion on food components in different matrices. On the other side, they have some drawbacks due to the oversimplified physiological factors applied. In particular, the role played by the intestinal microbiota is usually not taken into consideration, and the physiological conditions of the intestinal mucosa, the enterohepatic cycling and the immune system are not reproduced (González-Arias et al., 2013).

With the aim of taking into consideration the effect of intestinal microbiota, several colonic models involving anaerobic fermentation, have been proposed and successfully applied to mycotoxins (Dall'Erta et al., 2013; Gratz et al., 2013; Gratz et al., 2017). However, although the degradation of parent compounds can be easily monitored in the colonic assay, the identification of colonic metabolites is often challenging.

This work is therefore aimed to the evaluation of the catabolic fate of enniatin B upon human digestion, and colonic fermentation. In addition, a preliminary high resolution mass spectrometry (HRMS) identification of gastrointestinal metabolites of ENNB formed *in vitro*, have been tentatively performed.

2. Materials and methods

2.1. Chemicals

ENNB standard (purity ≥95%) was supplied by Sigma Aldrich (St. Louis, MO, USA). Bidistilled water was produced in our laboratory using an Alpha-Q System (Millipore, Marlborough, MA, USA). All sol- vents (HPLC grade) were obtained from Sigma (Stuttgart, Germany).

Potassium hydroxide, potassium chloride, sodium chloride, ammonium chloride, 37% hydrochloric acid, potassium dihydrogen phosphate, sodium hydrogen carbonate, and dried calcium chloride were obtained from Carlo Erba (Milan, Italy), potassium thiocyanate and sodium sulfate were purchased from Riedel de Haën (Hannover, Germany), sodium dihydrogen phosphate monohydrate was from Fluka (Chemika-Biochemika, Basil, Switzerland), magnesium chloride hexahydrate was supplied from Merck (Darmstadt, Germany). All chemicals for the preparation of the solutions mimicking the digestive juices (urea 98%, D-(þ)-glucose 99.5%, D-glucuronic acid, D-(+)-glucosamine hydrochloride 99%, type III mucin from porcine stomach, uric acid, type VIII A α-amylase from barley malt (20–80 units/mg protein) bovine serum albumin (BSA), pepsin from porcine gastric mucosa (3200–4500 units/mg protein), pancreatin from porcine pancreas (350 FIP-U/g Protease, 6000 FIP-U/g Lipase, 7500 FIP-U/g Amylase), type II lipase from porcine pancreas (100–400 units/mg protein), and bovine and ovine bile) were purchased from Sigma (Stuttgart, Germany). Moreover, other chemicals used for colonic fermentations such as (+)-arabinogalactan, bile salt, yeast extract,

tryptone, inuline, buffered peptone water, Dulbecco's phosphate buffer saline (PBS), casein sodium salt from bovine milk, pectin from citrus fruits, xylan from Birchwood, potassium hydrogen phosphate, magnesium sulfate monohydrate, guar gum and Tween 80 were obtained from Sigma-Aldrich (St. Louis, MO, USA), while Fe(II)-sulfate heptahydtate and L-cysteine hydrochloride were purchased from AppliChem (Darmstadt, Germany).

2.2. *In vitro* Gastrointestinal digestion of ENNB

For the *in vitro* digestion of ENNB, both Minekus' (M) and Versantvoort's (V) protocols have been applied in parallel on blank wheat flour, spiked with a proper amount of ENNB (5 µg and 6.16 µg for V and M protocol, respectively) to reach a concentration of 500 µg/L in the final digestion solution. The same protocols were applied to a 20 µg/L ENNB stock solution, further used for the identification of degradation products.

The M and V protocols are summarized in Fig. 1 and described in details as Supplementary Information (annex 5), together with the composition of digestive juices reported in Tables S1 and S2 (Supplementary Information, annex 5).

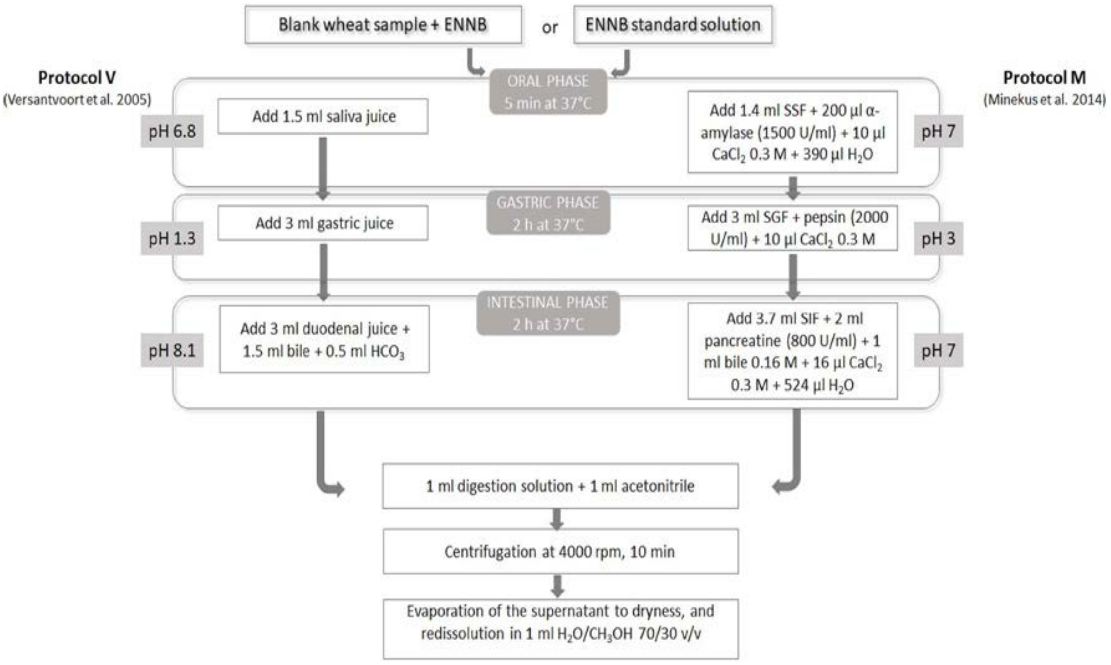

Fig. 1. Scheme of the digestion protocols (M vs V) applied within this study.

For each experiment a control sample was prepared by dissolving the same amount of analyte in 2 mL of a spent-blank digested sample prepared by performing the digestion experiment as previously described, excluding the target mycotoxin.

All experiments were performed in triplicate on different days. Data are given as mean values of independent experiments.

2.3. *In vitro* Faecal Fermentation of ENNB

Faecal fermentation of ENNB was performed in according to the protocol of Dall'Erta et al. (2013). At this purpose, samples of human fresh faeces were collected from three different healthy and non smoking donors and immediately stored under anaerobic conditions, as previously described (Dall'Erta et al., 2013).

For the colonic fermentation, 0.4 mL of ENNB stock solution was transferred into a vial and then added with 1.8 mL of faecal solution at 20% and 1.8 mL of growth medium, to reach a final toxin concentration of 800 μg/L. The sample was treated with a gently nitrogen flow in order to eliminate oxygen from the fermentation environment and then incubated in a water bath at 37 °C and mixed at 200 strokes/minute. Faecal fermentation was stopped by adding 4 mL of acetonitrile, before the addition of ENNB for time zero (T0), or after 30 min (T = 30 min) and 24 h (T = 24 h) from the beginning of the treatment. All the solutions were centrifuged at 21,952 g for 10 min and stored at −80 °C till analysis. For each incubation time also control samples (ENNB solution without fecal slurry) were prepared following the same protocol.

All experiments were performed in triplicate on different days. Data are given as mean values of independent experiments.

2.4. Targeted UHPLC-MS/MS analysis of ENNB

Targeted quantification of ENNB was performed using LC-MS/MS on a 2695 Alliance separation system (Waters Co., Milford, MA, USA) equipped with a Quattro API triple-quadrupole mass spectrometer with an electrospray source (Micromass, Waters, Manchester, UK). Chromatographic conditions were as follows: the column was a 50 × 2.00 mm inner diameter, 2.5 μm, Phenomenex Synergy Fusion C18, 100A; the flow rate was 0.2 mL/min; the column temperature was set at 30 °C; the injection volume was 10 μL; gradient elution was performed by using doubly-distilled water (eluent A) and methanol (eluent B), both acidified with 0.2% formic acid: initial condition at 30% B, 0–1 min isocratic step, 1–8 min linear gradient to 65% B, 8–20 min linear gradient to 90% B, 20–22 min isocratic step, 22–23 min linear gradient to 30% B and re-equilibration step at 30% B for 7 min (total analysis time: 30 min). MS parameters were the following: ESI + (positive ionization mode); capillary voltage, 4.0 kV; cone voltage, 40 V; extractor voltage, 2 V; source block temperature, 120 °C; desolvation temperature, 350 °C; cone gas flow and desolvation gas flow (nitrogen), 50 and 700 L/h, respectively. Detection was achieved by using the multiple reaction monitoring (MRM) mode, by monitoring three transitions, as follow: 640.8 → 196.2, 640.8 → 214.2 and 640.8 → 314.2 (CE 30 eV) for Enniatin B. The first transition reported was used for

quantification, while the second and the third transitions were chosen as qualifier. Retention time for ENNB was 12.20 ± 0.03 min. The analytical methods used were assessed for linearity and recovery. Limit of detection (LOD) and limit of quantification (LOQ) have been calculated as 10 μg/L (S/N = 3) and 30 μg/L (S/N = 10), respectively. Recovery experiments were performed by spiking a blank chyme at a target concentration level (500 μg/L), and a mean value of 95% has been calculated. All data reported in this study are corrected for recovery. Matrix-matched calibration experiments were performed in the range 100–1000 μg/Kg−1 for the target analyte in the original matrix. A good linearity was obtained over the selected range (r2 > 0.999).

2.5. Untargeted UHPLC-HRMS screening of ENNB degradation products

Digested samples were subjected to untargeted UHPLC-HRMS to identify catabolic metabolites of ENNB for which analytical standards were not available.

For the chromatographic separation, a reversed-phase C18 Kinetex EVO column (Phenomenex, Torrance, CA, USA) with 2.10 × 100 mm and a particle size of 2.6 μm heated to 40 °C was used. 10 μl of sample extract was injected into the system; the flow rate was 0.4 mL/min. Gradient elution was performed by using 1 mM ammonium acetate in water (eluent A) and methanol (eluent B) both acidified with 0.5% acetic acid. Initial conditions were set at 10% B followed by a linear change to 40% B in 4 min and to 90% B in 16 min. Column was then washed for 2 min with 90% B followed by a reconditioning step for 3 min using initial composition of mobile phases. The total run time was 25 min.

LC-HRMS full scan spectra were recorded using Q-ExactiveTM high resolution mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with electrospray ionization (ESI).

The Q-Exactive mass analyzer was operated in the full MS/data dependent MS/MS mode (full MS–dd-MS/MS) in positive ionization mode. The following parameters were set: sheath and auxiliary gas flow rates 40 and 10 arbitrary units, respectively; spray voltage 3.5 kV; heater temperature 250 °C; capillary temperature 300 °C. Following parameters were used in full MS mode: resolution 70,000 FWHM (defined for m/z 200), scan range 400 ¬ 900 m/z, automatic gain control (AGC) target 3e6, maximum inject time (IT) 200 ms. Parameters for dd- MS/MS mode: resolution 17,500 FWHM (defined for m/z 200) AGC target 2e5, maximum IT 50 ms, normalized collision energy (NCE) 35% with ± 25% step was used.

Putative identification of degradation products of ENNB was performed with the aid of Compound Discoverer™ software (v. 1.0; Thermo Scientific, Fremont, CA, USA). The software detects chromatographic peaks and the mass of the corresponding compound is compared with a list of generated theoretical metabolites. Potential metabolites were described by exact mass, HRMS/MS fragmentation, isotopic pattern and retention time with respect to the parent compound (as determined from *in silico* predictions).

2.6. In silico ADMET profile prediction

The ADMET (Absorption, Distribution, Metabolism, Excretion and Toxicity) profile of ENNB and the identified metabolites, was predicted using SwissADME (http://www.swissadme.ch/), a freely available web- based tool. The tool predicts human intestinal absorption (HIA), and blood-brain barrier (BBB) penetration, besides being able to classify compounds as targets of p-glycoprotein (P-gp) efflux, inhibitors of cytochrome P450 isoforms CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, as well as substrates for the metabolism by isoforms CYP2C9, CYP2D6, and CYP3A4.

3. Results

3.1. Stability of ENNB in the digestion and fecal fermentation assays

The effect of the digestion process on ENNB was evaluated on a blank wheat flour spiked with 5 μg and 6.16 μg of toxins, for M and V protocols respectively. Data are reported in Fig. 2a. No significant difference was observed among the data obtained using protocols M and V, with an overall degradation rate of 78.45 ± 5.39%.

Since ENNB remained partially stable after the digestion step, the possible cleavage exerted by human gut microbiota was evaluated as well. At this aim, the mycotoxin was incubated in faecal slurries for 30 min and 24 h and the results obtained were compared with those observed in the control. The time points were selected on the basis of our previous work (Dall'Erta et al., 2013),

considering t = 24 h as the average time for a realistic estimation of food permanence in the gut. Compared to the control, ENNB showed a significant degradation rate already after 30 min, reaching 70% at 24 h of observation (see Fig. 2b).

Overall, the data obtained in the applied assay demonstrated the low stability of ENNB under in vitro gastrointestinal conditions suggesting a significant degradation of the parent compound along the gastrointestinal tract.

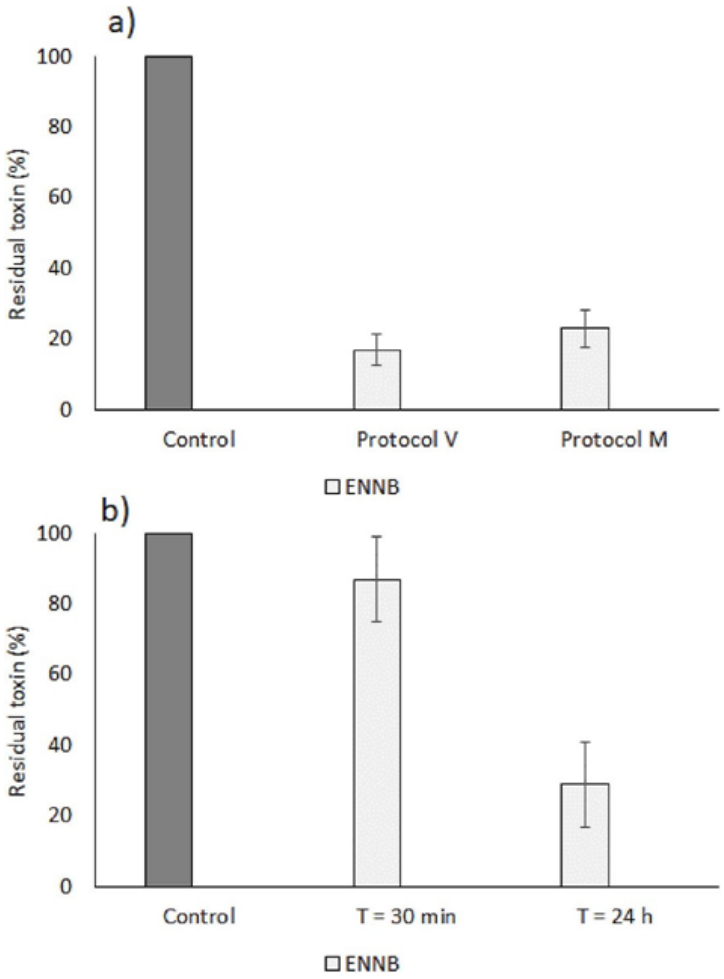

Fig. 2. a) Degradation rate of ENNB in wheat flour after digestive treatment; b) Degradation rate of ENNB in wheat flour after faecal fermentation. Different symbols indicate statistically significant differences ($p < 0.005$).

3.2. Screening of Metabolites

In order to identify the degradation products of ENNB during gastrointestinal digestion, LC-HRMS analysis was preferred, due to the limited knowledge on ENNB metabolism. A total of 5 catabolic metabolites plus isomers, named M1-M5 according to the retention time, have been annotated in both M and V protocols. Most of the metabolites eluted in front of ENNB using reverse phase column (see Fig. 3) indicating that they are more hydrophilic than the parent compound. The possible structures of metabolites are reported in Fig. 3.

Table 1 summarizes the molecular formulas, retention times, adduct ions, mean mass error of the putatively identified ENNB catabolites. Their HRMS/MS spectra are reported in Figs. S1–S5 (Supplementary Information, annex 5) and diagnostic fragments that testify the formation of these product are highlighted.

All the degradation products can be ascribed to the oxidation and opening of the depsipeptide ring (#M4 and #M5), followed by further fragmentation (#M1, #M2 and #M3) of the elongated ENNB structure.

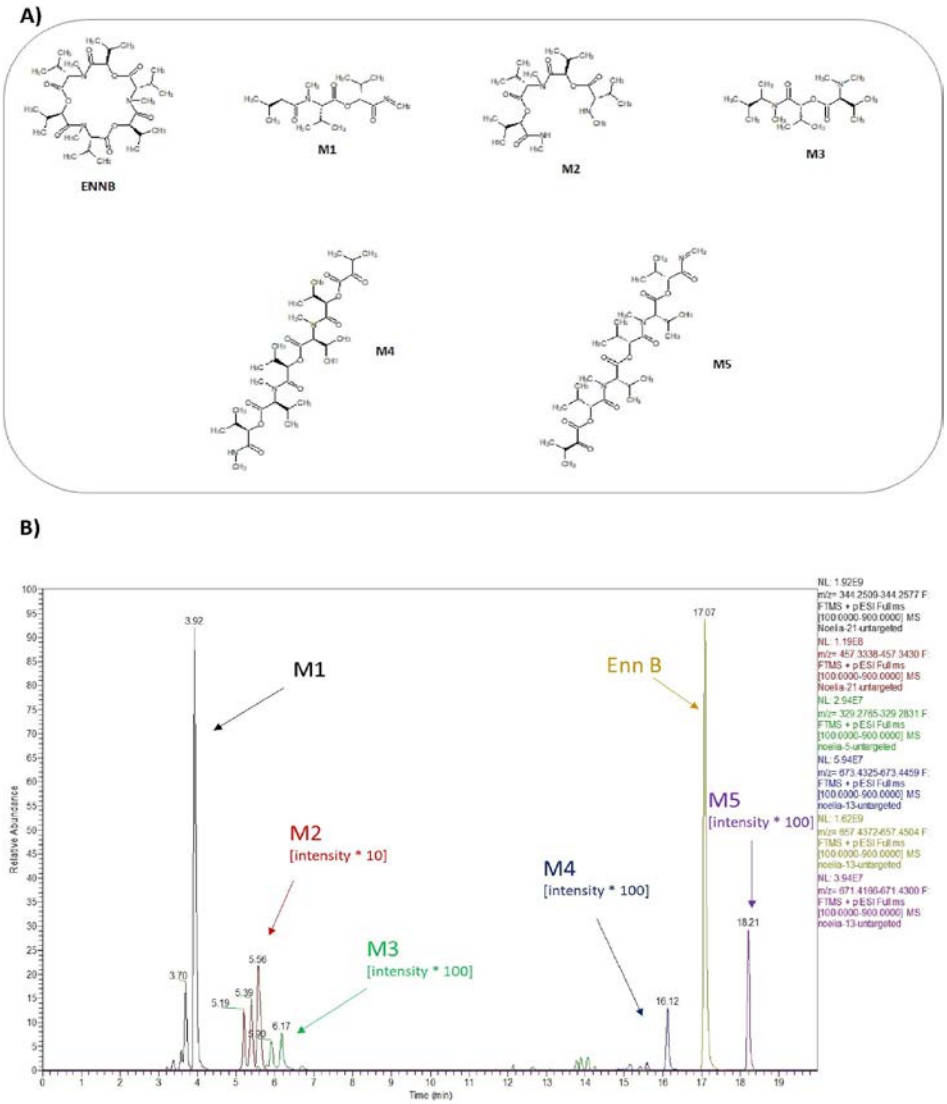

Fig. 3. A) Chemical structures of putative ENNB gastrointestinal metabolites identified within this work; B) LC-HRMS extracted ion chromatogram of accurate mass traces (± 3 ppm) of ENNB and its corresponding products. Due to low abundance compared to ENNB, EIC intensities of #M2, #M3, #M4 and #M5 were multiplied by a factor of 10 or 100.

Table 1. Enniatin B biotransformation/degradation products elucidated by UHPLC-HRMS.

Metabolite	Neutral Formula	Rt	Adduct	m/z	Error[a] ppm
Enniatin B	C33H57N3O9	17	[M + NH4]+	657.4438	−0.7
M1	C17H30N2O4	3.91	[M + NH4]+	344.2543	−0.2
M2	C23H41N3O5	5.56	[M + NH4]+	457.3384	0.9
M3	C18H36N2O3	6.16	[M+H]+	329.2798	0.8
M4	C33H57N3O10	16.11	[M + NH4]+	673.4392	1.4
M5	C33H55N3O10	18.21	[M + NH4]+	671.4225	2.7

[a] Resolution in full MS mode 70,000 FWHM, maximum acceptable error 5 ppm

3.3. In silico ADMET profile prediction

The putative metabolites underwent *in silico* ADME analysis using the BOILED-Egg predictive model (www.swissadme.ch), to investigate their pharmacokinetic properties compared to the parent compound. Calculated data according to the chemico-physical parameters from the *in silico* model are reported in Table 2.

The model indicated that #M4 and #M5 are likely not absorbed at gastrointestinal level, while #M2 as well as ENNB seem to be likely absorbed, as reported in Fig. 4. In addition, metabolites #M1 and #M3 are likely to show moderate blood-brain barrier (BBB) crossing and are predicted as not to be effluated from the central nervous system by the P-glycoprotein.

In the case of metabolism, the possible interaction of putative metabolites with various cytochrome P450 was evaluated *in silico*, showing different pattern for the parent compounds and its metabolites. While none of the considered

compounds is expected to act as CYP1A2, CYP3A4, CYP2D6, or CYP2C9 inhibitor, both #M1, #M2, #M4 and #M5 are likely to inhibit CYP2C19.

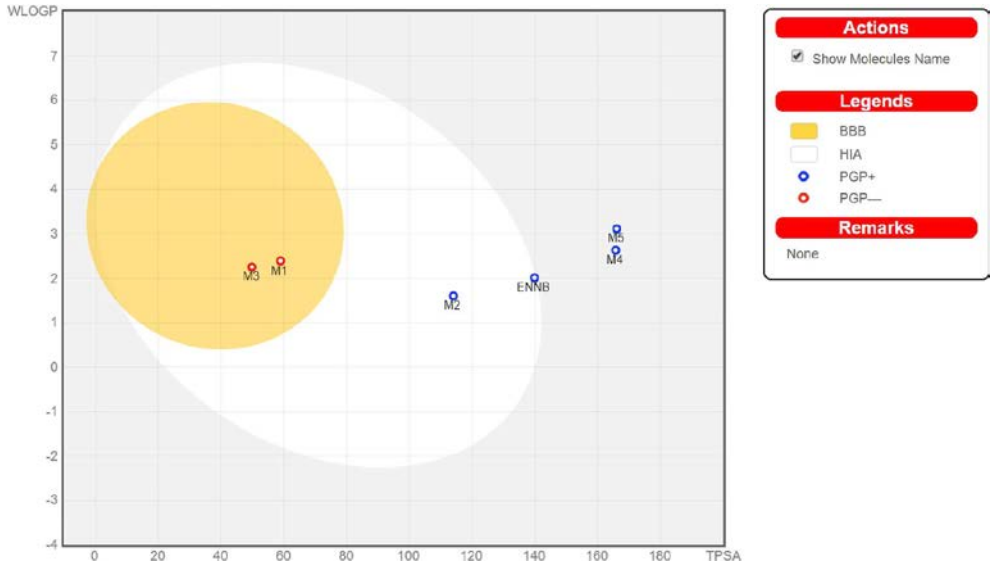

Fig. 4. BOILED-Egg model obtained for ENNB and its gastrointestinal metabolites. The model is expressed as a function of two chemico-physical descriptors, polarity ($pTSA/A^2$) and lipophilicity (WLOGP). The white region (Human Intestinal Absorption, HIA) is the physicochemical space of molecules with highest probability of being absorbed by the gastrointestinal tract, and the yellow region (Blood-Brain Barrier, BBB) is the physicochemical space of molecules with highest probability to permeate to the brain. Yellow and white areas are not mutually exclusive. Compounds predicted as showing an active efflux by P-glycoprotein (PGP+) are reported as blue dots, while compounds predicted as not able to be actively effluted (PGP-) are reported as red dots. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 2. ADMET parameters obtained *in silico* for ENNB and its putative gastrointestinal metabolites.

Molecule	M1	M2	M3	M4	M5	ENNB
Formula	C16H30N2O3	C23H43N3O6	C17H34N2O3	C33H57N3O10	C33H55N3O10	C33H57N3O9
MW	298.42	457.6	314.46	655.82	653.8	639.82
TPSA	58.97	114.04	49.85	165.69	166.02	139.83
WLOGP	2.39	1.6	2.25	2.63	3.11	2.01
GI absorption	***High***	***High***	***High***	Low	Low	***High***
BBB permeant	***Yes***	No	***Yes***	No	No	No
Pgp substrate	No	***Yes***	No	***Yes***	***Yes***	***Yes***
CYP1A2 inhibitor	No	No	No	No	No	No
CYP2C19 inhibitor	***Yes***	***Yes***	No	***Yes***	***Yes***	No
CYP2C9 inhibitor	No	No	No	No	No	No
CYP2D6 inhibitor	No	No	No	No	No	No
CYP3A4 inhibitor	No	No	No	No	No	No

4. Discussion

Scant and often contradictory information is available in the literature so far on the bioaccessibility of emerging mycotoxins, such as ENNB. In particular, Serrano et al. (2014) reported bioaccessibility for ENNB1, ENNA, ENNA1 in infant formulas during the duodenal digestion, with a mean value of 1.43% for ENNB1, 0.37% for ENNA and 22.41% for ENNA1, respectively. On the contrary, an higher bioaccessibility was reported by Prosperini et al. (2013), being the values observed for ENNB, ENNB1, ENNA, and ENNA1 in the range 40.4–79.9%, in breakfast cereals, biscuits and bread. Similar results were reported by Meca et al. (2012 a,b), with a mean bioaccessability of 80% for ENNB, ENNB1, ENNA, and ENNA1 in spiked commercial wheat crispy bread. This inconsistency is likely due to unstandardized *in vitro* digestive conditions, often applied on different commercial products, regardless the matrix composition. It has been demonstrated, indeed, that pH and fiber content may effectively modulate the bioaccessibility of contaminants, i.e. enniatins (Meca et al., 2012; Serrano et al., 2014; Manzini et al., 2015). In particular, lower pH may support a more effective degradation of the matrix, and thus a higher bioaccessibility of xenobiotics and bioactives. On the other hands, fiber can decrease the absorption rate of nutrients due to the entrapping mechanical effect. As an example, Serrano et al. (2014) observed that the macronutrient present in the matrix have the capacity to bind ENNs, which are later released in the gut by further matrix degradation due to colonic bacteria. As a further issue, Manzini et al. (2015) observed lower ENNs bioaccessibility when static digestive models have been applied with respect to the dynamic ones. For

overcoming possible differences due to the digestive conditions applied, we focused on two well-established protocols, the consensus one proposed by Minekus et al. (2014) and recently followed up by Brodkorb et al. (2019), and the one proposed by Versantvoort et al. (2005) for assessing the bioaccessibility of contaminants.

Both protocols were applied to a wheat blank flour spiked with ENNB, and performed under the same experimental conditions. Our results clearly demonstrated that ENNB is highly degraded under the applied conditions, without any significant difference between the protocols. In addition, our study confirmed that a preliminary transformation of ENNB may occur already in the digestive tract, before absorption and further metabolisation. Furthermore, when colonic fermentation is applied, data presented herein indicated a further degradation of residual ENNB after 24h of incubation.

Taken altogether, our results suggest that the first preliminary ENNB degradation step may occur at presystemic level. The parent compound together with its presystemic metabolites, once absorbed, may enter the liver where the extensive metabolism previously described may take place. Finally, the amount of ENNB which is not absorbed in the gastrointestinal tract, i.e. because of the entrapping in the matrix, can be released and further biotransformed in the gut by colonic bacteria. Our data may therefore provide relevant insight into ENNB bioavailability, explaining some discrepancy observed among *in vitro* and *in vivo* experiments in the literature (Faeste et al., 2011; Ivanova et al., 2011; Rodriguez-Carrasco et al. 2016, 2018; Ivanova et al. 2017). In order to better investigate the gastrointestinal degradation of ENNB, UHPLC-HRMS experiments have been

performed on standard solution and ENNB spiked flour. In both samples, 5 metabolites have been putatively identified. In all cases, their formation can be ascribed to the oxidation and opening of the depsipeptide ring, followed by N-de- methylation and further fragmentation. Worth of mention, compound #M4 was already described by Ivanova et al. (2011) and Fraeyman et al. (2016) as a major degradation product for ENNB. In our work, however, the main degradation product was #M1, which can be originated by degradation of all the reported metabolites as well as of the parent compound. Although liver microsomal metabolism *in vitro* was extensively described by Ivanova et al. (2011), the possible ENNB degradation occurring in the gastrointestinal tract at presystemic level have not been described so far. Devreese et al. (2014) performed an *in vivo* trials in pigs, upon intravenous and oral administration of ENNB1. Comparing the pharmacokinetic parameters obtained over the two administration routes, the authors did not observed any relevant difference. However, the biotransformation *in vivo* was not taken into consideration. Later, Ivanova et al. (2017) compared the metabolite formation of ENNB1 *in vivo* and *in vitro* in pigs. The authors stated that metabolite formation was higher when ENNB1 was absorbed from the gut compared to intravenous administration indicating pre-systemic metabolism of ENNB1 after oral uptake.

Compared to the previously results, our study showed the formation *in vitro* of 5 metabolites of ENNB already at presystemic level. Although only one out of five metabolites was already described in the literature, the main modifications described herein, i.e. oxidation and N-de- methylation, were consistent with the biotransformation routes already reported in previous studies

(Ivanova et al., 2011; Fraeyman et al., 2016; Ivanova et al. 2017; Rodrigues-Currasco et al. 2018).

It should be noticed that, although the phase I pathways involved are maintained, the metabolites identified over years suffer from inconsistency in terms of structure annotation, probably on account of the *in vitro* conditions applied. Ivanova et al. (2011) reported the formation of 12 ENNB metabolites upon hepatic biotransformation in vitro with rat, pig, and human microsomes. Later on, the same authors compared the metabolites occurring *in vitro* in experiments upon incubation of ENNB1 with pig liver microsomes to those found in the plasma of pigs after single oral or intravenous administration of the same toxins (Ivanova et al. 2017). From those described *in vitro*, the authors were able to find in pig plasma 6 out of 10 metabolites. Similarly, Rodriguez- Carrasco et al. (2018), who performed the first pilot biomonitoring study for ENNB, reported the occurrence of 3 metabolites in human plasma.

To assess whether the degradation compound found in our model assay might represent a detoxification step, the toxicological profile of the newly discovered metabolites has to be characterized.

In the present study, the SwissADME tool, previously developed and validated for drugs (Daina et al., 2017), has been used for the *in silico* investigation of the human absorption and metabolism of ENNB and its degradation products. The model, based on a large number of chemico- physical parameters obtained by structural analysis, was developed and validated for supporting ADME characterization of drugs, and is available as an open source web-tool

(Daina et al., 2017). It has been recently successfully applied to the investigation of mycotoxins, as reported by Taroncher et al. (2018).

In addition, the BOILED-Egg model was obtained from the derived chemico-physical descriptors. The Brain-Or-IntestinaL-EstimateD permeation method (BOILED-Egg) model is a computational model developed for the prediction of gastrointestinal absorption and brain penetration of small molecules. Based on lipophilicity (WLOGP) and polarity (topological polar surface area, pTSA) calculation, concomitant predictions for both brain and intestinal permeation are obtained and translated into molecular design, owing to a highly accurate but still intuitive output of the model (Daina and Zoete, 2016).

In silico analysis showed that, not only the parent compound but also 3 out of 5 gastrointestinal metabolites are likely to be readily absorbed within the human intestinal tract. This result suggests that gastrointestinal metabolites #M1, #M2, and #M3 could enter the sys temic circulation and, therefore, distributed to tissues and target organs. However, the parent compound and #M2 are predicted to interact with P-glycoprotein (Pgp), an ATP-dependent drug efflux pump for xenobiotic compounds with broad substrate specificity. Pgp is extensively distributed and expressed in several tissues, among them the intestinal epithelium where it pumps xenobiotics back into the intestinal lumen. While Pgp activity can negatively affect a pharmaceutical drug pharmacokinetics, in case of foodborne toxins this interaction could be seen as a protective factor leading to a lower cell accumulation of the xenobiotics, contributing i.e. to an *in vivo* decrease of ENNB cytotoxic effect.

According to the *in silico* model, some concerns could be derived for the possible blood-brain barrier (BBB) crossing by #M1 and #M3, especially in consideration of their unlikely interaction with the P- glycoprotein. In terms of further hepatic metabolism, while ENNB is known to undergo CYP-related metabolism, predictive data suggest that some of its gastrointestinal catabolites may act as CYP2C19 inhibitors. Considering that CYP2C19 is a liver enzyme responsible for the hepatic metabolism of at least 10% of current medical drugs (Flockhart DA, 2007), the inhibitory potential of the most bioavailable catabolites #M1 and #M2 should be further investigated.

Taken altogether, these data, only apparently inconsistent, provide actually a picture of the complex biotransformation of enniatins upon ingestion. After a first presystemic degradation in the gastrointestinal tract, ENNB and its catabolic products may be partially adsorbed and further extensively metabolised in the liver. Once reached the gut, residual ENNB may be liberated from the matrix and further bio- transformed by the colonic bacteria.

5. Conclusion

Overall, our results demonstrated that low stability of ENNB under in vitro gastrointestinal conditions, suggesting a significant degradation of the parent compound along the gastrointestinal tract. This outcome suggests that pharmacokinetic and toxicological studies, when comparing data obtained by *in vitro* and *in vivo* experiments, should focus not only on the hepatic metabolism of ENNB, but also on the degradative biotransformation occurring in the digestive tract as well as in the gut. Five degradation products of ENNB have been tentatively identified using HRMS, among those one already reported in the literature. Consistently with previous studies, oxidation and N-de- methylation were identified as the major degradation pathways. Furthermore, the ADME characterization of degradation products was performed *in silico*. The outcome suggested that 3 out of five metabolites likely to be absorbed within the human intestinal tract, and two of them may be of concern for crossing the blood-brain barrier. Further investigation will be focused on the *in vivo* characterization of the toxicological profile of these newly discovered degradation products, to investigate whether they might rise some concerns.

Acknowledgments

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References

Alonso-Garrido, M., Escrivá, L., Manyes, L., Font, G., 2018. Enniatin B induces expression changes in the electron transport chain pathway related genes in lymphoblastic T-cell line. Food Chem. Toxicol. 121, 437–443.

Behm, C., Degen, G.H., Föllmann, W., 2009. The Fusarium toxin enniatin B exerts no genotoxic activity, but pronounced cytotoxicity in vitro. Mol. Nutr. Food Res. 53 (4), 423–430.

Brodkorb, A., Egger, L., Alminger, M., et al., 2019. INFOGEST static in vitro simulation of gastrointestinal food digestion. Nat. Protoc. 14, 991–1014.

Daina, A., Zoete, V., 2016. A BOILED-egg to predict gastrointestinal absorption and brain penetration of small molecules. ChemMedChem 11, 1117–1121.

Daina, A., Michielin, O., Zoete, V., 2017. SwissADME: a free web tool to evaluate pharmacokinetics, drug-likeness and medicinal chemistry friendliness of small molecules. Sci. Rep. 7, 42717.

Dall'Erta, A., Cirlini, M., Dall'Asta, M., Del Rio, D., Galaverna, G., Dall'Asta, C., 2013. Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. Chem. Res. Toxicol. 26, 305–312.

Devreese, M., Broekaert, N., De Mil, T., Fraeyman, S., De Backer, P., Croubels, S., 2014. Pilot toxicokinetic study and absolute oral bioavailability of the Fusarium mycotoxin enniatin B1 in pigs. Food Chem. Toxicol. 63, 161–165.

Dornetshuber, R., Heffeter, P., Kamyar, M.R., Peterbauer, T., Berger, W., Lemmens- Gruber, R., 2007. Enniatin exerts p53-dependent cytostatic and p53-independent cytotoxic activities against human cancer cells. Chem. Res. Toxicol. 20 (3), 465–473.

Efsa Contam Panel (Efsa Panel on Contaminants in the Food Chain), 2014. Scientific Opinion on the risks to human and animal health related to the presence of beau- vericin and enniatins in food and feed. 2014. EFSA Journal 12 (8), 3802 174.

Efsa Supporting Publication, 2018. In vivo toxicity and genotoxicity of beauvericin and enniatins. Combined approach to study in vivo toxicity and genotoxicity of mycotoxins beauvericin (BEA) and enniatin B (ENNB). EN-1406. Istituto Superiore di Sanità (ISS), Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA) and French Agency for Food, Environmental and Occupational Health & Safety (ANSES) 2018, 183.

Fæste, C.K., Ivanova, L., Uhlig, S., 2011. In vitro metabolism of the mycotoxin enniatin B in different species and cytochrome p450 enzyme phenotyping by chemical inhibitors. Drug Metab. Dispos. 39, 1768e1776.

Feifel, S.C., Schmiederer, T., Hornbogen, T., Berg, H., Süssmuth, R.D., Zocher, R., 2007. In vitro synthesis of new enniatins: probing the α-d-hydroxy carboxylic acid binding pocket of the multienzyme enniatin synthetase. Chembiochem 8 (15), 1767–1770.

Flockhart, D.A., 2007. Drug Interactions: Cytochrome P450 Drug Interaction Table. Indiana University School of Medicine. https://drug-interactions.medicine.iu.edu.

Föllmann, W., Behm, C., Degen, G.H., 2009. The emerging Fusarium toxin enniatin B: in-vitro studies on its genotoxic potential and cytotoxicity in V79 cells in relation to other mycotoxins. Mycotoxin Res. 25 (1), 11–19.

Fraeyman, S., Devreese, M., Antonissen, G., De Baere, S., Rychlik, M., Croubels, S., 2016. Comparative oral bioavailability, toxicokinetics, and biotransformation of enniatin B1 and enniatin B in broiler chickens. J. Agric. Food Chem. 64 (38), 7259–7264.

Fredlund, E., Gidlund, A., Sulyok, M., Börjesson, T., Krska, R., Olsen, M., Lindblad, M., 2013. Deoxynivalenol and other selected Fusarium toxins in Swedish oats occurrence and correlation to specific Fusarium species. Int. J. Food Microbiol. 167, 276–283.

González-Arias, C.A., Marín, S., Sanchis, V., Ramos, A.J., 2013. Mycotoxin bioaccessibility/absorption assessment using in vitro digestion models: a review. World Mycotoxin J. 6 (2), 167–184.

Gratz, S.W., Duncan, G., Richardson, A.J., 2013. The human fecal microbiota metabolizes deoxynivalenol and deoxynivalenol-3-glucoside and may be responsible for urinary deepoxy-deoxynivalenol. Appl. Environ. Microbiol. 79 (6), 1821–1825.

Gratz, S.W., Dinesh, R., Yoshinari, T., Holtrop, G., Richardson, A.J., Duncan, G., MacDonald, S., Lloyd, A., Tarbin, J., 2017. Masked trichothecene and zearalenone mycotoxins withstand digestion and absorption in the upper GI tract but are efficiently hydrolyzed by human gut microbiota in vitro. Mol. Nutr. Food Res. 61 (4), 1600680.

Ivanova, L., Fæste, C.K., Uhlig, S., 2011. In vitro phase I metabolism of the depsipeptide enniatin B. Anal. Bioanal. Chem. 400 (9), 2889–2901.

Ivanova, L., Uhlig, S., Devreese, M., Croubels, S., Fæste, C.K., 2017. Biotransformation of the mycotoxin enniatin B1 in pigs: a comparative in vitro and in vivo approach. Food Chem. Toxicol. 105, 506e517.

Jestoi, M., Kokkonen, M., Uhlig, S., 2009. What about the 'other' Fusarium mycotoxins? World Mycotoxin J. 2 (2), 181–192.

Jonsson, M., Jestoi, M., Anthoni, M., Welling, A., Loivamaa, I., Hallikainen, V., et al., 2016. Fusarium mycotoxin enniatin B: cytotoxic effects and changes in gene expression profile. Toxicol. Vitro 34, 309–320.

Juan, C., Mañes, J., Raiola, A., Ritieni, A., 2013. Evaluation of beauvericin and enniatins in Italian cereal products and multicereal food by liquid

chromatography coupled to triple quadrupole mass spectrometry. Food Chem. 140 (4), 755–762.

Lindblad, M., Gidlund, A., Sulyok, M., Borjesson, T., Krska, R., Olsen, M., Fredlund, E., 2013. Deoxynivalenol and other selected Fusarium toxins in Swedish wheat occurrence and correlation to specific Fusarium species. Int. J. Food Microbiol. 167, 284–291.

Manyes, L., Escrivá, L., Serrano, A.B., Rodríguez-Carrasco, Y., Tolosa, J., Meca, G., Font, G., 2014. A preliminary study in Wistar rats with enniatin A contaminated feed. Toxicol. Mech. Methods 24 (3), 179–190.

Manzini, M., Rodriguez-Estrada, M.T., Meca, G., Mañes, J., 2015. Reduction of beauvericin and enniatins bioaccessibility by prebiotic compounds, evaluated in static and dynamic simulated gastrointestinal digestion. Food Contr. 47, 203–211.

Meca, G., Zinedine, A., Blesa, J., Font, G., Mañes, J., 2010. Further data on the presence of Fusarium emerging mycotoxins enniatins, fusaproliferin and beauvericin in cereals available on the Spanish markets. Food Chem. Toxicol. 48 (5), 1412–1416.

Meca, G., Font, G., Ruiz, M.J., 2011. Comparative cytotoxicity study of enniatins A, A1, A2, B, B1, B4 and J3 on Caco-2 cells, Hep-G2 and HT-29. Food Chem. Toxicol. 49 (9), 2464–2469.

Meca, G., Mañes, J., Font, G., Ruiz, M.J., 2012a. Study of the potential toxicity of commercial crispy breads by evaluation of bioaccessibility and bioavailability of minor Fusarium mycotoxins. Food Chem. Toxicol. 50 (2), 288–294.

Meca, G., Giri, A., Sagratini, G., Vittori, S., Font, G., Mañes, J., 2012b. The soluble dietary fiber inulin can influence the bioaccessibility of enniatins. Food Funct 3 (8), 853–858.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al., 2014. A standardised static in vitro digestion method suitable for food an international consensus. Food Funct 5 (6), 1113–1124.

Prosperini, A., Meca, G., Font, G., Ruiz, M.J., 2013. Bioaccessibility of enniatins A, A1, B, and B1 in different commercial breakfast cereals, cookies, and breads of Spain. J. Agric. Food Chem. 61 (2), 456–461.

Prosperini, A., Berrada, H., Ruiz, M.J., Caloni, F., Coccini, T., Spicer, L.J., Perego, M.C., Lafranconi, A., 2017. A review of the mycotoxin enniatin B. Front Public Health 5, 304.

Rodríguez-Carrasco, Y., Heilos, D., Richter, L., Süssmuth, R.D., Heffeter, P., Sulyok, M., Kenner, L., Berger, W., Dornetshuber-Fleiss, R., 2016. Mouse tissue distribution and persistence of the food-born fusariotoxins Enniatin B and Beauvericin. Toxicol. Lett. 247, 35–44.

Rodriguez-Carrasco, Y., Izzo, L., Gaspari, A., Graziani, G., Manes, J., Ritieni, A., 2018. Urinary levels of enniatin B and its phase I metabolites: first human pilot biomonitoring study. Food Chem. Toxicol. 118, 454–459.

Serrano, A.B., Meca, G., Font, G., Ferrer, E., 2014. Risk assessment of beauvericin, en- niatins and fusaproliferin present in follow-up infant formula by in vitro evaluation of the duodenal and colonic bioaccessibility. Food Contr. 42, 234–241.

Taroncher, M., Tolosa, J., Prosperini, A., Ruiz, M.J., 2018. In silico and in vitro prediction of the toxicological effects of individual and combined mycotoxins. Food Chem. Toxicol. 122, 194–202 2018 Dec.

Versantvoort, C.H., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J., Sips, A.J., 2005. Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem. Toxicol. 43 (1), 31–40.

4.GENERAL DISCUSSION

4. DISCUSIÓN GENERAL

Para alcanzar los objetivos planteados en el trabajo de investigación desarrollado en la presente Tesis Doctoral, se ha realizado una revisión bibliográfica acerca de la presencia de micotoxinas en productos botánicos y acerca del potencial de las tecnologías no térmicas de procesado de los alimentos (pulsos eléctricos de alta intensidad y altas presiones hidrostáticas) para mitigar los contenidos de micotoxinas. Por otra parte, se ha llevado a cabo la validación de métodos analíticos para la determinación de micotoxinas basados en Cromatografía líquida y Cromatografía de gases acopladas a espectrometría de masas en tándem de acuerdo a la normativa Europea (EC, 2002) y se han evaluado los niveles de concentración en los productos frescos (zumos, plantas medicinales y tés, en distintas formas) para evaluar la exposición de la población a micotoxinas través de su consumo. Se ha evaluado el comportamiento de las micotoxinas durante el proceso térmico de preparación de infusiones y tras la aplicación de tecnologías no térmicas de procesado de alimentos (pulsos eléctricos de alta intensidad y altas presiones hidrostáticas). Se han identificado productos de degradación de micotoxinas tras el procesado no térmico. Así mismo, se ha estudiado la bioaccesibilidad de ENNB tras la digestión *in vitro* e identificado metabolitos catabólicos tras la digestión.

4.1. Validación de la metodología analítica

Se ha optimizado la metodología analítica para la determinación de micotoxinas en alimentos según las recomendaciones establecidas por parte de la Comisión Europea relativas al funcionamiento de los métodos analíticos y la

interpretación de los resultados (EC, 2002). En dichas directrices, se establece que una sustancia puede ser identificada mediante LC-MS/MS y GC-MS/MS empleando al menos dos transiciones, una de cuantificación y una de confirmación. La fragmentación de los iones precursores se ha optimizado mediante la aplicación de distintas energías de colisión, seleccionando las dos transiciones con mayor proporción entre la señal cromatográfica y el ruido de fondo (S/N), considerando la mínima interferencia de los componentes de la matriz. La identificación de las micotoxinas se ha basado en el tiempo de retención del compuesto, su masa exacta o la de su correspondiente aducto y su patrón típico de fragmentación. La cuantificación se ha realizado con la transición de cuantificación, ratificada por la transición de confirmación.

La LC-MS/MS con trampa de iones (IT) en modo positivo se ha utilizado para el análisis de micotoxinas en todas las matrices estudiadas a excepción de los tricotecenos, PAT y ZEA en zumos. Así, se ha empelado para la determinación de las micotoxinas AFB1, AFB2, AFG1, AFG2, ZEA, OTA, ENNA, ENNA1, ENNB, ENNB1 y BEA en productos de plantas medicinales, para determinar 3aDON, 15aDON, NIV, HT-2, T-2, además de las micotoxinas mencionadas anteriormente en infusiones de tés y plantas medicinales y para determinar AFB1, AFB2, AFG1, AFG2, AOH, AME, OTA, FB1, FB2, ENNA, ENNA1, ENNB, ENNB1, BEA, STG en zumos. La separación se ha realizado empleando una columna Germini C18 (150 mm x 4.6 mm, 5 μm), utilizando como fases móviles agua y metanol con 0.1% de ácido fórmico y 5 mM de formeato de amonio, un tiempo total de 30 min de inyección y un flujo establecido en 0.25 mL/min.

La GC-MS/MS con triple cuadrupolo (QqQ) se ha empleado para el análisis de ZEA, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, PAT, T-2 y HT-2 en las muestras de zumos. Previamente al análisis, se ha llevado a cabo un proceso de derivatización mediante una mezcla de (BSA+ TMCS+ TMSI). La separación se ha realizado utilizando una columna capilar HP-5MS (30 m x 0.25 mm x 0.25 μm) en modo de vaporización programable por temperatura (PTV) a 250 °C empleando helio como gas transportador a una presión fija de 20.3 psi. El tiempo total de inyección empleado es de 23.15 min.

La cromatografía líquida acoplada a espectrometría de masas de alta resolución TOF (LC-ESI-qTOF-MS) en modalidad MS full scan se ha empleado para la identificación de los productos de degradación tras los tratamientos no térmicos. Los productos de degradación con sus respectivos aductos se identificaron con la masa exacta de cuatro cifras decimales. La columna utilizada para la separación consistió en una Germini C18 (150 mm x 4.6 mm, 5 μm), empelando como fases móviles agua y acetonitrilo al 0.1% de ácido fórmico. El tiempo de inyección fue de 20 min y el flujo 0.2 mL/min.

La cuantificación de ENNB tras la digestion *in vitro*, se ha realizado mediante cromatografía liquida acoplada a tripe quadrupolo (UHPLC-MS/MS). La columna utilizada consistió en una Synergy Fusion C18 (50 × 2.00 mm inner diameter, 2.5 μm), empelando como fases móviles agua y metanol acidificados con 0.2% de ácido fórmico. El tiempo total de análisis fue de 30 min y el flujo se fijó en 0.2 mL/min.

La cromatografía líquida acoplada a espectrometría de masas de alta resolución Orbitrap (UHPLC-HRMS) se ha utilizado para identificar los productos de degradación y metabolitos catabólicos de ENNB tras la digestión *in vitro*. El analizador de masas Q-Exactive se empleó en modo full MS/data dependiente de MS/MS (full MS–dd-MS/MS) en modo de ionización positiva. Para la separación cromatográfica, se empleó una columna de fase reversa C18 Kinetex EVO (2.10 x 100 mm, 2.6 μm). Las fases móviles utilizadas consistieron en agua y metanol 1 mM de acetato de amonio y 0.5% de ácido acético. El tiempo de inyección fue de 25 min y el flujo 0.4 mL/min.

4.1.1. Optimización de los métodos de extracción

Se han empelado distintos procedimientos de extracción en función de las características de la matriz alimentaria. Los métodos se han optimizado en términos de recuperación, repetibilidad (precisión intra-día), reproducibilidad (precisión inter-día), efecto matriz (ME), linealidad, límites de detección (LOD) y límites de cuantificación (LOQ) según la Decisión de la Comisión (EC, 2002).

4.1.1.1. Extracción de micotoxinas en matrices sólidas

El método QuEChERS se ha utilizado para la extracción de micotoxinas de la materia en crudo de plantas medicinales y de cápsulas de plantas medicinales.

La metodología Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS), consiste en un procedimiento de extracción simple, rápido y económico basado en una separación inicial con acetonitrilo, seguida de extracción dispersiva en fase sólida mediante sales mixtas como aminas secundaria primarias y sulfato de magnesio anhidro utilizadas para eliminar el

agua residual y la purificación de las muestras. Se han utilizado 2 g de muestra, junto con 10 ml de agua acidificada al 2% con ácido fórmico y 10 ml de acetonitrilo, empleando posteriormente las sales NaCl y MgSO4 y el sorbente Octadecyl C18 en la fase de purificación, para eliminar lípidos, proteínas, carbohidratos y pigmentos.

Plantas medicinales

Para las plantas medicinales en crudo se ha optimizado el método QuEChERS para la extracción de las micotoxinas (AFB1, AFB2, AFG1, AFG2, ZEA, ENNA, ENNA1, ENNB, ENNB1 y BEA). Las recuperaciones obtenidas para un nivel de concentración 10 × LOQ oscilaron entre 62 y 101% en el caso de la precisión intra-día y entre 58 y 110% para la precisión inter-día y estuvieron dentro de la desviación estándar relativa (<20%). Respecto al efecto matriz obtenido, se observó una supresión de la señal para las AFs (23-37%). Para minimizar el efecto matriz, los parámetros analíticos se obtuvieron utilizando curvas de calibración preparadas en matriz. Los coeficientes de correlación lineal (r^2), con valores desde 0.990- 0.999 mostraron una linealidad adecuada para todas las micotoxinas estudiadas. Los LOD obtenidos se encuentran entre 0.1 y 5 µg/kg, y los LOQ entre 0.3 y 17 µg/kg.

Cápsulas de plantas medicinales

El método QuEChERS se ha optimizado para la extracción de las micotoxinas AFB1, AFB2, AFG1, AFG2, OTA, ZEA, ENNA, ENNA1, ENNB, ENNB1 y BEA a partir de cápsulas de plantas medicinales. Los parámetros analíticos obtenidos revelan recuperaciones intra-día e inter-día entre un 73 y 119% con una desviación estándar relativa menor del 20% para un nivel

de concentración de 100xLOQ. El efecto matriz de supresión de la señal para AFs oscila entre 46-62% y los límites de detección y cuantificación entre 0.15 μg/kg y 3 μg/kg y entre 0.5 μg /kg y 10 μg/kg, respectivamente. La linealidad en el rango de 0.991 a 0.999. Estos parámetros se obtuvieron utilizando curvas de calibración preparadas en matriz, para compensar el efecto matriz observado.

4.1.1.2. Extracción de micotoxinas en matrices líquidas

Para la extracción de micotoxinas en las infusiones de té y plantas medicinales y en los zumos se ha utilizado el método de micro extracción líquido-líquido dispersiva (DLLME).

La extracción DLLME consiste es un sistema ternario formado por una solución acuosa, un disolvente extractante orgánico (frecuentemente con alta densidad) y un disolvente dispersante (miscible en las fases extractante y acuosa), que permite obtener altas recuperaciones.

Infusiones de té y plantas medicinales

Para las infusiones de té y plantas medicinales el método DLLME se ha optimizado utilizando distintas alternativas de disolvente dispersante (acetonitrilo o metanol), de disolvente extractante (cloroformo, acetato de etilo y tetracloruro de carbono) y distintos volúmenes de disolvente extractante (500, 620 y 700 μl) junto con 950 μl de disolvente dispersante. Los mejores resultados se han obtenido empleando las combinaciones de disolvente dispersante/extractante acetonitrilo-acetato de etilo (950 μl/ 620 μl) en un primer paso y metanol-cloroformo (950 μl/ 620 μl) en un segundo paso partiendo de 5 ml de volumen de muestra junto con 1 g de NaCl.

Los parámetros analíticos obtenidos para la optimización del método DLLME para el análisis de 16 micotoxinas (AFB1, AFB2, AFG1, AFG2, 3aDON, 15aDON, NIV, HT-2, T-2, ZEA, OTA, ENNA, ENNA1, ENNB, ENNB1 y BEA) estuvieron de acuerdo con lo establecido por la Decisión de la Comisión (EC, 2002). Las recuperaciones oscilaron entre 65 y 127%, y estuvieron dentro de la desviación estándar relativa para un nivel de concentración de 10xLOQ. Respecto al efecto matriz, se observó una supresión de la señal de las micotoxinas 3aDON y ZEA y un aumento de la señal de NIV, por lo que el resto de los parámetros analíticos se determinaron utilizando curvas de calibrado elaboradas con matriz. Así, los límites de detección y cuantificación oscilaron de 0.05 a 10 μg/l y de 0.2 a 33 μg/l, respectivamente. Finalmente, la linealidad obtenida fue adecuada, con coeficientes de correlación (r^2) entre 0.992 y 0.999.

Zumos

El método DLLME se ha optimizado para el análisis 30 micotoxinas (AFB1, AFB2, AFG1, AFG2, AOH, AME, OTA, FB1, FB2, ENNA, ENNA1, ENNB, ENNB1, BEA, STG, ZEA, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, PAT, T-2 y HT-2). Posteriormente AFB1, AFB2, AFG1, AFG2, AOH, AME, OTA, FB1, FB2, ENNA, ENNA1, ENNB, ENNB1, BEA y STG se determinaron por cromatografía líquida acoplada a espectrometría de masas en tándem, mientras que ZEA, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, PAT, T-2 y HT-2 se determinaron por cromatografía de gases acoplada a la espectrometría de masas en tándem.

Los parámetros analíticos obtenidos mostraron una supresión de la señal (del 41 al 64%) para α-ZOL, AFB1, AFG1, AFG2, OTA, ENNA1, ENNB1 y BEA. Por lo tanto, se utilizaron curvas de calibración preparadas en matriz para obtener el resto de los parámetros. Los LOD obtenidos variaron de 0.15 a 2.34 μg/l y los LOQ de 0.5 a 7.81 μg/l. Para evaluar la linealidad, los coeficientes de regresión obtenidos fueron superiores a 0.990. Las recuperaciones se estudiaron a tres niveles de concentración (50, 100 y 200 μg/l). Los valores obtenidos oscilaron entre 61 y 115% y las precisiones intra-día e inter-día fueron inferiores al 14% y al 19%, respectivamente, para todas las micotoxinas estudiadas.

Debido al efecto matriz observado en ambos métodos la cuantificación de las micotoxinas en las distintas muestras se ha realizado mediante curvas preparadas con extractos de matriz adicionadas de los distintos estándares, para compensar las variaciones de la señal y llevar a cabo una cuantificación adecuada.

4.2. Presencia de micotoxinas en alimentos

Una vez validados los métodos de extracción se ha procedido al análisis de micotoxinas en las matrices alimentarias estudiadas. La tabla 1 (anexo 6) muestra la clasificación de todas las muestras analizadas. Se han estudiadas 85 muestras de cápsulas medicinales elaboradas a base de un solo tipo de planta medicinal (65): cola de caballo, alcachofa, valeriana, diente de león, cardo mariano, fucus, boldo, ginkgo, jengibre, pasiflora, harpagofito, espino blanco, melisa, té rojo, té verde y a base de combinaciones de plantas para tratar el insomnio o perder peso (21). Así mismo se han estudiado 224 muestras de plantas medicinales en crudo (detalladas en la taba 1 del anexo 6), de las que

posteriormente se han analizado las infusiones resultantes con el fin de evaluar los contenidos finales. También se han analizado 44 muestras de infusiones té negro, rojo, verde y té verde con menta y 52 muestras de infusiones de manzanilla, manzanilla con miel, manzanilla con anís, tila, poleo menta, tomillo, valeriana y cola de caballo. Finalmente se han analizado 80 muestras de zumos elaborados a partir de una sola fruta (frescos y a base de concentrados) y a partir de combinaciones de zumos, purees e ingredientes lácteos y funcionales (detallados en la tabla 1 del anexo 6).

4.2.1. Presencia de micotoxinas en matrices sólidas

Cápsulas de plantas medicinales

Se ha analizado la presencia de AFs, ZEA, OTA, ENNs y BEA en 85 muestras de suplementos dietéticos de plantas medicinales más utilizados como remedios naturales en España. De estas muestras, 64 estaban elaboradas a base de un solo tipo de planta medicinal y 21 estaban compuestas de combinaciones de plantas medicinales para tratar el insomnio o para perder peso.

Todas las micotoxinas estudiadas se han detectado en las muestras analizadas excepto las AFs, con incidencias entre el 1 al 34% y niveles desde <LOQ a 3850.49 μg/kg. ENNB ha sido la micotoxina que presentó mayor incidencia (34%), pero con una concentración media en las muestras positivas de 88,7 μg / kg. ZEA aunque solo se ha detectado en el 8% de las muestras, ha presentado la concentración media más alta (1340.1 μg/kg). OTA solo se ha detectado en una muestra de mezcla de hierbas para tratar el insomnio a una concentración de 799 μg/kg.

Por tipo de planta medicinal, las cápsulas de alcachofa, té verde, té rojo y ginkgo no han mostrado contaminación por micotoxinas. Las cápsulas de mezcla de plantas medicinales para perder peso tampoco han resultado contaminadas, estos comprimidos estaban hechos principalmente a base de té verde y fucus. Solo una de las cinco muestras de cápsulas de fucus analizadas ha sido positiva para ZEA (659.73 μg / kg). En los resultados obtenidos en la presente tesis en infusiones de *Camellia sinensis* y en un estudio previo Di Mavungu et al., (2009) tampoco se observaron presencia de micotoxinas a niveles superiores al límite de cuantificación en muestras de té verde y rojo y ginkgo, respectivamente.

Por otra parte, al menos una de las muestras analizadas de cápsulas de valeriana, diente de león, boldo, jengibre, pasiflora, cola de caballo, cardo mariano, harpagofito, espino blanco, melisa, fucus y mezcla de plantas medicinales utilizadas para tratar el insomnio han mostrado contaminación por una o más micotoxinas. Las cápsulas de boldo, cardo mariano, cola de caballo y jengibre han mostrado mayor contaminación, con co-ocurrencia de micotoxinas a niveles de hasta 1000 μg/kg. En cardo mariano, las ENNs se han detectado con alta incidencia (75%) y concentraciones máximas entre 109.19 y 1378.21 μg/Kg. Veprikova et al. (2015) también observaron alta incidencia de tricotecenos (13-78%), toxinas de Alternaria (22-97%), ZEA (78%) y ENNs (84-91%) en cardo mariano, con concentraciones máximas de ENNs desde 2340 a 9260 μg/Kg y de 751 μg/Kg para ZEA, mientras que Arroyo-Manzanares et al. (2013) no encontraron presencia de micotoxinas en un extracto de cardo mariano.

El estudio no ha sido diseñado para analizar diferencias entre las muestras ecológicas y las convencionales, por eso el número de muestras de cada tipo no es equitativo. Al comparar el contenido de micotoxinas entre las cápsulas ecológicas y las convencionales, el 58.3% de las muestras ecológicas frente al 41.1% de las muestras convencionales han resultado contaminadas por al menos una micotoxina. En muestras ecológicas, se han observado co-ocurrencias de dos y cinco micotoxinas frente a co-ocurrencias de dos, tres, cuatro y cinco micotoxinas en muestras convencionales.

Plantas medicinales

Se ha estudiado la presencia de AFs, ZEA, OTA, ENNs y BEA en un total de 224 muestras de plantas medicinales en crudo pertenecientes a 56 especies de plantas.

La incidencia de las micotoxinas estudiadas en las plantas medicinales en crudo ha variado del 1% (ENNA y BEA) al 15% (ENNB), mientras que la concentración media del total de las muestras ha variado desde <LOQ (BEA) a 520.74 μg/kg (ZEA). ZEA y AFG2 son las micotoxinas que presentan contenidos más altos.

ZEA se ha determinado en el 11% de las muestras, principalmente en salvia, stevia, eucalipto, mejorana, poleo y rabo de gato. Kong et al. (2013) observaron una incidencia similar (12%) en plantas medicinales procedentes de la medicina China pero a una concentración media de 11.096 μg/kg, inferior a la del presente estudio. Veprikova et al. (2015) indicaron una concentración máxima de 824 μg/kg para ZEA en suplementos dietéticos a base de plantas medicinales.

Las AFs se han detectado en muestras de salvia, hierba luisa, manzanilla, eucalipto, menta, tomillo, orégano, artemisa, romero, melisa, albahaca y ajedrea a concentraciones medias entre 64.76 y 383.53 µg /Kg pero con incidencias muy bajas (4-11%). En un estudio realizado en España, Santos et al. (2009) también observaron niveles altos de AFs (<1.4 a 855 µg/kg) con una incidencia mayor que en el presente estudio (96%), en cambio Liu et al., (2012) observaron incidencias similares al presente estudio: AFB1 (16%), AFB2 (14%), AFG1 (6%) y AFG2 (2%) en 174 muestras comerciales de plantas medicinales procedentes de la medicina China, pero menores contenidos (concentración media de 3,85 µg/kg para AFs).

Para las micotoxinas emergentes, salvia, rabo de gato, ortiga, diente de león, regaliz, anís, melisa, eufrasia, sauce, llantén, pulmonaria, verbena, sanguinaria, cola de caballo, espino blanco y tila han sido las especies de plantas medicinales positivas. Las incidencias observadas oscilan entre 1 y 15%, y las concentraciones medias entre <LOQ y 42,43 µg/kg. Estos resultados han sido similares a los indicados por Hu & Rychlik (2014b) en plantas medicinales procedentes de la medicina China, con contenidos totales desde 2.5 a 751 µg/kg.

4.2.2. Presencia de micotoxinas en matrices líquidas

Infusiones de té "*Camellia sinensis*"

Se ha analizado la presencia de 16 micotoxinas (AFB1, AFB2, AFG1, AFG2, 3aDON, 15aDON, NIV, HT-2, T-2, ZEA, OTA, ENNA, ENNA1, ENNB, ENNB1 y BEA) en 44 muestras de té preparadas para su consumo, pertenecientes a té negro, té rojo, té verde y té verde con menta.

De las micotoxinas analizadas, AFB1, 3aDON, NIV, HT2, T2, ZEA, OTA, ENNB1, ENNA1, ENNA y BEA no se han detectado en ninguna de las muestras. Las micotoxinas que presentaron mayor incidencia han sido AFG2 (18%) y AFB2 (14%).

Por tipo de té, el té negro y el té rojo son positivos para AFG2 pero a concentraciones por debajo de los límites de cuantificación. El té verde es positivo para ENNB a niveles inferiores al límite de cuantificación. El té verde con menta contiene la mayor contaminación por micotoxinas, con niveles > LOQ para AFB2, AFG2 y 15aDON. AFB2 ha presentado niveles que oscilan entre 14.4-32.2 µg/l, AFG2 está en el rango de 1.9-2.6 µg/l y 15aDON presenta niveles de 60.5-61 µg/l. AFG1 se detecta a niveles <LOQ. Los niveles obtenidos para AFB2 han sido superiores a los límites establecidos para AFs en especias en el Reglamento de la Comisión (EC, 2006a) en las muestras positivas de té verde con menta. En un estudio previo, a diferencia del presente trabajo, Monbaliu et al. (2010) no encontraron micotoxinas en ninguna muestra de infusion de té.

Infusiones de plantas medicinales

Se ha estudiado la presencia de 16 micotoxinas (AFB1, AFB2, AFG1, AFG2, 3aDON, 15aDON, NIV, HT-2, T-2, ZEA, OTA, ENNA, ENNA1, ENNB, ENNB1 y BEA) en 52 muestras de infusiones preparadas para su consumo, clasificadas en manzanilla, manzanilla con miel, manzanilla con anís, tila, poleo menta, tomillo, valeriana y cola de caballo.

Así mismo, se ha estudiado la presencia de AFs, ZEA, OTA, ENNs y BEA en 224 infusiones preparadas a partir de las plantas medicinales analizadas

previamente en crudo, con el fin de evaluar los contenidos finales de micotoxinas.

De las micotoxinas estudiadas se han detectado AFB2, AFG1, AFG2, 15aDON, ZEA, ENNB y ENNB1 en el total de las muestras analizadas, con incidencias inferiores al 10% en todos los casos y concentraciones no superiores a 100 µg/l, en general.

Las micotoxinas que se han encontrado con mayor frecuencia en las 52 muestras de infusiones han sido las AFs, AFB2 (10%), AFG2 (10%) y AFG1 (8%). ENNB se ha detectado en dos muestras y 15aDON en una. En relación con los diferentes tipos de plantas analizadas, las micotoxinas no se detectaron en ninguna muestra de manzanilla, manzanilla con anís, manzanilla con miel y tila. En contraste, poleo menta, tomillo, valeriana y cola de caballo fueron positivas para más de una micotoxina. Las AFs, se han detectado con concentraciones medias de las muestras positivas desde < LOQ a 112.2 µg/l. 15aDON se ha detectado con una concentración media de 112.5 µg/l, pero solamente en una muestra de valeriana. La ENNB se ha detectado en dos muestras de cola de caballo a una concentración de <LOQ. Los niveles obtenidos para AFB2 y AFG2 en algunos casos han sido superiores a los límites establecidos (10 µg/kg para la suma de AFs) en especias en el Reglamento de la Comisión (EC, 2006a).

Al analizar las infusiones de las 224 muestras de plantas medicinales, se han detectado AFB2, AFG1, AFG2, ZEA, ENNB y ENNB1 con incidencias del 1 al 6% y concentraciones que variaron desde niveles <LOQ a 82.2 µg/l. Al comparar los contenidos de micotoxinas en las infusiones de plantas medicinales con las mismas muestras analizadas en crudo, los niveles de micotoxinas son más

bajos en las infusiones, y las micotoxinas AFB1, AFB2, ENNA, ENNA1, ENNB1 y BEA no se han detectado a niveles superiores al LOQ.

Comparando los resultados, se han obtenido valores similares en infusiones de té "*Camellia sinensis"* en la presente tesis, al igual que en infusiones de plantas medicinales AFB2, AFG1, AFG2, 15aDON y ENNB, también han sido las micotoxinas detectadas en las muestras y AFB2 (14.4-32.2 μg/l) y 15aDON (60.5-61 μg /l), presentan también los niveles más altos. Sin embargo, con respecto a estudios previos disponibles la bibliografía, Monbaliu et al. (2010) no observaron presencia de micotoxinas en infusiones preparadas a partir de muestras de tés y plantas medicinales contaminadas con varias micotoxinas a niveles <80 μg / Kg.

Zumos

Se ha evaluado la presencia de 30 micotoxinas (AFB1, AFB2, AFG1, AFG2, AOH, AME, OTA, FB1, FB2, ENNA, ENNA1, ENNB, ENNB1, BEA, STG, ZEA, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, PAT, T-2 y HT-2 toxinas) en 80 muestras de zumos de fruta. De ellas 40 pertenecen a zumos de un solo tipo de fruta, de los cuales 7 son zumos de naranja naturales y 33 son zumos a base de concentrados de fruta (naranja, pera, manzana, piña y melocotón). 40 están elaborados a base de mezclas de zumo de frutas, pures de verduras y algunos contienen ingredientes lácteos o ingredientes funcionales como semillas, plantas medicinales etc.

De las 30 micotoxinas analizadas, 9 (AOH, AME, PAT, OTA, AFB1, AFB2, AFG2, β-ZAL y HT-2) se han detectado en cantidades cuantificables en

el 49% de los zumos analizados. AOH y PAT, son las micotoxinas más detectadas con incidencias del 29% y 18%, respectivamente, mientras que β-ZAL y HT-2 se han detectado solo en el 3% de las muestras. Respecto a los contenidos, la concentración media de las muestras positivas oscila entre 3,75 y 207,01 μg/l. AOH y PAT también han sido las micotoxinas detectadas con contenidos más altos. Los resultados obtenidos son consistentes con la información disponible en la bibliografía, donde PAT, AOH y AME son las micotoxinas detectadas más frecuentemente en zumos (Fernández-Cruz et al., 2010). Se ha observado co-ocurrencia de 2, 3, 5 o 7 micotoxinas en el 12% de las muestras analizadas.

PAT se ha detectado en los zumos con una incidencia del 18%, y concentraciones entre <LOQ y 50.95 μg/l. Solo una muestra ha superado ligeramente el límite máximo establecido para PAT en zumos de frutas por la Comisión Europea (50 μg/l) (EC, 2006a). En Irán, Rahimi et al. (2015) obtuvieron resultados similares, detectaron PAT en el 16% de 161 muestras de zumos de frutas con niveles entre 5 y 190.7 μg/kg. OTA se ha determinado en el 9% de las muestras estudiadas a concentraciones entre 2.93 y 10.81 μg/l. La Comisión Europea ha establecido 2 μg/kg como límite para OTA en el zumo de uva (EC, 2006a). Asadi et al. (2018) observaron menores contenidos que en el presente estudio, con el 15% de 20 zumos de manzana y el 25% de 20 zumos de uva, contaminados por OTA a niveles desde 0.06 a 0.1 μg /l y desde 0.06 a 0.12 μg/l, respectivamente. AME y AOH se han detectado en el 10 y 29% de las muestras analizadas a una concentración media de 8,5 y 207 μg/l, respectivamente. Zwickel et al. (2016) determinaron AOH y AME con una incidencia similar (27 y 5% de 78 muestras de zumo, respectivamente), pero con contenidos más bajos: AOH (de 0.81 a 8.16 μg/l) y AME (de 0,89 a 1,54 μg/l).

AFB1, AFB2 y AFG2 se han detectado en el 8, 6 y 4% de los zumos analizados respectivamente, a niveles entre 1.24 y 18.1 μg/l. En un estudio realizado en Egipto, Abdel-Sater et al. (2001) informaron que el 100% de cinco bebidas de manzana analizadas resultaron positivas para AFB1 y AFG1 en un rango de 20 a 30 μg/l y dos de cinco zumos de guayaba presentaron AFB1 a un nivel de 12 μg/l.

Solo 2 muestras de zumo de manzana han resultado contaminadas por β-ZAL con concentraciones de 22.59 y 23.85 μg/l, respectivamente. Existe poca información disponible en la literatura sobre la presencia de ZEA y sus metabolitos en los zumos de frutas. En un estudio previo, Carballo et al. (2018) no encontraron presencia de ZEA y β-ZAL en un estudio multimicotoxina realizado en 42 muestras de zumos de frutas. La toxina HT-2 soló se ha detectado en 2 muestras de mezclas de zumos de frutas con concentraciones de 21.38 y 24.15 μg / L, respectivamente. HT-2 tampoco se encuentra comúnmente en frutas y sus productos derivados y hay poca información disponible en la bibliografía.

Comparando los resultados por tipo de muestras de zumo, las muestras de un solo tipo de fruta presentan contaminación de hasta 8 micotoxinas diferentes (AOH, AME, PAT, AFB1, AFB2, AFG2, OTA, β-ZAL) a niveles inferiores a 50 μg /l, mientras que los zumos a base de mezclas presentan contaminación por AOH, PAT y HT-2. Las muestras de zumo de naranja fresco han sido las contaminadas por el mayor número de micotoxinas, hasta 7 (AFB1, AFB2, AFG2, AOH, AME, PAT y OTA). EL contenido de AOH, es mayor en los zumos a base de mezclas, tal vez por la adición de otros ingredientes, como el jengibre, verduras como pepino y espinacas o frutas de la pasión. Algunos

autores han reportado contaminación por AOH en verduras como tomate y frutos rojos (Qiao et al. 2018; Rodríguez-Carrasco et al. 2016).

4.3. Evaluación del riesgo por exposición a micotoxinas

La evaluación de la exposición de la población a micotoxinas se ha llevado a cabo desde un enfoque determinista, obteniendo los valores de Ingesta Diaria Estimada (IDE) a partir de las concentraciones de micotoxinas obtenidas en alimentos y los datos disponibles de consumo de alimentos en las hojas de balance alimentario del MAPA y de la FAO o los datos de dosis recomendadas por el fabricante en el caso de los suplementos de plantas medicinales. Para el cálculo del consumo de alimentos expresado en kg de peso corporal (kg/pc), se ha considerado un peso medio de 70 kg para la población adulta y de 25 kg para la población infantil.

Para obtener las concentraciones medias de micotoxinas en los alimentos, los datos por debajo del LOQ, se han procesado según las recomendaciones de la EFSA considerando dos escenarios de exposición (EFSA, 2010). En el escenario de nivel bajo de exposición (LB) se ha asignado el valor de cero a las muestras donde las micotoxinas no se han detectado o se han detectado a niveles por debajo del límite de cuantificación, mientras que en el escenario de nivel alto de exposición (UB), se ha asignado el valor del LOD a las muestras donde no se detectaron las micotoxinas y el valor de LOQ a las muestras donde se detectaron por debajo del LOQ.

Para llevar a cabo la evaluación del riesgo se han comparado los valores de IDE calculados con los valores de ingesta diaria tolerable (IDT) establecidos

para las distintas micotoxinas. La caracterización del riego se expresa como % de IDT. Para las micotoxinas estudiadas en la presente tesis se han establecido las siguientes IDT por parte de la Autoridad Europea de Seguridad Alimentaria (EFSA), el Comité Científico sobre la Alimentación Humana (SCF) y el Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios. Se ha fijado una ingesta diaria tolerable máxima provisional de 0,4 μg / kg pc / día para PAT (EFSA, 2002), una IDT de 0,25 μg / kg pc / día para ZEA y de 0,1 μg / kg pc / día para la suma de HT2 y T2 (EFSA, 2014) y una ingesta semanal tolerable (TWI) de 0,12 μg / kg pc / semana para OTA (EFSA, 2006). Para aquellas micotoxinas para las cuales aún no se ha establecido un valor de IDT, como las micotoxinas emergentes de *Fusarium*, los valores de IDE obtenidos se han comparado con los valores de IDT más bajos y altos fijados para otras micotoxinas de *Fusarium* como DON (1 μg / kg pc / día) (SCF, 2002) y la suma de las toxinas HT2 y T2 (0.1 μg / kg pc / día) (EFSA, 2014). Para las AFs, al tratarse de compuestos genotóxicos y cancerígenos, no existe un valor de IDT establecido para realizar la caracterización del riesgo y se aplica el principio ALARA (As Low As Reasonably Achievable), se deben reducir su presencia a niveles tan bajos como sea razonablemente posible.

4.3.1. Evaluación del riesgo por exposición a micotoxinas por el consumo de cápsulas de plantas medicinales

Se ha evaluado el riesgo de exposición a las micotoxinas a través del consumo de las cápsulas de plantas medicinales que han sido positivas al analizar la presencia de las micotoxinas estudiadas, considerando el consumo recomendado por parte de los fabricantes.

Para ZEA, los valores de IDE obtenidos representan un porcentaje del 0.21 al 11.89% de la IDT. Por tipo de cápsula, las cápsulas de boldo son el principal contribuyente a la exposición dietética de ZEA. OTA, solo se ha determinado en las cápsulas de mezclas de plantas medicinales para tratar el insomnio, con una IDE calculada que representa el 3.57% (LB) y el 3.92% (UB) de la TWI establecida para OTA.

Para las micotoxinas emergentes, se han comparado los valores de IDE con los fijados para otras micotoxinas de *Fusarium* (DON (1 μg / kg pc / día) (SCF, 2002) y la suma de las toxinas HT2 y T2 (0.1 μg / kg pc / día) (EFSA, 2014)). Las IDE obtenidas para BEA en los diferentes escenarios varían del 0.23 al 3.46% de la IDT establecida para HT-2 y T-2 y del 0.02 al 0.34% de la IDT establecida para DON. Los comprimidos de cardo mariano han sido el tipo de cápsula que más contribuyó a la exposición dietética de BEA. Las IDE obtenidas para la suma de ENNs han alcanzado del 0.0048 a 22.2% de la IDT fijada para la suma de HT-2 y T-2, representando un riesgo potencial en el caso de los suplementos de cola de caballo y cardo mariano con unas IDE que alcanzan el 15.6% y el 22.2% de la IDT, respectivamente. Los porcentajes obtenidos disminuyeron a valores menos alarmantes (de 0,00048 a 2,2%) cuando se comparan las IDE con la IDT establecida para DON. En general, el consumo de suplementos de plantas medicinales a la dosis recomendada por el fabricante no supone un riesgo considerable, aunque en algunos casos se alcancen porcentajes considerables de la IDT. No obstante, las cápsulas de plantas medicinales pueden constituir una fuente adicional de exposición a micotoxinas, por lo que su control es aconsejable.

4.3.2. Evaluación del riesgo por exposición a micotoxinas por el consumo de infusiones de tés "*Camellia sinensis*".

Los datos del consumo anual de té se han consultado en la base de datos del Ministerio de Agricultura, Pesca y Alimentación de España (MAPA, 2013). El consumo anual fue de 0,12 kg, equivalentes a 16 l de té listo para el consumo por persona y año.

Las AFs, aunque se han determinado en las bebidas de tés son compuestos cancerígenos genotóxicos por lo que no se puede recomendar una IDT y se recomienda que su presencia en los alimentos se reduzca a niveles tan bajos como sea razonablemente posible (ALARA).

Las IDE calculadas para 15aDON han sido 1,74 ng / kg pc / día (enfoque LB) y 1.80 ng / kg pc / día (enfoque UB), los % de IDT (comparando con la IDT fijada para DON) son 0.17 y 0.18% en el enfoque LB y UB, respectivamente.

Las IDE para ENNB solo se ha calculado en el enfoque UB, porque se ha detectado en las muestras a niveles inferiores a los límites de cuantificación. Para la caracterización del riesgo, la IDE para ENNB (0.038 ng/kg pc/día) se ha comparado con los valores de IDT más bajo y alto establecidos para las micotoxinas de *Fusarium* (0.1 µg / kg pc / día y 1 µg / kg pc / día), respectivamente (EFSA, 2014; SCF, 2002). Los porcentajes obtenidos han sido el 0.0038% y el 0.038% de las IDT, respectivamente. En ambas micotoxinas, 15aDON y ENNB, los valores de IDE están muy por debajo de los valores de IDT.

4.3.3. Evaluación del riesgo por exposición a micotoxinas por el consumo de infusiones de plantas medicinales.

Para llevar a cabo la evaluación del riesgo de la población a micotoxinas a través del consumo de infusiones preparadas a partir de plantas medicinales, el consumo anual se ha estimado en 0.12 kg para la población española (MAPA, 2017; 2018). Debido a que la población española no es una gran consumidora de infusiones, también se ha llevado a cabo la evaluación del riesgo considerando los datos de consumo de la población británica, estimado en 1.84 kg (FAO, 2013). Estos valores son equivalentes a un consumo de 16 y 245 l, respectivamente por persona y año de promedio. Así mismo, debido a que 16 litros por persona y año sería un consumo muy bajo, menor a una taza por día, se ha contemplado también la situación de mayores consumidores de infusiones de plantas medicinales para la población española, considerando el consumo de una taza (200 ml/día) y tres tazas (600 ml/día) por día, para así llevar a cabo la evaluación del riesgo de una forma más realista.

De las micotoxinas analizadas, AFB2, AFG1, AFG2, ZEA, 15aDON y ENNB y ENNB1 se han detectado en las muestras analizadas. En el caso de las aflatoxinas, no hay fijada una IDT, ya que son compuestos genotóxicos y cancerígenos, por lo que no es posible realizar una caracterización de riesgo. Respecto a 15aDON, el valor de IDE se ha comparado con la IDT de DON. En el caso de ENNB y ENNB1 al no tener un valor de IDT establecido, los valores de IDE calculados se han comparado con las IDT más alta y baja fijadas para otras micotoxinas de *Fusarium* (DON y la suma de HT-2 y T-2). En todos los casos, los valores de IDE están muy por debajo de las IDT establecidas, con porcentajes inferiores al 0.15% tanto en el escenario LB como en el UB para la

población española y con porcentajes inferiores al 2.2% para la población británica. En el caso de grandes consumidores (3 tazas al día) considerando la población Española, donde el consumo medio es inferior a una taza al día, la IDE obtenida para las ENNs representa un 1.42% del valor más bajo de IDT establecido para las micotoxinas de *Fusarium*.

Para ZEA, las IDE obtenidas no superan el 3.5% en el escenario LB y el 3.67% en el UB de la IDT establecida para ZEA, considerando a grandes consumidores de infusiones de plantas medicinales.

En general los valores de IDE calculados están muy por debajo de las IDT establecidas, por lo que el riesgo de exposición a micotoxinas a través del consumo de infusiones de plantas medicinales no es elevado, aunque puede aumentar grandes consumidores.

4.3.4. Evaluación del riesgo por exposición a micotoxinas por el consumo de zumo

Para evaluar la exposición de niños y adultos a micotoxinas a través del consumo de zumos de frutas se ha considerado un peso corporal de 70 kg para la población adulta y de 25 kg para la población infantil. El consumo diario de zumos consultado en la base de datos del MAPA es de 9.2 l por persona y año de promedio (MAPA, 2017). Para la población infantil al tratarse de mayores consumidores de zumos, se ha considerado un consumo diario de 200 ml correspondiente a un brik de zumo, para llevar a cabo un enfoque más realista.

Las IDE se han calculado para aquellas micotoxinas positivas en las muestras que tenían un valor de IDT establecido (PAT, OTA, βZAL y HT-2). Teniendo en cuenta la media de las muestras positivas para calcular la concentración media, los valores de IDE obtenidos representan un porcentaje notable de los valores de IDT fijados. Para la población adulta, se han obtenido porcentajes de (2.5% TDI), (11.5% TDI), (3.3% TDI) y (8.2% TDI) respectivamente para PAT, OTA, βZAL y HT-2. Sin embargo, cuando se usa la media de las muestras totales, los valores de IDE obtenidos para la población adulta están muy por debajo de las IDT fijadas, representando menos del 2% de las IDT, tanto empleando el enfoque LB como el UB. Para la población infantil, se ha obtenido una tasa de exposición del 56.4% de la IDT para PAT y del 74.3% de la IDT para βZAL, y IDE superiores a la IDT para OTA y HT-2, considerando como concentración media la media de las muestras positivas. Estos valores disminuyen al 13.8% de la IDT para PAT, al 35.3% de la IDT para OTA, al 9.15% de la IDT para βZAL y al 9% de la IDT para HT-2 considerando la media del total de las muestras desde el enfoque UB. Estos resultados evidencian un riesgo considerable para los niños. Torovic et al. (2017) observaron una exposición de riego similar para PAT en niños a través del consumo de zumos de manzana, al utilizar la media de muestras totales y considerar el mismo consumo que en el presente trabajo (0.2 l).

4.4. Efecto del procesado térmico de preparación de infusiones de plantas medicinales en los contenidos de micotoxinas.

Se ha evaluado el efecto del proceso de infusionado en las micotoxinas AFs, ZEA, ENNs y BEA, comparando los contenidos de las plantas medicinales

en crudo con los contenidos en las infusiones resultantes. Los resultados obtenidos muestran que durante la preparación de las infusiones de plantas medicinales, se logra una reducción importante en los contenido de micotoxinas. Los porcentajes de reducción oscilan entre 74% y 100%. AFB1, ENNA, ENNA1 y BEA no se han detectado en las infusiones resultantes, y AFB2 y ENNB1 se han detectado a niveles <LOQ. La tasa de reducción oscila entre 48 y 100% (media de 82%) para AFG1, entre 63-100% (media de 93%) para AFG2, entre 41 y 100% (media de 74%) para ZEA, y entre 25 y 100% (media de 95%) para ENNB. Para ZEA se observa una tasa de reducción menor, lo que podría explicarse debido a las cantidades elevadas detectadas en las muestras en crudo. Las reducciones alcanzadas durante el proceso de infusionado podrían no deberse a la temperatura, ya que la mayoría de las micotoxinas estudiadas son resistentes a temperaturas de 100 ° C, como las AFs y la ZEA, pero podrían deberse a la baja solubilidad y tendencia de estas micotoxinas a migrar a las bebidas resultantes durante el proceso de infusionado. Iha & Trucksess (2010), también obtuvieron porcentajes de reducción elevados al investigar la migración de AFs y OTA al preparar infusiones de jengibre, con tasas de reducción de hasta un 70% para AFs y un 80% para OTA. Ariño et al. (2007) solo observaron un 1% de transferencia de OTA desde la raíz de regaliz a la infusión resultante. Reinholds et al. (2019) observaron que del 32 al 100% de DON y ZEA presentes en los tés en crudo se extraían en las infusiones resultantes, obteniendo menores porcentajes de reducción que en el presente estudio. Respecto ZEA, Ryu et al., (2003) estudiaron su estabilidad a temperaturas de 100 a 225ºC y concluyeron que era relativamente estable a temperaturas de ebullición, tal y como también se ha observado en el presente estudio. En la literatura no hay información acerca del efecto del proceso de infusionado en los contenidos de micotoxinas

emergentes. En el presente estudio, se observa una alta reducción de las micotoxinas emergentes (del 95 al 100%). Debido a su baja solubilidad en agua, la tendencia de estas micotoxinas a migrar hacia las bebidas resultantes de plantas medicinales es escasa. Esta tendencia también fue indicada por Serrano et al. (2016) y Hu et al. (2014a), quienes observaron baja transferencia de ENNs al agua durante la cocción de la pasta y durante la producción de cerveza, respectivamente.

4.5. Efecto del procesado por altas presiones hidrostáticas (HPP) en los contenidos de micotoxinas.

Las muestras de zumo de uva se han adicionado con las micotoxinas AFB1, AFB2, AFG1, AFG2, ENNA, ENNA1, ENNB, ENNB1 y BEA de forma individual a una concentración de 100 μg/l y han sido tratadas por HPP a 500 MPa durante 5 min. Después del tratamiento, se han obtenido concentraciones de 83.1 ± 2 μg/l (AFB1), 86.3 ± 16 μg/l (AFB2), 80.8 ± 6 μg /l (AFG1), 71 ± 10 μg/l (AFG2), 82.89± 3 μg/l (ENNA), 87.78± 7 μg/l (ENNA1), 90.16± 6 μg/l (ENNB), 92.11±2 μg/l (ENNB1), 86.38±5 μg/l (BEA) correspondientes a porcentajes de reducción del 17% (AFB1), 14% (AFB2), 19% (AFG1), 29% (AFG2), 17% (ENNA), 12% (ENNA1), 10% (ENNB), 8% (ENNB1) y 14% (BEA), respectivamente. Con respecto a la información disponible en la bibliografía no hay estudios disponibles sobre el efecto del procesamiento por HPP sobre los contenidos de AFs y las micotoxinas emergentes en zumos, sin embargo, se ha estudiado el efecto en otras micotoxinas como PAT en zumos, o DON, ZEA y CIT en otras matrices.

En zumos con distinta composición, Hao et al., (2016) estudiaron la degradación de PAT bajo diferentes condiciones del tratamiento HPP (de 400 a 600 MPa, con tiempo de procesamiento de 0-300 s, y temperatura de 11 ° C) adicionada a una concentración de 200 μg /l. Estos autores observaron el nivel más alto de degradación en una disminución de 60 μg /l bajo el tratamiento a 600 MPa durante 300 s, equivalente a una reducción del 30%, similar a las reducciones observadas en el presente estudio. Avsaroglu et al. (2015) observaron porcentajes más altos de reducción, de hasta el 60% en zumos de manzana adicionados de PAT. En otras matrices alimentarias, en aceitunas enriquecida con concentraciones de 1; 1.25; 2.5; 10; 25 y 100 μg/Kg de CIT y tratadas con HPP a 250 MPa durante 5 minutos, Tokusoğlu et al. (2010) observaron una reducción promedio de 100, 98, 55, 37, 9 y 1.3% respectivamente. A la misma concentración analizada (100 μg/Kg), se obtuvieron mayores reducciones en el presente estudio, pero bajo un tratamiento de 500 MPa. Kalagatur et al. (2018) observaron una reducción completa de DON y ZEA en maíz después del tratamiento con HPP (550 MPa) a 45 ° C durante 20 minutos. Por lo tanto, los porcentajes de reducción pueden depender de la matriz alimentaria, la micotoxina estudiada y las condiciones de tratamiento aplicadas.

El mismo tratamiento aplicado en paralelo a muestras de agua empleadas como control, ha producido mayores porcentajes de reducción que en las muestras de zumo, del 61 al 87% para AFs y del 30 al 83% para las micotoxinas emergentes, poniendo de manifiesto un posible efecto de los componentes de la matriz en los resultados obtenidos.

4.6. Efecto del procesado por pulsos eléctricos de alta intensidad (PEF) en los contenidos de micotoxinas.

Las muestras de zumo de uva y de smoothie se han adicionado con las micotoxinas AFB1, AFB2, AFG1, AFG2, ENNA, ENNA1, ENNB, ENNB1 y BEA de forma individual a una concentración de 100 μg/l y han sido tratadas en el equipo de pulsos eléctricos bajo un campo de intensidad de 3 kv/cm y energía específica de 500 KJ/Kg. Después del tratamiento PEF, se ha observado una reducción significativa de las micotoxinas estudiadas en ambos tipos de muestras. En las muestras de zumo de uva, los contenidos de micotoxinas emergentes obtenidos han sido 46.81 ± 0.8 μg/l (ENNA), 52.23 ± 4.4 μg/l (ENNA1), 56.98 ± 10.88 μg/l (ENNB), 56.8 ± 2.79 μg/l (ENNB1) y 54.04 ± 0.4 μg/l (BEA), que se corresponden con porcentajes de reducción entre el 43 y el 53%. En las muestras de smoothies los contenidos han sido 44.35 ± 0.21 μg/l (ENNA), 40.84 ± 2.12 μg/l (ENNA1), 42.94 ± 14.56 μg/l (ENNB), 40.12 ± 6.58 μg/l (ENNB1) y 29.8 ± 9.8 μg/l (BEA), equivalentes a porcentajes de reducción entre el 56 y el 70%, ligeramente más elevados que en zumos.

Respecto a AFs, en las muestras de zumo de uva, los contenidos tras el tratamiento han sido 74.35± 10 μg/l (AFB1), 28.47± 5 μg/l (AFB2), 16.41± 6 μg/l (AFG1) y 76.65 ± 5 μg/l (AFG2), correspondiéndose con porcentajes de reducción comprendidos entre el 24 y el 84%. En smoothies, los contenidos han sido de 84.13±5 μg/l (AFB1), 23.31± 11 μg/l (AFB2), 21.04± 1 μg/l (AFG1) y 61.88 ± 2 μg/l (AFG2), equivalentes a porcentajes de reducción comprendidos entre un 16 y un 79%.

Existe poca información disponible en la literatura sobre el efecto del tratamiento PEF en los contenidos de micotoxinas. Vijayalakshmi et al. (2017)

optimizaron la combinación del procesamiento térmico con el tratamiento por PEF para reducir los contenidos de AFs adicionadas artificialmente a un sistema de patata dextrosa agar, a diferentes valores de pH y observaron que el tratamiento combinado era más efectivo que los procesos individuales, con porcentajes de reducción del 92.3 al 96.9% para AFB1 en función del pH y del 82 al 95.7% para AFs. En otro estudio, estos autores (Vijayalakshmi et al. (2018)) observaron una reducción de los contenidos de AFB1 y AFs del 77 y 97%, tras optimizar la metodología PEF. Las reducciones observadas por estos autores fueron similares a las observadas en la presente tesis para BEA en muestras de smoothie (70%) y para AFB2 y AFG1 tanto en muestras de zumos (72 y 84%, respectivamente) como en smoothies (77 y 79%, respectivamente), no obstante, se han obtenido menores reducciones para las ENNs (43-60%) y para AFB1 y AFG2 (16-38%).

Al aplicar el mismo tratamiento a muestras de agua contaminada con las mismas condiciones y empeladas como controles, se han observado menores porcentajes de reducción en el caso de las ENNs y BEA (21-45%) y similares en el caso de las AFs (30-84%) poniendo de manifiesto un posible efecto de la matriz en los resultados obtenidos. Como han observado otros autores (Avsaroglu et al., 2015; Hao et al., 2016), la degradación obtenida tras el tratamiento podría depender de los componentes del zumo. Este hecho podría explicar las diferentes reducciones observadas para las micotoxinas emergentes tras el tratamiento PEF en los zumos y smoothies en comparación con el agua.

4.7. Productos de degradación tras el procesamiento no térmico

Durante el procesamiento de alimentos, se pueden formar algunos productos de degradación o micotoxinas modificadas. La mayoría de estos compuestos generados después del procesamiento de alimentos no han sido testados para evaluar sus posibles efectos adversos para la salud humana, lo que dificulta la realización de una evaluación del riesgo adecuada (Cotterill et al., 2008). En este caso, los enfoques computacionales se pueden usar como una herramienta preliminar para identificar el potencial tóxico de estos productos de degradación o formas modificadas de las micotoxinas mediante el uso de su estructura química y se pueden emplear como un método de selección para determinar qué compuestos serán analizados posteriormente mediante ensayos *in vitro*.

Tras el tratamiento mediante PEF de las muestras de zumo de uva y smoothie adicionadas con las micotoxinas emergentes se han obtenido porcentajes de reducción comprendidos entre un 43 y 70%. Posteriormente, se ha llevado a cabo el análisis mediante cromatografía líquida acoplada a espectrometría de masas de alta resolución TOF (LC-ESI-qTOF-MS) para la identificación tentativa de productos de degradación de ENNs y BEA, tras el tratamiento. Así mismo, también se ha llevado a cabo la identificación de productos de degradación en las muestras de zumo de uva adicionadas con AFs con mayores porcentajes de reducción tras el tratamiento PEF. Finalmente, el servidor web ProTox-II (método de predicción *in silico*) se ha utilizado para evaluar y comparar la toxicidad de los productos de degradación identificados con la micotoxina precursora.

Varios productos de degradación de ENNS y BEA se han identificado en las matrices de zumo de uva y smoothie. Los productos de degradación observados confirman la reducción del efecto del tratamiento por PEF en las micotoxinas emergentes. Los productos de degradación se originaron por la pérdida de fragmentos aminoacídicos estructurales de las moléculas originales como ácido hidroxivalerico (HyLv), Valina (Val), Isoleucina (Ile) o fenilalanina (Phe). Para BEA, se ha identificado el producto de degradación con m/z 517.3705 que corresponde a BEA con la pérdida de una unidad de Phe y HyLv. En un estudio previo, Meca et al. (2012a) también observaron un producto de degradación de BEA con la pérdida de estos dos componentes estructurales (Phe + HyLv). Para ENNA, se ha identificado el producto de degradación m/z 475.3261, que corresponde a la pérdida de una unidad de Ile y HyLv. Este producto de degradación también fue indicado previamente por Serrano et al., (2013). Para ENNA1, se ha detectado el producto de degradación m/z 475.3244, correspondiente a la pérdida de Val y HyLv. Para ENB, se han observado dos productos de degradación. El producto de degradación 1 (m/z 527.2000) corresponde a la pérdida de Val y fue previamente identificado por Serrano et al., (2013). El producto de degradación 2 (m/ z 437.1936), se caracteriza como el aducto de sodio de ENNB, con la pérdida de Val y HyLv. Finalmente, para ENNB1, el producto de degradación m/z 443.1674 se ha identificado como la pérdida de Val y HyLv.

Para las AFs, se ha identificado un producto de degradación de AFB2 en zumo tras el tratamiento por PEF con m/z 355.0711, correspondiéndose con una de las muestras con mayor reducción después del tratamiento (aproximadamente 72%). El producto m/z 355.0711 se puede originar debido la adición de grupos de reacción OH^- y H^+ a los dobles enlaces de la estructura

de AFB2 y la pérdida del grupo metileno (-CH_2). Wang et al. (2015) observaron previamente un producto de degradación similar para AFB1.

Respecto a los productos de degradación de ENNs y BEA, el servidor web ProTox-II, de acuerdo con las predicciones obtenidas, ha asignado una toxicidad aguda oral DL50 de 3 mg / kg, con un 100% de similitud promedio y precisión de predicción, para ambos productos de degradación de ENNB identificados, asignándolos en la categoría de toxicidad I. El resto de los productos de degradación se clasifican en las categorías III y IV. Según este resultado, se debe prestar especial atención a los productos de degradación de ENNB identificados, ya que su toxicidad prevista es comparable a la de ENNB y también a la de la toxina T-2. Con respecto a la toxicidad calculada para diversos puntos toxicológicos, los resultados obtenidos muestran que los productos de degradación identificados se predicen como compuestos inactivos para hepatotoxicidad. Sin embargo, el producto de degradación identificado para ENNB1 se predice como un compuesto activo desde el punto de vista inmunotoxico, aunque el porcentaje de precisión es bajo (51%).

El producto de degradación identificado para AFB2 se ha clasificado en la categoría de toxicidad IV por el servidor web ProTox-II, siendo menos tóxico que su compuesto original. Además, ha demostrado ser "inactivo" para carcinogenicidad, hepatotoxicidad y mutagenicidad, con un 62%, 72% y 70% de probabilidad, respectivamente. Por otro lado, se predice que este producto de degradación se trata de un compuesto inmunotóxico con un 99% de probabilidad.

Los resultados de predicción obtenidos para las rutas toxicológicas, las rutas de señalización del receptor nuclear y las rutas de respuesta al estrés revelan

que los productos de degradación identificados en la presente tesis se predicen como inactivos para todas las vías analizadas.

4.8. Evaluación de la bioaccesibilidad y la estabilidad de ENNB durante la digestión

Se ha evaluado la estabilidad de ENNB en el tracto gastrointestinal humano durante la digestión. Para ello se ha simulado la digestión *in vitro* y la fermentación colónica. Para evaluar la digestión *in vitro* de ENNB, se han aplicado los protocolos Minekus (2014) y Versantvoort (2005) en paralelo a una muestra de harina de trigo libre de micotoxinas y enriquecida con una cantidad de ENNB de 5 µg para el protocolo Versantvoort y de 6.16 µg para el protocolo Minekus con el fin de alcanzar una concentración de 500 µg/l en la solución de la digestión final. La fermentación fecal de ENNB se ha realizado de acuerdo con el protocolo de Dall'Erta et al. (2013).

Tras la aplicación de ambos protocolos de digestión no se observan diferencias significativas, con una tasa de degradación global de 78.45 ± 5.39%. El posible efecto de la microbiota intestinal también se ha evaluado, para ello la ENNB se ha incubado en lodos fecales durante 30 min y 24 h comparando los resultados obtenidos con los observado en el control. En comparación con el control, la ENNB muestra una tasa de degradación significativa después de 30 min, alcanzando el 70% a las 24 h de estudio. Por lo que los datos obtenidos reflejan una baja estabilidad de ENNB en condiciones gastrointestinales *in vitro,* sugiriendo una degradación significativa del compuesto original a lo largo del tracto gastrointestinal. En estudios previos, Serrano et al. (2014) informaron

valores de bioaccesibilidad en fórmulas infantiles durante la digestión duodenal, del 1.43% para ENNB1, 0.37% para ENNA y 22.41% para ENNA1, respectivamente, correspondiéndose con una elevada degradación como la observada en la presente tesis. Por el contrario, mayor bioaccesibilidad fue reportada por Prosperini et al. (2013), con valores observados para ENNB, ENNB1, ENNA y ENNA1 en el rango 40.4–79.9%, en muestras de cereales de desayuno, galletas y pan. Estas diferencias de resultados pueden deberse a condiciones de digestión *in vitro* no estandarizadas, a menudo aplicadas a diferentes productos comerciales y con distinta composición de la matriz. Además, se ha demostrado que el pH y el contenido de fibra también pueden modular la bioaccesibilidad (Meca et al., 2012b; Serrano et al., 2014; Manzini et al., 2015). En particular, un pH más bajo puede resultar en una degradación más eficaz de la matriz, y por lo tanto en una mayor bioaccesibilidad de xenobióticos y compuestos bioactivos. Por otro lado, la fibra puede disminuir la tasa de absorción de nutrientes debido al efecto mecánico de atrapamiento. En resumen, nuestros resultados sugieren que el paso preliminar de degradación de ENNB puede ocurrir a nivel presistémico. El compuesto padre junto con sus metabolitos presistémicos, una vez absorbidos, pueden ingresar al hígado donde el metabolismo extenso tiene lugar. Finalmente, la cantidad de ENNB que no se absorbe en el tracto gastrointestinal, debido a la retención por parte de la matriz, puede liberarse y biotransformarse aún más en el intestino por parte de las bacterias del colon.

4.9. Productos de degradación y metabolitos catabólicos de ENNB tras la digestión gastrointestinal

Tras la digestión in *vitro* por los protocolos Minekus y Versantvoort, las muestras digeridas se han sometido a un análisis mediante cromatografía líquida acoplada a la espectrometría de masas de alta resolución Orbitrap (UHPLC-HRMS) para identificar los productos de degradación y metabolitos catabólicos de ENNB durante la digestión.

Un total de 5 metabolitos catabólicos nombrados M1-M5, según el tiempo de retención, han sido observados en ambos protocolos. La mayoría de los metabolitos se han eluido antes de la ENNB usando una columna de fase inversa, lo que indica que son más hidrófilos que el compuesto original. Todos los productos de degradación identificados pueden atribuirse a la oxidación y apertura del anillo depsipéptido (metabolitos # M4 y # M5), seguidos de una fragmentación adicional de la estructura alargada (metabolitos # M1, # M2 y # M3). El compuesto # M4 ya fue descrito anteriormente por Ivanova et al. (2011) y Fraeyman et al. (2016) como un producto de degradación importante para ENNB. En la presente tesis, sin embargo, el principal producto de degradación es # M1, que puede originarse debido a la degradación de los metabolitos reportados, así como del compuesto original.

Adicionalmente, se ha estudiado el perfil toxicológico de los metabolitos catabólicos identificados. Para ello se ha empleado la herramienta Swiss ADME de análisis *in silico* para predecir el ADMET (Absorción, Distribución, Metabolismo, Excreción y Toxicidad) de ENNB y sus metabolitos catabólicos identificados. La herramienta predice la absorción intestinal humana (HIA) y la penetración a través de la barrera hematoencefálica (BBB), además de clasificar

los compuestos que interactúan con la Glucoproteína-P (Pgp) (bomba de flujo de salida de xenobióticos dependiente de ATP) y los inhibidores de las isoformas del citocromo P450: CYP1A2, CYP2C9, CYP2C19, CYP2D6 y CYP3A4, así como los sustratos del metabolismo por parte de las isoformas CYP2C9, CYP2D6 y CYP3A4.

El modelo indica que probablemente los metabolitos # M4 y # M5 no se absorban a nivel gastrointestinal, mientras que el compuesto original y # M1, # M2 y # M3 podrían absorberse fácilmente dentro del tracto intestinal humano, ingresando al sistema circulatorio y, por lo tanto, distribuirse a tejidos y órganos diana. Sin embargo, se predice que ENB y # M2 interactuarían con la glucoproteína-P (Pgp), llevando a una menor acumulación en las células. Se predice que # M1 y # M3, posiblemente sean capaces de cruzar la barrera hematoencefálica (BBB), especialmente en consideración de su improbable interacción con la P- glucoproteína. En el caso del metabolismo, la posible interacción de los productos de ENNB con varias isoformas del citocromo P450 se ha evaluado *in silico*, mostrando un patrón diferente para la ENNB y sus metabolitos. Mientras que se sabe que ENNB sufre metabolismo relacionado con CYP, ninguno de sus metabolitos catabólicos actúa como inhibidor de CYP1A2, CYP3A4, CYP2D6 o CYP2C9, pero es probable que # M1, # M2, # M4 y # M5 inhiban CYP2C19. CYP2C19 es una enzima hepática responsable del metabolismo en el hígado de al menos el 10% de los fármacos (Flockhart, 2007).

4.10. Referencias

- Abdel-Sater, M.A., Zohri, A.A., & Ismail, M.A. (2001). Natural contamination of some Egyptian fruit juices and beverages by mycoflora and mycotoxins. J*ournal of Food Science and Technology-MYSORE* 38: 407–411.

- Ariño, A., Herrera, M., Estopañan, G., & Juan, T. (2007). High levels of ochratoxin A in licorice and derived products. *International journal of food microbiology* 114(3): 366-369. https://doi.org/10.1016/j.ijfoodmicro.2006.08.005

- Arroyo-Manzanares, N., García-Campaña, A. M., & Gámiz-Gracia, L. (2013). Multiclass mycotoxin analysis in Silybum marianum by ultra high performance liquid chromatography–tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid–liquid microextraction. *Journal of Chromatography A* 1282: 11-19. https://doi.org/10.1016/j.chroma.2013.01.072

- Asadi, M. (2018). Determination of ochratoxin A in fruit juice by high-performance liquid chromatography after vortex-assisted emulsification microextraction based on solidification of floating organic drop. *Mycotoxin research* 34: 15–20. https://doi.org/10.1007/s12550-017-0294-x.

- Avsaroglu, M. D., Bozoglu, F., Alpas, H., Largeteau, A., & Demazeau, G. (2015). Use of pulsed-high hydrostatic pressure treatment to decrease patulin in apple juice. *High pressure research*, 35(2): 214-222. https://doi.org/10.1080/08957959.2015.1027700.

-Carballo, D., Pinheiro-Fernandes-Vieira, P., Tolosa, J., Font, G., Berrada, H. & Ferrer, E. (2018). Dietary exposure to mycotoxins through fruits juice consumption. *Revista Toxicologia*. 35: 2–6.

-Cotterill, J. V., Chaudhry, M. Q., Matthews, W., & Watkins, R. W. (2008). *In silico* assessment of toxicity of heat-generated food contaminants. *Food and Chemical Toxicology*, 46(6): 1905-1918. https://doi.org/10.1016/j.fct.2008.01.030.

-Dall'Erta, A., Cirlini, M., Dall'Asta, M., Del Rio, D., Galaverna, G., & Dall'Asta, C. (2013). Masked mycotoxins are efficiently hydrolyzed by human colonic microbiota releasing their aglycones. *Chemical Research in Toxicology* 26: 305–312. https://doi.org/10.1021/tx300438c.

-Di Mavungu, J. D., Monbaliu, S., Scippo, M. L., Maghuin-Rogister, G., Schneider, Y. J., Larondelle, Y., Callebaut, A., Robbens, J., Van Peteghem, C., & De Saeger, S. (2009). LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. *Food additives and contaminants* 26(6): 885-895. https://doi.org/10.1080/02652030902774649

-EC 2002. European Commission (2002). Decision 2002/657/EC of 12 August 2002, implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union*. L221: 8−36.

-EC 2006a. European Commission (2006). Regulation (EC) 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*. L364, 20.12.2006, pp. 5-24.

- EFSA (2002). Report of Experts Participating in Task 3.2.8. Assessment of Dietary Intake of Patulin by the Population of EU Member States. Available online:https://ec.europa.eu/food/sites/food/files/safety/docs/cs_contaminants_catalogue_patulin_3.2.8_en.pdf

-EFSA (2006). Opinion of the Scientific panel on contaminants in the food chain on a request from the commission related to Ochratoxin A in food. *EFSA Journal* 365: 1 – 56.

-EFSA (2010). Management of left-censored data in dietary exposure assessment of chemical substances. *EFSA Journal* 8(3): 1557.

- EFSA (2014). Scientific Opinion on the risks for human and animal health related to the presence of modified forms of certain mycotoxins in food and feed. *EFSA Journal* 12(12): 3916-4023.

- Fernández-Cruz, M.L., Mansilla, M.L., & Tadeo, J.L. (2010). Mycotoxins in fruits and their processed products: Analysis, occurrence and health implications. *Journal of advanced research* 1: 113–122. https://doi.org/10.1016/j.jare.2010.03.002.

- Food and Agriculture Organization (FAO). 2013. *Food Balance Sheets*. http://www.fao.org/faostat/en/#data/FBS.

- Flockhart, D.A. (2007). Drug Interactions: Cytochrome P450 Drug Interaction Table. Indiana University School of Medicine. https://drug-interactions.medicine.iu.edu.

-Fraeyman, S., Devreese, M., Antonissen, G., De Baere, S., Rychlik, M., & Croubels, S. (2016). Comparative oral bioavailability, toxicokinetics, and biotransformation of enniatin B1 and enniatin B in broiler chickens. *Journal of agricultural and food chemistry*. 64 (38): 7259–7264. https://doi.org/10.1021/acs.jafc.6b02913.

-Hao, H., Zhou, T., Koutchma, T., Wu, F., & Warriner, K. (2016). High hydrostatic pressure assisted degradation of patulin in fruit and vegetable juice blends. *Food control*, 62: 237-242. https://doi.org/10.1016/j.foodcont.2015.10.042.

-Hu, L., Gastl, M., Linkmeyer, A., Hess, M., & Rychlik, M. (2014a). Fate of enniatins and beauvericin during the malting and brewing process determined by stable isotope dilution assays. *LWT-Food Science and Technology* 56: 469-477. https://doi.org/10.1016/j.lwt.2013.11.004.

- Hu, L., & Rychlik, M. (2014b) Occurrence of enniatins and beauvericin in 60 Chinese medicinal herbs. *Food Additives & Contaminants: Part A* 31: 1240-1245. https://doi.org/10.1080/19440049.2014.913813.

- Iha MH, & Trucksess MW. (2010) Aflatoxins and ochratoxin A in tea prepared from naturally contaminated powdered ginger. *Food Additives and Contaminants* 27: 1142-1147. https://doi.org/10.1080/19440041003795319.

-Ivanova, L., Faste, C.K., & Uhlig, S. (2011). In vitro phase I metabolism of the depsipeptide enniatin B. *Analytical and bioanalytical chemistry* 400 (9): 2889–2901. https://doi.org/10.1007/s00216-011-4964-9.

- Kalagatur, N. K., Kamasani, J. R., Mudili, V., Krishna, K., Chauhan, O. P., & Sreepathi, M. H. (2018). Effect of high pressure processing on growth and mycotoxin production of *Fusarium* graminearum in maize. *Food bioscience* 21: 53-59. https://doi.org/10.1016/j.fbio.2017.11.005.

-Kong, W.J., Shen, H.H., Zhang, X.F., Yang, X.L., Qiu, F., Ou-yang, Z., & Yang, M.H. (2013) Analysis of zearalenone and α-zearalenol in 100 foods and medicinal plants determined by HPLC-FLD and positive confirmation by LC-

MS-MS. *Journal of the Science of Food and Agriculture* 93: 1584- 1590. https://doi.org/10.1002/jsfa.5926.

- Liu, L., Jin, H., Sun, L., Ma, S., & Lin, R (2012). Determination of Aflatoxins in Medicinal Herbs by High-performance Liquid Chromatography–Tandem Mass Spectrometry. *Phytochemical Analysis* 23: 469-476. https://doi.org/10.1002/pca.2343.

-Ministry of Agriculture, Food and Environment (MAGRAMA). 2013. *Data on food consumption, Tea consumption. Statistics database-home consumption*. Madrid, Spain. Available: http://www.mapama.gob.es/

- Ministry of Agriculture, Fisheries and Food (MAPAMA). (2017). *Data on Food Consumption, Tea Consumption. Statistics Database-home Consumption*. Madrid, Spain. Available: http://www. mapama.gob.es/.

- Ministry of Agriculture, Fisheries and Food (MAPAMA). (2017). *Data on Food Consumption, Fruit Juices. Statistics Database-Home Consumption*. Madrid, Spain. Available: https://www.mapa.gob.es/app/consumo-en-hogares/consulta10.asp.

- Ministry of Agriculture, Food and Environment. (MAGRAMA). (2018). *Data on Food Consumption*. Madrid, Spain. Available: http://www. mapama.gob.es/.

-Manzini, M., Rodriguez-Estrada, M.T., Meca, G., & Manes, J. (2015). Reduction of beauvericin and enniatins bioaccessibility by prebiotic compounds, evaluated in static and dynamic simulated gastrointestinal digestion. *Food Control*. 47: 203–211. https://doi.org/10.1016/j.foodcont.2014.07.016.

-Meca, G., Ritieni, A., & Mañes, J. (2012a). Influence of the heat treatment on the degradation of the minor Fusarium mycotoxin beauvericin. *Food Control*, *28*(1): 13-18. https://doi.org/10.1016/j.foodcont.2012.04.016.

-Meca, G., Giri, A., Sagratini, G., Vittori, S., Font, G. & Manes, J. (2012b). The soluble dietary fiber inulin can influence the bioaccessibility of enniatins. *Food & Function* 3 (8): 853–858. https://doi.org/10.1039/C2FO00004K.

-Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D. J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G. E., Wickham, M.S.J., Weitschies, W., & Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food–an international consensus. *Food & Function* 5 (6): 1113–1124. https://doi.org/10.1039/C3FO60702J.

- Monbaliu, S., Wu, A., Zhang, D., Van Peteghem, C., & De Saeger, S. (2010). Multimycotoxin UPLC− MS/MS for tea, herbal infusions and the derived drinkable products. *Journal of Agricultural and Food Chemistry* 58: 12664-12671. https://doi.org/10.1021/jf1033043.

- Prosperini, A., Meca, G., Font, G., & Ruiz, M.J., (2013). Bioaccessibility of enniatins A, A1, B, and B1 in different commercial breakfast cereals, cookies, and breads of Spain. *Journal of agricultural and food chemistry*. 61 (2): 456–461. https://doi.org/10.1021/jf3044023.

- Qiao, X., Yin, J., Yang, Y., Zhang, J., Shao, B., Li, H., & Chen, H. (2018). Determination of Alternaria Mycotoxins in Fresh Sweet Cherries and Cherry-

Based Products: Method Validation and Occurrence. *Journal of agricultural and food chemistry 66*: 11846–11853. https://doi.org/10.1021/acs.jafc.8b05065

- Rahimi, E., & Rezapoor Jeiran, M. (2015). Patulin and its dietary intake by fruit juice consumption in Iran. *Food Additives & Contaminants: Part B* 8: 40–43. https://doi.org/10.1080/19393210.2014.967814.

- Reinholds, I., Bogdanova, E., Pugajeva, I., Bartkevics, V. (2019). Mycotoxins in herbal teas marketed in Latvia and dietary exposure assessment. *Food Additives & Contaminants: Part B* 12(3): 199-208. https://doi.org/10.1080/19393210.2019.1597927

- Rodríguez-Carrasco, Y., Mañes, J., Berrada, H., & Juan, C. (2016). Development and validation of a LC-ESI-MS/MS method for the determination of Alternaria toxins alternariol, alternariol methyl-ether and tentoxin in tomato and tomato-based products. *Toxins 8*: 328. https://doi.org/10.3390/toxins8110328.

- Ryu, D., Hanna, M.A., Eskridge, K.M., Bullerman, L.B. (2003). Heat stability of zearalenone in an aqueous buffered model system. *Journal of agricultural and food chemistry* 51: 1746-1748. https://doi.org/10.1021/jf0210021.

- Santos, L., Marín, S., Sanchis, V., & Ramos, A.J. (2009) Screening of mycotoxin multicontamination in medicinal and aromatic herbs sampled in Spain. *Journal* of the *Science* of *Food* and *Agriculture* 89: 1802-1807. https://doi.org/10.1002/jsfa.3647.

-Scientific 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 by the SCF on 26 February 2002). Report SCF/CS/ CNTM/MYC/27; European Commission Scientific Committee on Food: Brussels, Belgium.

-Serrano, A. B., Meca, G., Font, G., & Ferrer, E. (2013). Degradation study of enniatins by liquid chromatography–triple quadrupole linear ion trap mass spectrometry. *Food chemistry 141*(4): 4215-4225. https://doi.org/10.1016/j.foodchem.2013.05.030.

-Serrano, A.B., Meca, G., Font, G., Ferrer, E. (2014). Risk assessment of beauvericin, enniatins and fusaproliferin present in follow-up infant formula by in vitro evaluation of the duodenal and colonic bioaccessibility. *Food Control* 42: 234–241. https://doi.org/10.1016/j.foodcont.2014.02.014.

- Serrano, A.B., Font, G., Mañes, J., & Ferrer, E. (2016) Development a mitigation strategy of enniatins in pasta under home-cooking conditions. *LWT-food science and technology* 65: 1017- 1024. https://doi.org/10.1016/j.lwt.2015.09.036.

-Tokuşoğlu, Ö., Alpas, H., & Bozoğlu, F. (2010). High hydrostatic pressure effects on mold flora, citrinin mycotoxin, hydroxytyrosol, oleuropein phenolics and antioxidant activity of black table olives. *Innovative food science & emerging technologies* 11(2): 250-258. https://doi.org/10.1016/j.ifset.2009.11.005.

- Torović, L., Dimitrov, N., Assunção, R., Alvito, P. (2017). Risk assessment of patulin intake through apple-based food by infants and preschool children in Serbia. *Food Additives & Contaminants: Part A 34*, 2023–2032. https://doi.org/10.1080/19440049.2017.1364434.

-Veprikova, Z., Zachariasova, M., Dzuman, Z., Zachariasova, A., Fenclova, M., Slavikova, P., Vaclavikova, M., Mastovska, K., Hengst, D., Hajslova, J. (2015). Mycotoxins in plant-based dietary supplements: Hidden health risk for consumers. *Journal of agricultural and food chemistry* 63(29): 6633-6643. https://doi.org/10.1021/acs.jafc.5b02105.

-Versantvoort, C.H., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J., & Sips, A.J., (2005). Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. *Food and Chemical Toxicology*. 43 (1): 31–40. https://doi.org/10.1016/j.fct.2004.08.007.

-Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., Kumar, S & Reddy, R. (2017). Effect of combination processing on aflatoxin reduction: process optimization by response surface methodology. *Journal of Food Processing and Preservation 41*(6): e13230. https://doi.org/10.1111/jfpp.13230.

-Vijayalakshmi, S., Nadanasabhapathi, S., Kumar, R., & Kumar, S. S. (2018). Effect of pH and pulsed electric field process parameters on the aflatoxin reduction in model system using response surface methodology. *Journal of food science and technology 55*(3): 868-878. https://doi.org/10.1007/s13197-017-2939-3.

-Wang, S. Q., Huang, G. Q., Li, Y. P., Xiao, J. X., Zhang, Y., & Jiang, W. L. (2015). Degradation of aflatoxin B 1 by low-temperature radio frequency plasma and degradation product elucidation. *European Food Research and Technology*, 241(1): 103-113. https://doi.org/10.1007/s00217-015-2439-5.

-Zwickel, T., Klaffke, H., Richards, K., & Rychlik, M. (2016). Development of a high performance liquid chromatography tandem mass spectrometry based analysis for the simultaneous quantification of various Alternaria toxins in wine, vegetable juices and fruit juices. *Journal of Chromatography A 1455*: 74–85. https://doi.org/10.1016/j.chroma.2016.04.066.

5.CONCLUSIONS

5. CONCLUSIONES

1. Los niveles de micotoxinas emergentes de *Fusarium* en plantas medicinales suelen ser bajos, con algunas excepciones, que superan valores de 1000 μg/kg. A pesar de sus efectos perjudiciales, algunos estudios están investigando la posible aplicación de las micotoxinas emergentes en la terapia anticancerosa y como antimicrobianos e insecticidas.
2. La revisión bibliográfica muestra que las tecnologías no térmicas de procesado de los alimentos, Altas Presiones Hidroestáticas (HPP) y Pulsos Eléctricos de Alta Intensidad (PEF) constituyen una herramienta prometedora para reducir las micotoxinas en diferentes alimentos, ya sea reduciendo los hongos micotoxigénicos o disminuyendo la cantidad de micotoxinas.
3. La validación de la metodología analítica para la determinación de micotoxinas ha permitido el análisis multimicotoxina en plantas medicinales y tés en crudo, cápsulas o infusiones listas para su consumo, y en zumo.
4. AFs, ZEA, ENNB y ENNB1 han sido las micotoxinas más detectadas en las matrices de plantas medicinales y tés, con contenidos más elevados en la materia en crudo y en las cápsulas que en las infusiones resultantes con niveles inferiores a 100 μg/L.
5. En los zumos, las micotoxinas más detectadas han sido AOH y PAT, con incidencias del 29 y 18% respectivamente y concentraciones medias de las muestras positivas de 207 y 28.18 μg/L, respectivamente.

6. La evaluación de la exposición a micotoxinas revela un riesgo potencial muy bajo para la salud a través del consumo de productos botánicos, y un riesgo potencial bajo para niños por el consumo de zumos.
7. El proceso térmico de infusionado produce reducciones en el contenido de micotoxinas en un rango entre el 74% al 100%, observándose una baja tendencia de las micotoxinas a migrar desde la materia prima a las infusiones resultantes.
8. Las tecnologías no térmicas de procesado de los alimentos (HPP y PEF) producen reducciones en los contenidos de micotoxinas en un rango entre un 8 al 84%.
9. Se han identificado varios productos de degradación de micotoxinas emergentes y AFB2 tras el tratamiento por PEF. La aplicación de metodología *in silico* (Prot Tox II) muestra para la mayoría de ellos una menor toxicidad que para los compuestos originales.
10. El estudio *in vitro* de la estabilidad de ENNB en el tracto gastrointestinal humano refleja una baja estabilidad de ENNB, sugiriendo una degradación significativa del compuesto original a lo largo del tracto gastrointestinal al identificarse 5 productos de degradación.

5. CONCLUSIONS

1. The bibliographic review about the presence of emerging *Fusarium* mycotoxins in medicinal plants shows low levels in general. However, in some occasions levels higher than 1000 μg/kg have been observed in raw materials. Despite the attributed harmful health effects, some studies are exploring the possible application of emerging mycotoxins in antigenic therapy and as antimicrobials and insecticides.
2. The bibliographic review on the effect of non-thermal food processing technologies HPP and PEF, reveals that these treatments can be a promising tool to reduce mycotoxins in several food products, either by reducing mycotoxigenic fungi or by reducing the amount of mycotoxins.
3. The validation of the analytical methodology has allowed the multimycotoxin analysis in medicinal plants and teas as raw materials, tablets, beverages and juices.
4. AFs, ZEA, ENNB and ENNB1 have been the most detected mycotoxins in medicinal plant and teas matrices, with contents that were higher in raw materials and capsules than in the resulting beverages, where in general the contents do not exceed 100 μg / L.
5. For juices, the most detected mycotoxins are AOH and PAT, with incidences of 29 and 18%, respectively, and with mean concentrations of positive samples of 207 and 28.18 μg / L, respectively.
6. Mycotoxin exposure assessment through consumption of botanicals do not reveal an appreciable health risk, while exposure through consumption of juices may represent a potential risk in children considering a 200 mL of juice intake.

7. The thermal infusion process produces reductions in the mycotoxins contents in a range of 74% to 100%, observing a low tendency of mycotoxins to migrate from the raw materials to the resulting beverages.
8. Non-thermal food processing technologies (HPP and PEF) cause reductions in mycotoxin contents in a range of 8 to 84%.
9. Several emerging mycotoxins and AFB2 degradation products have been identified after PEF treatment, most of which are predicted to be less toxic than the original compounds using the Pro Tox II server.
10. The *in vitro* study of ENNB stability in the human gastrointestinal tract reflects a low stability of ENNB, suggesting a significant degradation of the original compound throughout the gastrointestinal tract by identifying 5 degradation products.

ANNEX

Anexo 1. International Journal of Food Science and Technology (under review)

Mycotoxins occurrence in herbal medicines dietary supplements and exposure assessment

Table S1. Description of the analyzed samples.

Main health effect	Assigned number	Ingredients	Tablets recommended dosage (total g)
Containing one herbal ingredient			
Control cholesterol, protect liver, detox, weight loss	1 2 (ecologic) 3 4	Artichoke (*Cynara scolymus)*	6-9 (2.4-3.6 g) 2 (1.01 g) 4 (0.84 g) 2-3 (1.2-1.8 g)
	5 (ecologic) 6 7 8	Boldus *(Peumus boldus)*	2 (0.95 g) 4 (2.4 g) 4 (2g) 4 (2g)
	9 10 (ecologic) 11 12	Cardus Marianus *(Silybum marianum)*	3-6 (1.5-3g) 2 (1g) 3 (1.4 g) 3 (1.68 g)
	13 14 15 16 17	Dandelion *(Taraxacum officinale)*	3 (1.5 g) 4 (2 g) 2 (1.38 g) 4-6 (2-3 g) 2-3 (0.9- 1.35)

Maintain mobility and joint flexibility	18 19 20 21	Devil's clawroot *(Harpagophytum procumbens)*	2-3 (1-1.5 g) 2 (1.02 g) 1-2 (0.47-0.98 g) 3 (1.2 g)
Anti-inflamatory	22 (ecologic) 23 24 25	Ginger *(Zingiber officinale)*	2 (1.2 g) 4 (1.84 g) 2 (1.12 g) 3 (1.8 g)
	26 (ecologic) 27 28 29	Ginkgo *(Ginkgo biloba)*	2 (0.96 g) 2 (0.9g) 2 (0.75 g) 1-2 (0.47- 0.95 g)
Detox, weight loss, reduce blood sugar levels	30 31 (ecologic) 32 33 34	Green tea *(Camellia sinensis)*	6 (2.4 g) 2-3 (0.94-1.41 g) 3 (1.83 g) 3 (1.6 g) 4 (2.62 g)
	35 (ecologic) 36 37 38	Red tea *(Aspalathus linearis)*	2-3 (1.14- 1.71 g) 2-6 (0.7- 2.1) 4 (2 g) 6-9 (3-4.5 g)

	39 (ecologic) 40 41 42 43	Fucus *(Fucus vesiculosus)*	2 (0.55 g) 2 (0.7 g) 1-2 (0.47-0.948 g) 6-9 (3-4.5 g) 4 (1.33 g)
Diuretic, improve skin and nails conditions, strengthen bones and tendons	44 45 46 (ecologic) 47 48	Horsetail *(Equisetum arvense L.)*	3-6 (1.5-3 g) 3 (1.8 g) 2 (1.5 g) 3-6 (1.5-3 g) 6 (1.35 g)
Reduce anxiety, stress and insomnia	49 50 51	Lemon balm (*Melissa officinalis)*	3 (1.05 g) 2 (0.8 g) 2 (0.94 g)
	52 (ecologic) 53 54 55	Passionflower *(Passiflora incarnata L.)*	2 (1.14 g) 3 (1.11 g) 2-4 (1.12-2.24 g) 4 (2.4 g)
	56 57 58 59 60	Valerian *(Valeriana officinalis)*	3 (1.05 g) 3 (1.05 g) 3 (1.5 g) 4 (1.4 g) 4 (2 g)
Regulate blood pressure, regulate heart rate, control cholesterol	61 62 63 64	Whitethorn *(Crataegus monogyna*)	2-3 (0.8-1.2 g) 1-2 (0.47-0.94 g) 3 (0.9 g) 4-6 (1.6-2.4 g)

Containing more than one herbal ingredient			
Treat insomnia	65	Californian tail, californian poppy, hop, whitethorn	2 (1.2 g)
	66	Lemon balm, orange flower, valerian	3 (1.2 g)
	67	Californian poppy, passionflower, lemon balm, linden, valerian	1 (0.54 g)
	68	Californian poppy, passionflower, lemon balm, linden, valerian	1 (0.43 g)
	69	Passionflower, hop	1 (0.54 g)
	70	Californian poppy, passionflower, lemon balm, linden, valerian	1 (0.54 g)
	71	Lemon balm, saffron, melatonin	1 (0.66 g)
	72	Whitethorn, passionflower, valerian, rhodiola griffonia	1 (0.61 g)
	73	Passionflower, valerian, lemon balm, hop, Californian poppy	1 (0.76 g)
	74	Passionflower, Californian poppy, valerian	1 (0.51 g)
	75	Passionflower, valerian, eschscholtzia	2 (0.86 g)
	76	Lemon balm, passionflower, Californian poppy	1 (0.49 g)
	77	Lemon balm, whitethorn, vervain, Californian poppy, linden, chamomile	1 (0.48 g)
	78 (ecologic)	Lemon balm, orange flower, linden	3 (1.92 g)
	79	Passionflower, withethorn, hop, orange flower, valerian	6 (1.8 g)
	80	Californian poppy, passionflower, lemon balm, linden, valerian	2 (1.08 g)

Weight loss	81 (ecologic) 82 83 84 85	Green tea, moringa, Spirulina, algae, Chorella algae Artichoke, hop, senna, licorice, rhubarb Green tea, mate, fucus Senna, anise, fennel Fucus, green tea, mate	4 (1.91 g) 2 (0.97 g) 4 (2.06 g) 2 (0.93 g) 4 (2.06 g)

Anexo 2. Journal of Natural Products (2019)

Occurrence of Mycotoxins in Botanical Dietary Supplement Infusion Beverages

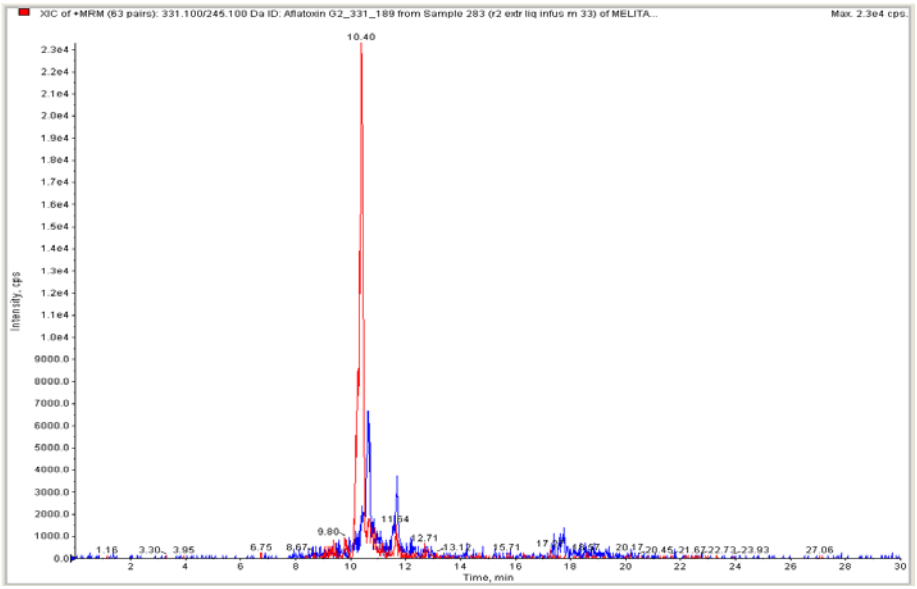
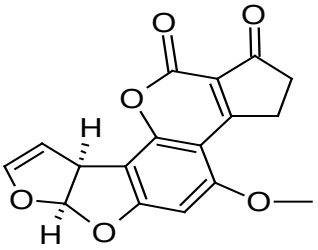
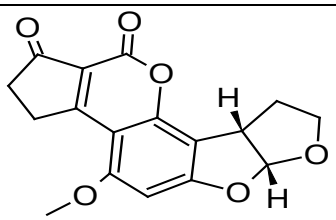
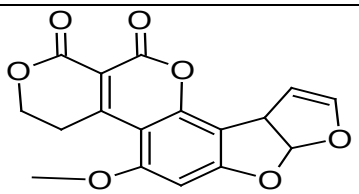
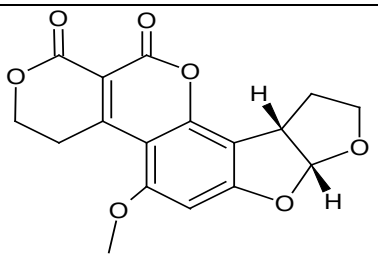
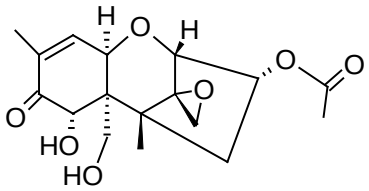
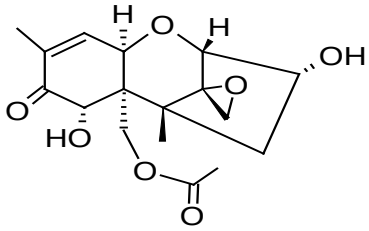
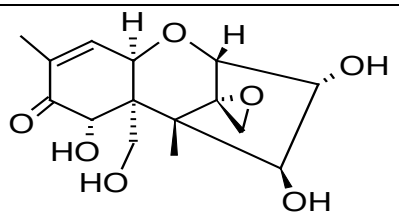
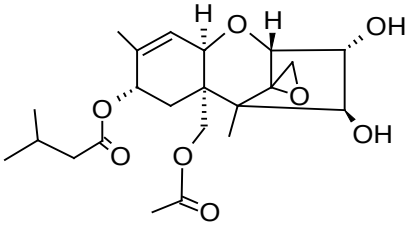
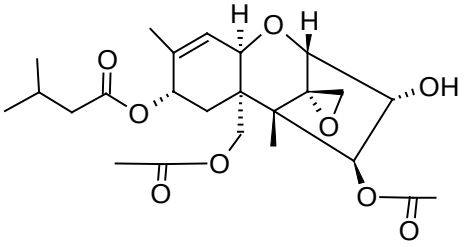
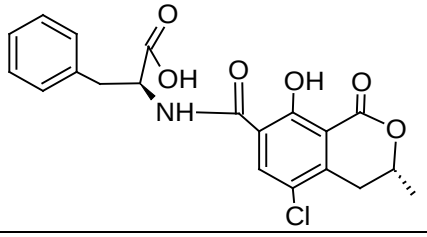
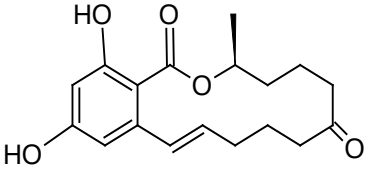


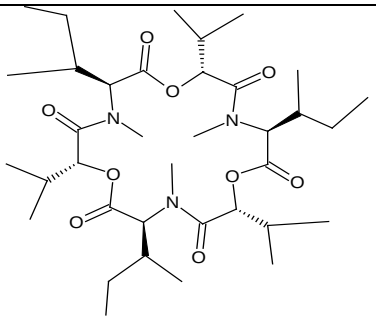
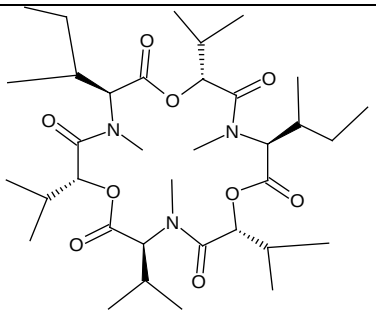
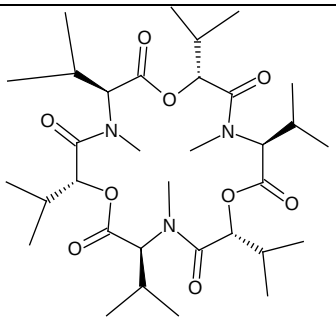
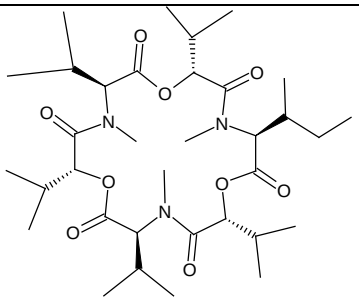
Figure S1. Chromatogram obtained from a sample of Thyme naturally contaminated by AFG2 (**4**) (6.6 µg/L) Retention Time= 10.40 min.

Table S1. Structure sheet of Mycotoxins Investigated with the numeral code and Molecular Structure.

numeral code	mycotoxin	molecular structure
1	aflatoxin B1	

2	aflatoxin B2	
3	aflatoxin G1	
4	aflatoxin G2	
5	3-acetyldeoxynivalenol (3aDON)	
6	15-acetyldeoxynivalenol (15aDON)	

7	nivalenol	
8	HT-2	
9	T-2	
10	ochratoxin A (OTA)	
11	zearalenone (ZEA)	

12	enniatin A (ENA)	
13	enniatin A1 (ENA1)	
14	enniatin B (ENB)	
15	enniatin B1 (ENB1)	

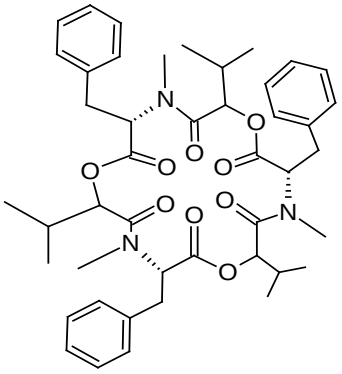
16	beauvericin (BEA)	

Table S2. Risk Assessment of Spanish and British population to Mycotoxins through the Consumption of Herbal Tea Beverages.

	spanish population				
mycotoxin	LB approach[c]				
	mean concentration (µg/L)	daily consumption L/kg bw/day)	estimate daily intakes (ng/kg bw/day)	tolerable daily intakes (ng/kg bw/day)	risk characterization (EDI/TDI)*100
15aDON (**6**)	2.16	0.00063	1.35	1000[a]	0.13%
	UB approach[c]				
	mean concentration (µg/L)	daily consumption (L/kg bw/day)	estimate daily intakes (ng/kg bw/day)	tolerable daily intakes (ng/kg bw/day)	risk characterization (EDI/TDI)*100
15aDON (**6**)	2.26	0.00063	1.42	1000[a]	0.14%
ENB (**14**)	0.06	0.00063	0.038	1000[a]	0.0038%
ENB (**14**)	0.06	0.00063	0.038	100[b]	0.038%
	british population				
mycotoxin	LB approach[c]				
	mean concentration (µg/L)	daily consumption Ll/kg bw/day)	estimate daily intakes (ng/kg bw/day)	tolerable daily intakes (ng/kg bw/day)	risk characterization (EDI/TDI)*100
15aDON (**6**)	2.16	0.0096	20.7	1000[a]	2.1%
	UB approach[c]				
	mean concentration (µg/L)	daily consumption (L/kg bw/day)	estimate daily intakes (ng/kg bw/day)	tolerable daily intakes (ng/kg bw/day)	risk characterization (EDI/TDI)*100

15aDON (**6**)	2.26	0.0096	21.7	1000[a]	2.2%
ENB (**14**)	0.06	0.0096	0.6	1000[a]	0.06%
ENB (**14**)	0.06	0.0096	0.6	100[b]	0.6%

[a] TDI DON (1 µg/kg bw/day)[27].

[b] TDI sum of T-2 and HT-2 toxins (0.1 µg/kg bw/day)[28].

[c] UB: upper bound levels, LB: lower bound levels.

Table S3. Numeral Code and Manufacturing Companies of each Botanical Sample.

numeral code	botanical sample	common name	manufacturing companies (city and country location)	voucher number
A	*Matricaria chamomilla* L.	chamomile	D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-04-141-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-04-142-17
			R.G.S.E.A.A. nº 25.00381/A (Alicante, Spain)	MUV-04-143-17
			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-04-144-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-04-145-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-04-146-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-04-147-17
			RSI 2500381 for CMI 2 (Les Ulis, France)	MUV-04-148-17
B	*Matricaria chamomilla* L.	chamomile with honey	Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-04-149-17
			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-04-150-17

			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-04-151-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-04-152-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-04-153-17
C	*Matricaria chamomilla* L.	chamomile with anise	R.G.S.E.A.A. nº 25.00381/A (Alicante, Spain)	MUV-04-154-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-04-155-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-04-156-17
			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-04-157-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-04-158-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-04-159-17
D	*Tilia europaea* L.	linden	Martínez y Cantó, S.L. (Novelda, Spain)	MUV-05-7-17
			R.G.S.E.A.A. nº 25.00381/A (Alicante, Spain)	MUV-05-8-17

			R.G.S.E.A.A. nº 25.00381/A (Alicante, Spain)	MUV-05-9-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-10-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-11-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-12-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-13-17
			RSI 2500381 for CMI 2 (Les Ulis, France)	MUV-05-14-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-05-15-17
E	*Mentha pulegium* L.	Pennyroyal with mint	Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-16-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-17-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-18-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-05-19-17
			R.G.S.E.A.A. nº 25.00381/A (Alicante, Spain)	MUV-05-20-17

			R.G.S.E.A.A. nº 25.00381/A (Alicante, Spain)	MUV-05-21-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-05-22-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-05-23-17
			RSI 2500381 for CMI 2 (Les Ulis, France)	MUV-05-24-17
			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-05-25-17
F	*Thymus vulgaris* L.	thyme	Martínez y Cantó, S.L. (Novelda, Spain)	MUV-06-14-17
			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-06-15-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-06-16-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-06-17-17
			M. David Cano Barrachina (Orihuela, Spain)	MUV-06-18-17
			M. David Cano Barrachina (Orihuela, Spain)	MUV-06-19-17
G	*Valeriana officinalis* L.	valerian	Martínez y Cantó, S.L. (Novelda, Spain)	MUV-06-20-17

			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-06-21-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-06-22-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-06-23-17
H	*Equisetum arvense* L.	horsetail	Martínez y Cantó, S.L. (Novelda, Spain)	MUV-06-24-17
			Martínez y Cantó, S.L. (Novelda, Spain)	MUV-06-25-17
			Pompadour Ibérica, S.A. (Alicante, Spain)	MUV-06-26-17
			D.E Master Blenders 1753 (Amsterdam, The Netherlands)	MUV-06-27-17

Table S4. LC-MS/MS parameters.

mycotoxin	DP[a]	precursor ion	quantification ion Q			confirmation ion q		
			CE[b]	product ion	CXP [c]	CE	product ion	CXP
AFB1(**1**)	46	313.1	39	284.9	4	41	241.0	4
AFB2 (**2**)	81	315.1	33	286.9	6	39	259.0	6
AFG1(**3**)	76	329.0	39	243.1	6	29	311.1	6
AFG2 (**4**)	61	331.1	27	313.1	6	39	245.1	4
HT2 (**8**)	21	442.2	19	262.8	4	19	215.4	8
T2 (**9**)	21	484.3	29	215.1	4	22	185.1	4
3aDON (**5**)	44	339.2	20	231.1	3	20	203.1	3
15aDON (**6**)	50	339.2	20	137.0	3	20	261.1	3
NIV (**7**)	50	313.4	80	115.1	3	27	175.1	3
OTA (**10**)	55	404.3	97	102.1	6	27	239.0	6
ZEA (**11**)	26	319.0	15	301.0	10	19	282.9	4
ENA (**12**)	76	699.4	35	210.1	14	59	228.2	16
ENA1(**13**)	66	685.4	37	210.2	8	59	214.2	10
ENB (**14**)	51	657.3	39	196.1	8	59	214.0	10
ENB1 (**15**)	66	671.2	61	214.1	10	57	228.1	12
BEA (**16**)	116	801.2	27	784.1	10	39	244.1	6

[a] DP: decluster potential (volts)

[b] CE: collision energy (volts)

[c] CXP: cell exit potential (volts)

Table S5. Analytical parameters for Method Validation (Recoveries, Intra-Day and Inter-Day Precisions, Matrix Effects and Limits of Detection and Quantification).

mycotoxin	recovery[c] ± RSD[d] (%)		SSE (%)[b]	LOD[a]	LOQ[a]
	intra-day precision	inter-day precision			
AFB1 (**1**)	114± 7	111± 10	78	0.7	2.3
AFB2 (**2**)	93± 5	88± 6	102	2.4	8.0
AFG1 (**3**)	96± 6	91± 8	91	0.7	2.4
AFG2 (**4**)	68± 9	67± 11	82	0.5	1.6
HT2 (**8**)	124 ± 5	119 ±9	87	10	33
T2 (**9**)	81± 8	84± 11	81	9	30
3aDON (**5**)	127±9	121± 12	47	8	27
15aDON (**6**)	125 ± 5	117± 9	113	0.1	0.3
NIV (**7**)	119± 11	115± 15	119	9.6	32
ZEA (**11**)	79± 7	76± 9	60	0.05	0.2
OTA (**10**)	66± 3	65± 5	69	5	17
ENA (**12**)	96± 5	93± 8	75	0.4	1.4
ENA1 (**13**)	105± 5	104± 7	78	0.2	0.7
ENB (**14**)	97± 4	94±5	107	0.05	0.2
ENB1 (**15**)	113± 6	110±9	70	0.1	0.3
BEA (**16**)	90±8	87± 12	96	1	3.2

[a] LOD and LOQ are limits of detection and quantification, respectively (µg/L).

[b] SSE: Signal Suppression- Enhancer.

[c] Recoveries: experimental data from analysis performed at concentrations of 10xLOQ.

[d] RSD: Relative standard deviation (calculated injecting samples in triplicate).

Anexo 3. Toxins (2019)

Mycotoxin Dietary Exposure Assessment through Fruit Juices Consumption in Children and Adult Population

Table S1. Description of the analyzed samples.

Mono-fruit juices (n=40)		**Blended beverages (n=40)**		
Fresh juices (n=7)	**Packed juices (n=33)**	**Ecological label (n=5)**	**Health claims label (n=24)**	**Common label (n=11)**
-Orange (n=7)	-Orange (n=3)	-Apple, mango, banana, passion fruit	**Containing herbs and spices**	-Pear, apple, pineapple, lemon
			-Apple, mango, tomato, lemon, beetroot, pepper, goji berries, pomegranate	
	-Apple (n=12)	-Apple, strawberry, raspberry, blueberries, banana	-Pumpkin, mango, carrot, passion fruit, ginger, chili extract	-Apple, pear, raspberry, strawberry, beetroot
	-Peach (n=7)	-Apple, pineapple, banana, coconut milk	-Apple, parsnip, celery, cucumber, Matcha tea, safflower	-Apple, grape, carrot, mango, lemon, passion fruit
	-Pear (n=4)	-Apple, cucumber, celery, kale, spinach, lemon, ginger	-Apple, carrot, beetroot, lemon, ginger	-Apple, carrot, beetroot, grape, lemon, strawberry, raspberry, blueberries
	-Pineapple (n=7)	-Carrot, apple, orange, ginger, lemon	-Apple, pumpkin, carrot, pineapple, lemon, ginger, cinnamon	-Pineapple, orange, apple, mango, passion fruit, grape, banana, pear, peach
			-Apple, spinach, celery, lemon, fennel, kiwi	-Apple, pineapple, mango, milk (n=6)
			-Apple, cucumber, kale, spinach, lemon, ginger, spirulina, lettuce	
			-Apple, spinach, celery, ginger, lemon	
			-Apple, Chia seeds, mango, banana, passion fruit	
			-Apple, pear, banana, cinnamon, yogurt, oats	
			-Apple, carrot, passion fruit, ginger, lemon, ginseng	

			-Apple, banana, pear, mango, spices -Pineapple, apple, banana, pineapple, orange, coconut, lemon, valerian -Apple, orange, aloe vera, raspberry, blueberries, strawberry, pomegranate -Apple, pear, grape, ginger, lemon, spinach, artichoke, sprirulina **Fiber rich** -Orange, apple, carrot, pineapple, pumpkin, citric fiber, guarana, agave syrup -Strawberry, banana, apple, beetroot, raspberry, citric fibre, pea protein, agavae syrup -Apple, pineapple, mango, banana, lettuce, coconut, carrot, corn, kiwi, citric fibre, hemp seeds -Apple, beetroot, pineapple, lemon, ginger, citric fibre **Antioxidant rich** -Apple, carrot, peach, pumpkin, ginger, vitamin C -Apple, carrot, strawberry, beetroot, vitamin C -Orange, carrot, lemon, vitamin C, E, β-carotene -Apple, carrot, beetroot, lemon, vitamin C, E	

Anexo 4. Plant Foods for Human Nutrition (2020)

Risk assessment and mitigation of the mycotoxin content in medicinal plants by the infusion process

Reagents and chemicals

Acetonitrile (ACN) and methanol (MeOH), HPLC grade, were supplied by Merck (Darmstadt, Germany). Chloroform ($CHCl_3$) (99%) was obtained from Merck (Darmstadt, Germany). Ethyl acetate (EtOAc) (HPLC grade 99,5+ %) was purchased from Alfa Aesar (Karlsruhe, Germany). Carbon tetrachloride (CCl_4) (99%) was obtained from Panreac (Barcelona, Spain). Deionized water (resistivity >18 MΩ cm-1) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore Corporation Bedford, USA). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Formic acid (reagent grade ≥ 95%) was supplied by Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride (NaCl) was supplied by VWR Chemicals (Leuven, Belgium). Anhydrous magnesium sulfate ($MgSO_4$), 99.5% min powder was obtained from Alfa Aesar (Karlsruhe, Germany). Octadecyl C18 sorbent was supplied by Phenomenex (Madrid, Spain). All solvents were filtered through a 0.45 μm cellulose filter supplied by Scharlau (Barcelona, Spain). All samples were passed through a 13 mm/0.22.μm nylon filter from Membrane Solutions (TX, USA) before injection. The AFB1, AFB2, AFG1, AFG2, ZEA, BEA, ENNA, ENNA1, ENNB and ENNB1 standards were purchased from Sigma (St. Louis, MO, USA). Individual stock solutions of each mycotoxin at a concentration of 100 mg/L were prepared in methanol. The appropriate working solutions were prepared starting from the individual stock solutions. All solutions were prepared and stored in darkness at −20°C until LC–MS/MS-IT analysis.

LC-MS/MS-IT analysis

For the LC-MS/MS analysis, an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with turbo ion spray (ESI) electrospray ionization was used. The analyzer combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. A Gemini-NX column C18 (Phenomenex, 150 mm × 4.6 mm, 5 particle size) preceded by a guard column was used for separating the analytes. The flow rate was fixed at 0.25 mL/min, and the oven temperature was set at 40°C. The two elution mobile phases employed were water acidified with 5 mM ammonium formate and 0.1% formic acid (mobile phase A) and methanol acidified with 5 mM ammonium formate and 0.1% formic acid (mobile phase B). The elution gradient started with a proportion of 0% eluent B; in 10 min, this proportion was increased to 100%, then decreased to 80% in 5 min, and finally decreased to 70% in 2 min. Then, in the next 6 min, the column was cleaned, readjusted to the initial conditions, and equilibrated for 7 min.

The analysis was performed using the turbo ion spray in positive ionization mode (ESI+). Nitrogen served as both the nebulizer and collision gas. The ion spray voltage was 5500 V; curtain gas flow was 20 arbitrary units; GS1 and GS2 were 50 and 50 psi, respectively; and a probe temperature (TEM) of 450°C was used during the analysis.

The spectrometric parameters (declustering potential, collision energy and cell exit potential) and the fragments monitored (quantification ions and confirmation ions) are shown in Table S2.

Method Validation

The DLLME method was optimized according to Pallarés et al. [24]. Intraday and interday recoveries ranged from 68±9 to 114±7% and from 67±11 to 111±16%, respectively, at the 10× LOQ level. The signal suppression-enhancer (SSE) for matrix effects was between 60 and 107%, indicating that SSE were important for ZEA (60%). Linearity was obtained with correlation coefficients (r^2) between 0.992-0.999. The LODs and LOQs calculated ranged from 0.05 μg/L (ENNB and ZEA) to 2.4 μg/L (AFB2) and from 0.2 μg/L (ENNB and ZEA) to 8 μg/L (AFB2), respectively.

For optimization of the QuEChERS extraction protocol (Table S3), the recoveries were determined by spiking triplicate blank samples with each analyzed mycotoxin at two concentration levels (10× LOQ and 100× LOQ) before and after the extraction procedure and comparing the absolute peak areas for each analyte. To assess the intraday precision, three determinations were performed on the same day and then again on nonconsecutive days to assess the interday precision. The results obtained for the 10× LOQ concentration level ranged from 62 to 101% in the case of the intraday precision and from 58 to 110% for the interday precision and were within the relative standard deviation (<20%).

Matrix effects (MEs) were assessed to evaluate their possible suppression or enhancement of the signal (SSE) and was performed by comparing the slope of the calibration curve dissolved in the blank sample with the slope of the calibration curve dissolved in methanol. SSE (%) was obtained as follows: SSE(%)= 100×slope with matrix/slope without matrix. The results obtained were great for all studied mycotoxins except for AFs, which presented a

comprising suppression of the signal (23-37%). To minimize these matrix effects, all analytical parameters were determined using matrix matched calibration curves.

To determine the linear correlation coefficients (r^2), two calibration curves were constructed, one in methanol and the other in the blank sample at concentration points ranging from the LOQ of each mycotoxin to 1000 μg/kg. The r^2 values obtained (0.990- 0.999) showed great linearity for all studied mycotoxins.

The LODs and LOQs were calculated by spiking a blank sample with decreasing concentrations of the studied mycotoxins using the criterion of S/N ≥ 3 for calculating the LOD and S/N ≥ 10 for the LOQ. The LODs obtained ranged from 0.1 to 5 μg/kg, and the LOQs ranged from 0.3 to 17 μg/kg.

Tables and Figures

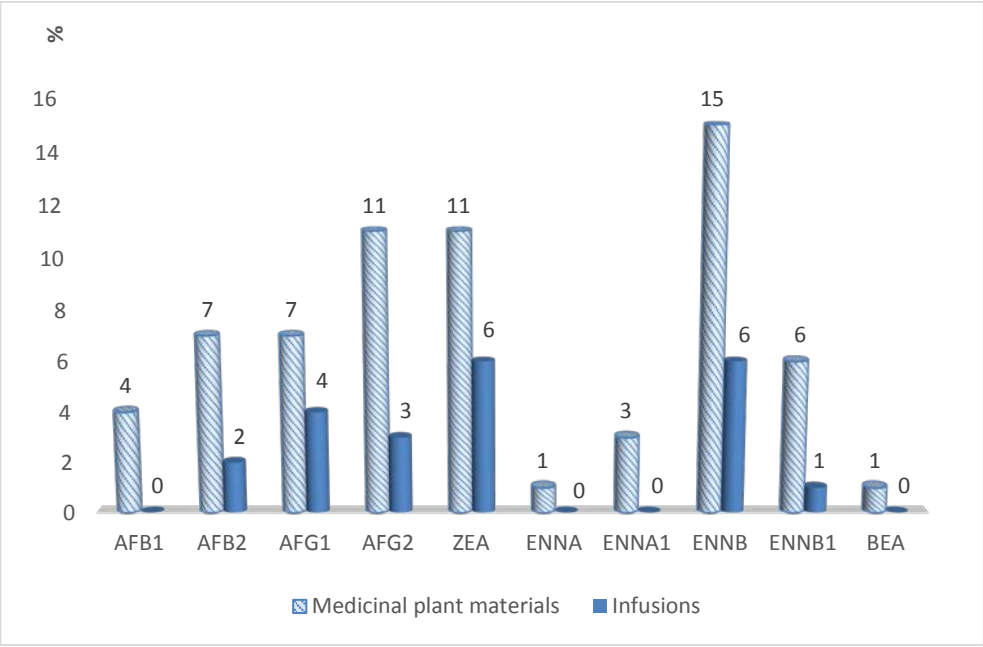


Figure S1. Incidence of mycotoxins in medicinal plant materials *vs* infusions (%). Aflatoxin B1 (AFB1); aflatoxin B2 (AFB2); aflatoxin G1 (AFG1); aflatoxin G2 (AFG2); zearalenone (ZEA); enniatin A (ENNA); enniatin A1 (ENNA1); enniatin B (ENNB); enniatin B1 (ENNB1); beauvericin (BEA).

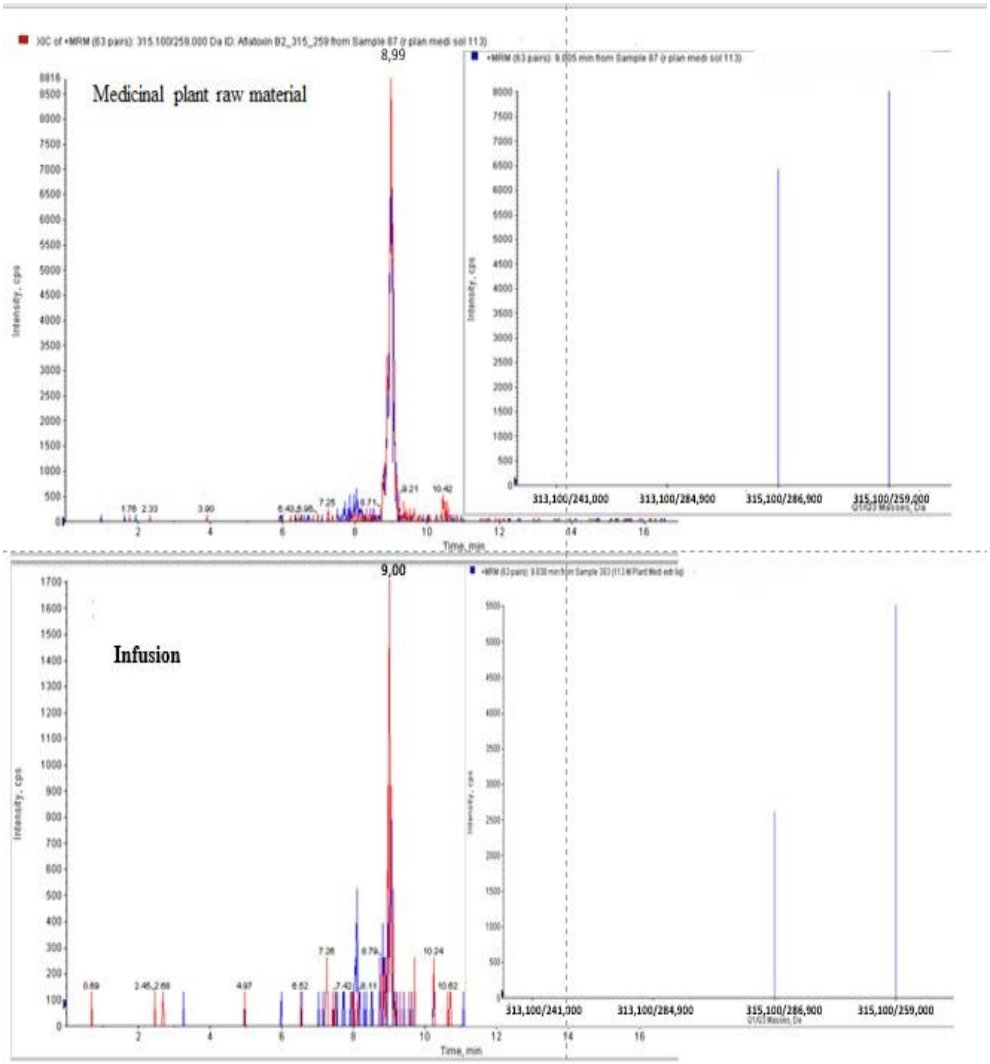


Figure S2. The exampled chromatogram of one medicinal plant sample positive for AFB2, the raw material *vs* the infusion.

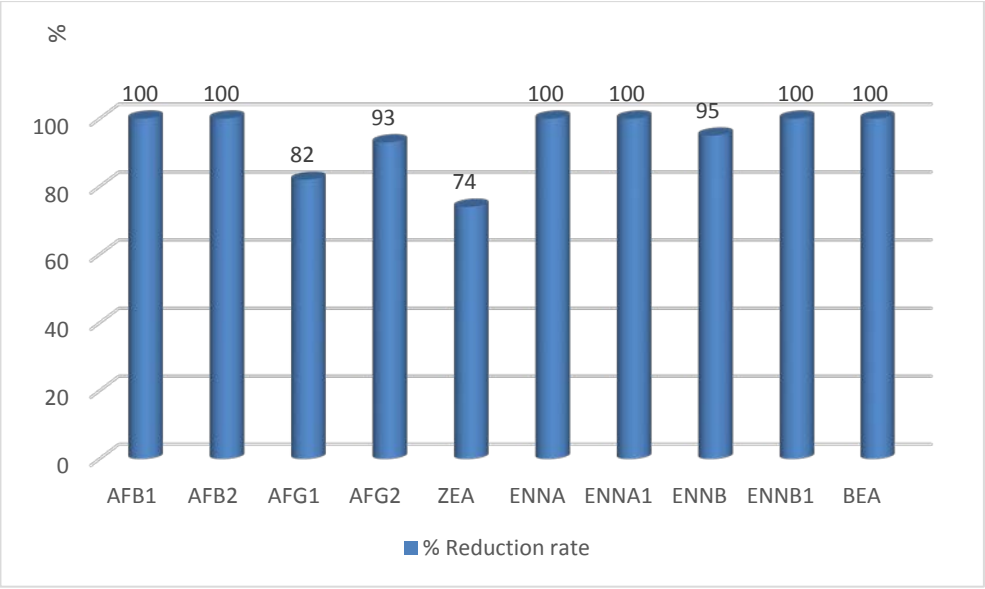


Figure S3. Rate reduction of studied mycotoxins in plant material infusions with boiled water.

Table S1. Detailed common and scientific names of the types of medicinal plants analyzed.

Scientific name	Common name
Salvia officinalis	Sage
Sideritis tragoriganum Lag	*Mursalski* Tea
Foeniculum vulgare	Fennel
Crataegus monogyna	Whitethorn
Origanum majorana	Marjoram
Melissa officinalis	Lemon balm leaves
Peumus boldus	Boldus leaves
Valeriana officinalis	Valerian root
Passiflora incarnata	Incarnata passion-flower plant
Matricaria chamomilla	Chamomile flower
Mentha pulegium	Pennyroyal
Urtica dioica	Common nettle
Stevia rebaudiana	Stevia
Taraxacum officinale	Dandelion plant
Aloysia citriodora	Lemon verbena
Citrus aurantium	Orange flower
Equisetum arvense L.	Horsetail
Silybum marianum	Cardus marianum
Glycyrrhiza glabra	Licorice root
Illicium verum	Star anise fruit
Mentha piperita	Peppermint
Eucalyptus sp	Eucalyptus leaves
Humulus lupulus	Common hop
Hibiscus sabdariffa	Roselle
Ginkgo biloba	Ginkgo tree
Thymus vulgaris	Thyme
Althaea officinalis	Marshmallow
Mentha spicata	Spearmint
Arctostaphylos uva-ursi	Bearberry leaves
Cassia angustifolia	Senna leaves
Euphrasia officinalis	Eyebright
Cuminum cyminum	Cumin
Agrimonia eupatoria	Agrimony
Calendula officinalis	Pot marigold
Satureja hortensis	Summer savory
Hemidesmus indicus	Indian Sarsaparilla
Malva sylvestris	Common Mallow
Tilia officinalis	Linden
Salix Alba	White Willow
Rosmarinus officinalis	Rosemary leaves
*Anethum graveo*lens	Dill

Table S2. Spectrometric parameters of liquid chromatography ion trap tandem mass spectrometry (LC-MS/MS-IT).

Mycotoxin	Retention Time (min)	DP[a]	Precursor ion	Quantification ion Q			Confirmation ion q		
				CE[b]	Product ion	CXP[c]	CE	Product ion	CXP
AFB1	9.13	46	313.1	39	284.9	4	41	241.0	4
AFB2	9.03	81	315.1	33	286.9	6	39	259.0	6
AFG1	8.86	76	329.0	39	243.1	6	29	311.1	6
AFG2	9.37	61	331.1	27	313.1	6	39	245.1	4
ZEA	10.40	26	319.0	15	301.0	10	19	282.9	4
ENNA	12.62	76	699.4	35	210.1	14	59	228.2	16
ENNA1	12.22	66	685.4	37	210.2	8	59	214.2	10
ENNB	11.60	51	657.3	39	196.1	8	59	214.0	10
ENNB1	11.89	66	671.2	61	214.1	10	57	228.1	12
BEA	12.00	116	801.2	27	784.1	10	39	244.1	6

[a] DP: declustering potential (volts); [b] CE: collision energy (volts); [c] CXP: cell exit potential (volts)

Table S3. Analytical parameters for QuEChERS method validation: recoveries, intraday and interday precision values, matrix effects and limits of detection and quantification.

Mycotoxin	Recovery[c] ± RSD[d] (%)		SSE (%)[b]	LOD	LOQ[a]
	Intraday precision	Interday precision		µg/kg	µg/kg
AFB1	68± 15	79± 14	37	3.0	10.0
AFB2	97± 13	92± 8	34	5.0	16.7
AFG1	62± 4	58± 1	34	3.0	10.0
AFG2	101±10	110± 13	23	5.0	16.7
ZEA	79± 7	87± 5	98	3.0	10.0
ENNA	70± 5	72± 6	112	0.1	0.3
ENNA1	90± 5	91± 7	119	0.1	0.3
ENNB	76± 1	80±6	99	0.1	0.3
ENNB1	69± 2	69±1	106	0.1	0.3
BEA	75± 8	76± 4	114	0.5	1.7

[a] LOD and LOQ are limits of detection and quantification, respectively (µg/kg)

[b] SSE: Signal Suppression-Enhancer

[c] Recoveries: Analysis performed at concentrations of 10× LOQ

[d] RSD: Relative standard deviation (calculated by injecting samples in triplicate)

Anexo 5. Food and Chemical Toxicology (2020)

Investigating the *in vitro* catabolic fate of Enniatin B in a human gastrointestinal and colonic model

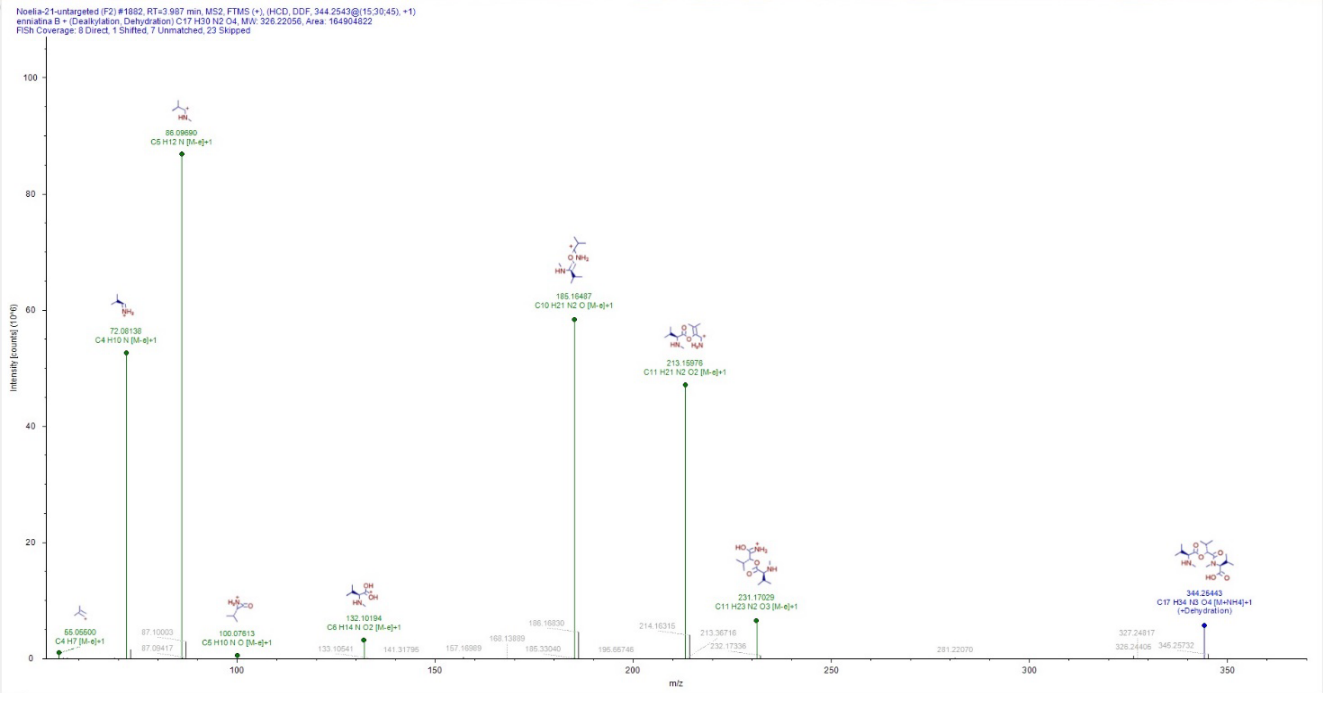


Figure 1. LC-HRMS/MS spectrum of #M1. Ammonium adduct was fragmented giving rise to most of the typical Enniatin B fragments highlighted in green (i.e. *m/z* 196,13309; *m/z* 186,14362; *m/z* 214,14362; *m/z* 640,4165; *m/z* 314,19592).

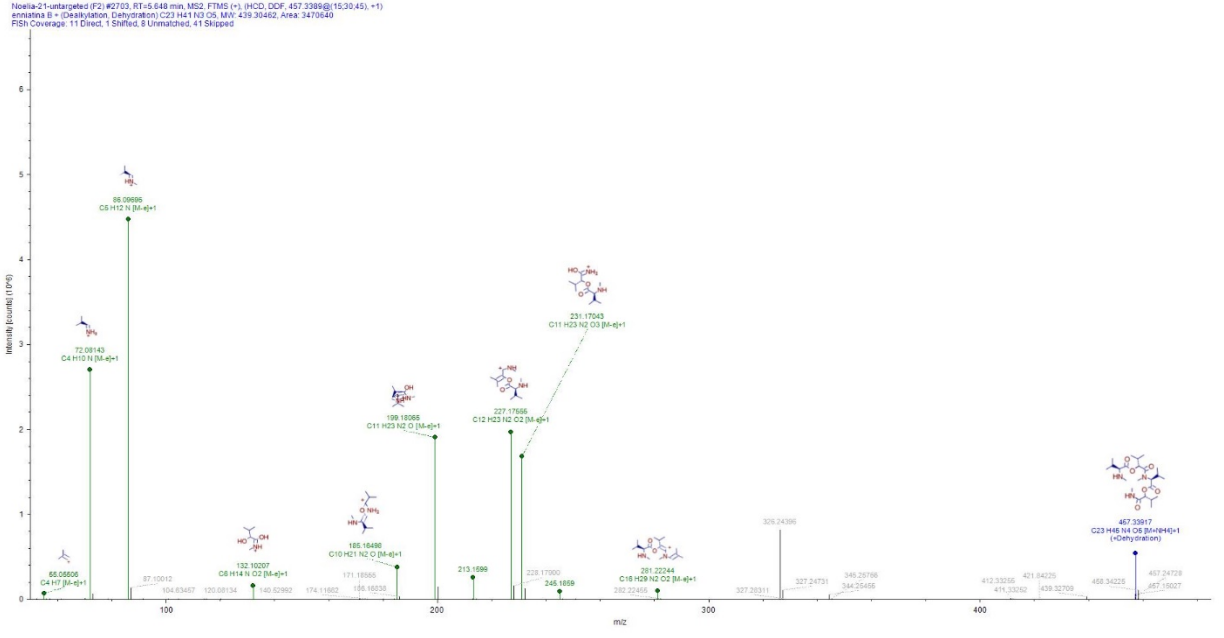


Figure 2. LC-HRMS/MS spectrum of #M2. Ammonium adduct was fragmented giving rise to most of the typical Enniatin B fragments highlighted in green (i.e. *m/z* 196,13309; *m/z* 186,14362; *m/z* 214,14362; *m/z* 640,4165; *m/z* 314,19592).

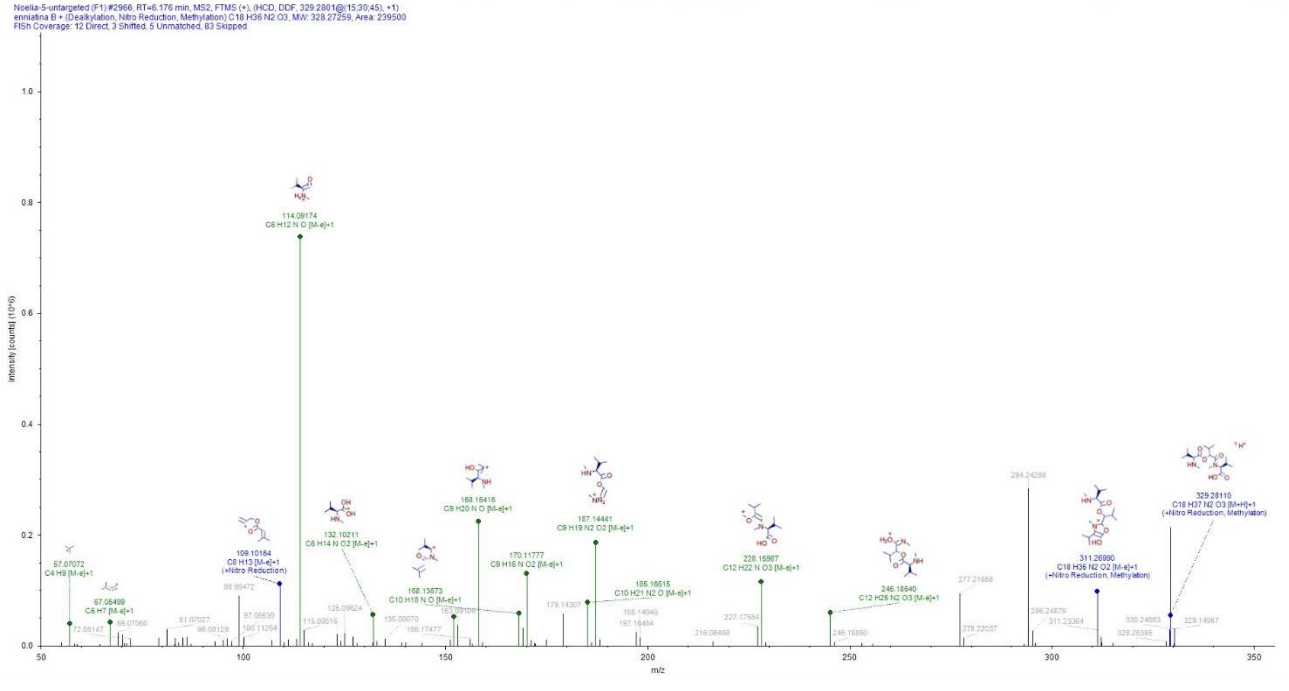


Figure 3. LC-HRMS/MS spectrum of #M3. Protonated adduct was fragmented giving rise to most of the typical Enniatin B fragments highlighted in green (i.e. *m/z* 196,13309; *m/z* 186,14362; *m/z* 214,14362; *m/z* 640,4165; *m/z* 314,19592).

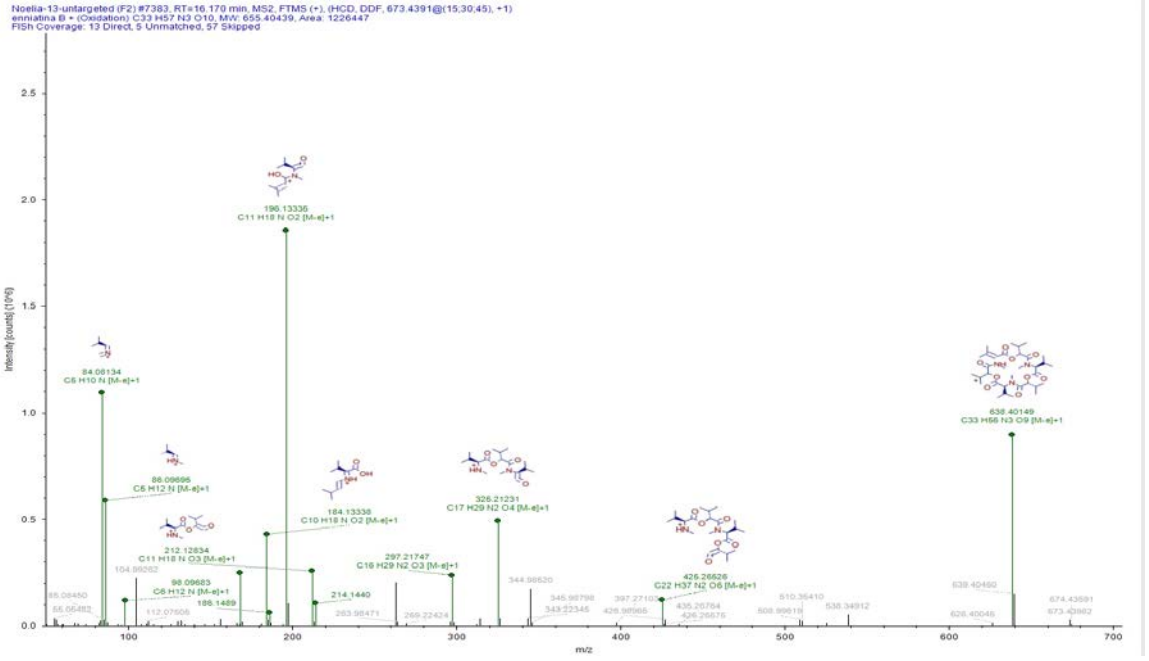


Figure 4. LC-HRMS/MS spectrum of #M4. Ammonium adduct was fragmented giving rise to most of the typical Enniatin B fragments highlighted in green (i.e. *m/z* 196,13309; *m/z* 186,14362; *m/z* 214,14362; *m/z* 640,4165; *m/z* 314,19592).

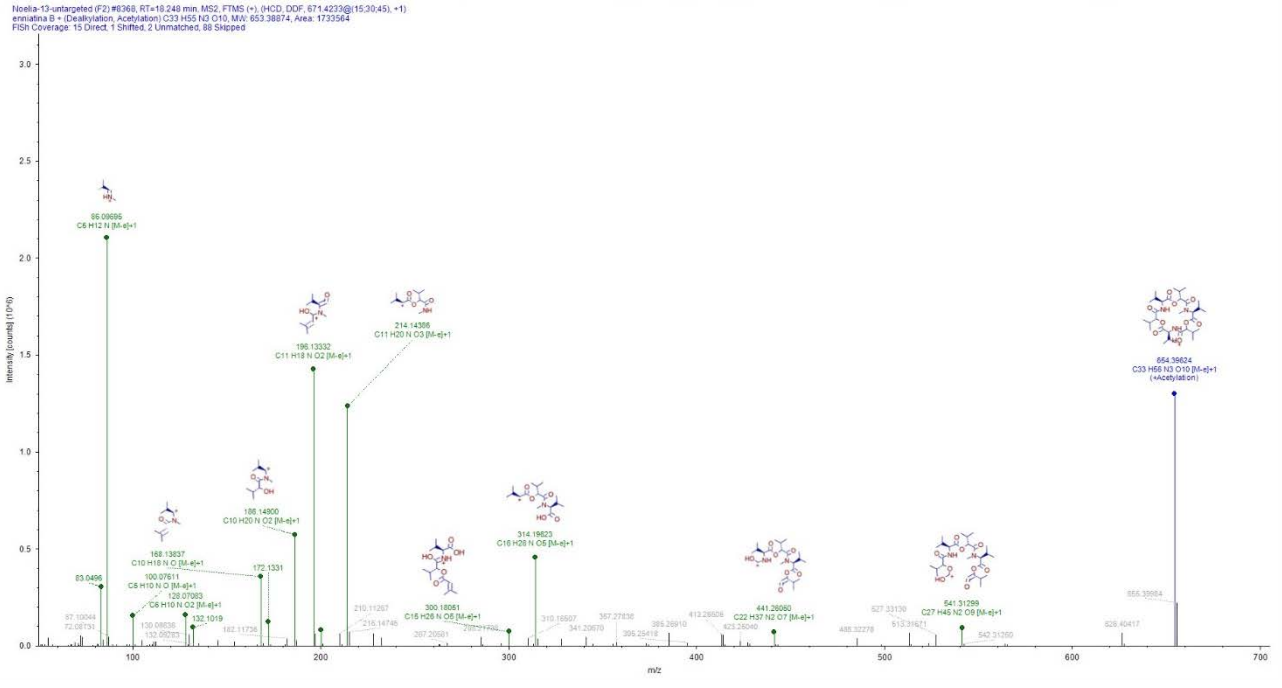


Figure 5. LC-HRMS/MS spectrum of #M5. Ammonium adduct was fragmented giving rise to most of the typical Enniatin B fragments highlighted in green (i.e. *m/z* 196,13309; *m/z* 186,14362; *m/z* 214,14362; *m/z* 640,4165; *m/z* 314,19592).

Detailed description of protocols

In the present work, the protocol proposed by Versantvoort et al. (2005) and by Minekus et al. (2014) were followed with some modifications, as reported below.

Versantvoort's protocol (V)

All the digestion juices (saliva, gastric juice, duodenal juice and bile) were prepared according the specifications reported in Table S1, reaching a final total volume of 500 ml for each synthetic juice. The final digestion mixtures were heated at 37 °C; the pH was readjusted, if required, at the target values (6.8 ± 0.2 (saliva), 1.30 ± 0.02 (gastric juice), 8.1 ± 0.2 (duodenal juice), 8.2±0.2 (bile)) using HCl (6M) or NaOH (1M).

Blank wheat flour spiked with 5 μg ENNB (1 g) or ENNB stock solution (100 μl containing 2 μg of mycotoxin) was added with 1.5 ml of saliva juice, stirred at 200 rpm on a stirrer (Orbital shaker-Incubator ES-20) for 5 min, and then added with 3 ml of gastric juice. The mixture was incubated for 2 hours at 37 °C, then added with 3 ml of duodenal juice, 1.5 ml of bile and 0.5 ml of HCO_3^- (1M) and incubated at 37 °C for 2 hours. Afterwards, an aliquot of the mixture was mixed with acetonitrile 1:1 v/v, and centrifuged at 4000 rpm for 10 minutes. The chyme was separated from the pellet and dried under N_2 stream. Finally, it was reconstituted in 1ml of H_2O/MeOH (70/30 v/v), centrifuged at 4000 rpm for 10 minutes, and analysed by LC-MS/MS.

Minekus' protocol (M)

Simulated Salivary Fluid (SSF), Simulated Gastric Fluid (SGF) and Simulated Intestinal Fluid (SIF) were prepared in a volume of 500 ml, as reported in Table S2. The rest of enzymes and constituents were prepared at concentrations indicated below in the protocol and then were added to the correspond step. The simulated digestive juices (SSF, SGF and SIF) were heated at 37 °C. The pH was readjusted to 7 (SSF), 3 (SGF) and SIF (7) with HCl (6M) or NaOH (1M) prior to be used.

Blank wheat flour spiked with 6.16 µg ENNB (1 g) or ENNB stock solution (100 µl containing 2 µg of mycotoxin) incubated with 1.4 ml of SSF, 0.2 ml of α-amylase (prepared at concentration 1500 U/ml in SSF), 10 µl of $CaCl_2$ and 390 µl bidistilled water for 2 min. Then, in the gastric phase, 3 ml of SGF were added, followed by porcine pepsine (calculated in order to achieved 2000 U/ml in the final volume of the digestion) and 10 µl of $CaCl_2$. The solution was stirred for 2 hours, by continuously adjusting the pH at 3 with HCl (6M) or NaOH (1M). For the intestinal step, 3.7 ml of SIF, 2 ml of pancreatin solution (prepared in SIF at concentration of 800 U/ml), 1 ml of bile (160 mM), 16 µl of $CaCl_2$ and 524 µl of water were added to the sample. The pH was then increase at 7 by dropping 60 µl NaOH (1M), and keep constant for the following 2 hours of incubation.

Afterwards, an aliquot of the mixture was mixed with acetonitrile 1:1 v/v, and centrifuged at 4000 rpm for 10 minutes. The chyme was separated from the pellet and dried under N_2 stream. Finally, it was reconstituted in 1ml of H_2O/MeOH (70/30 v/v), centrifuged at 4000 rpm for 10 minutes, and analysed by LC-MS/MS.

References

- Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., ... Brodkorb, A., 2014. A standardised static in vitro digestion method suitable for food–an international consensus. Food Funct., 5(6), 1113-1124.
- Versantvoort, C. H., Oomen, A. G., Van de Kamp, E., Rompelberg, C. J., Sips, A. J., 2005. Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem. Toxicol, 43(1), 31-40.

Table S1. Constituents of synthetic digestive juices used in protocol V (Versantvoort et al. 2005).

Constituent	Saliva	Gastric Juice	Duodenal Juice	Bile
Inorganic Solution	5 mL KCl (8.96 g/100 mL)	7.85 mL NaCl (17.5g/ 100 mL)	20 mL NaCl (17.5g/100 mL)	15 mL NaCl (17.5 g/100 mL)
	5 mL KSCN (2 g/100 mL)	1.5 mL NaH_2PO_4 (8.88g/100 mL)	20 mL $NaHCO_3$ (8.47g/100mL)	34.15 mL $NaHCO_3$ (8.47g/100mL)
	5 mL NaH_2PO_4 (8.88 g/100 mL)	4.6 mL KCl (8.96g/100 mL)	5 mL KH_2PO_4 (0.8g/100 mL)	2.1 mL KCl (8.96g/100 mL)
	5 mL Na_2SO_4 (5.7 g/100 mL)	9 mL $CaCl_2$ (1.66 g/100 mL)	3.15 mL KCl (8.96g/ 100mL)	75 µL HCl (37%)
	850 µL NaCl (17.5g/100 mL)	5 mL NH_4Cl (3.06g/100 mL)	5 mL $MgCl_2$ (1.06g/100mL)	
	10 mL $NaHCO_3$ (8.47g/100 mL)	3.25 mL HCl (37%)	90 µL HCl (37%)	
Organic Solution	4 mL Urea (2.5g/100 mL)	5 mL Glucose (6.5g/100 mL)	2 mL Urea (2.5g/100 mL)	5 mL Urea (2.5g/100 mL)
		5 mL Glucuronic acid (0.2g/100 mL)		
		1.7 mL Urea (2.5g/100 mL)		
		5 mL Glucosamine-HCl (3.3g/100 mL)		
Other constituents	α- amylase (29 mg/100 mL)	BSA (0.1g/ 100 mL)	$CaCl_2$ (0.9 mL/100mL)	$CaCl_2$ (1 mL/100mL)
	Uric acid (1.5 mg/100 mL)	Mucin (0.3g/100 mL)	Pancreatin (0.9g/100mL)	BSA (0.18g/100 mL)
	Mucin (2.5 mg/100 mL)	Pepsin (0.25g/100 mL)	Lipase (0.15g/100 mL)	Bile (3g/100 mL)
			BSA (0.1g/100mL)	
pH	**6.8± 0.2**	**1.3± 0.02**	**8.1± 0.2**	**8.2± 0.2**

Table S2. Constituents of synthetic digestive juices used in protocol M (Minekus et al. 2014).

Constituent	Simulated Salivary Fluid (SSF)	Simulated Gastric Fluid (SGF)	Simulated Intestinal Fluid (SIF)
KCl (3.7 g/100 mL)	15.1 mL	6.9 mL	6.8 mL
KH_2PO_4 (6.8 g/100 mL)	3.7 mL	0.9 mL	0.8 mL
N a HCO_3(8.4 g/100 mL)	6.8 mL	12.5 mL	42.5 mL
NaCl (11.7 g/100 mL)	--	11.8 mL	9.6 mL
$MgCl_2$ $(H_2O)_6$ (3.05 g/100 mL)	0.5 mL	0.4 mL	1.1 mL
NaOH 1M	--	--	--
HCl 6 M	0.09 mL	1.3 mL	0.7 mL
pH	**7**	**3**	**7**

Anexo 6. General Discussion

Tabla 1. Clasificación de las muestras analizadas.

Tipo de muestra	Número de muestras (n) y clasificación
Muestras sólidas	
Cápsulas plantas medicinales	Plantas individuales (64) Cola de caballo (5) Alcachofa (4) Valeriana (5) Diente de león (5) Cardo mariano (4) Fucus (5) Boldo (4) Ginkgo (4) Jengibre (4) Pasiflora (4) Harpagofito (4) Espino blanco (4) Melisa (3) Té rojo (4) Té verde (5) Combinaciones de plantas (21) Tratar el insomnio (16) Perder peso (5)
Plantas medicinales en crudo	(224 muestras) (4 muestras x cada especie) Especies de plantas: Salvia, Rabo de gato, Hinojo, Espino blanco, Mejorana, Melisa, Boldo, Valeriana, Pasiflora, Manzanilla, Poleo, Ortiga Verde, Stevia, Diente de León, Hierba Luisa, Azahar, Cola de Caballo, Cardo Mariano, Regaliz, Anís, Menta, Eucalipto, Lúpulo, Hibisco, Ginkgo, Tomillo, Malvavisco, Hierbabuena, Gayuba, Sen, Eufrasia, Cominos, Agrimonia, Caléndula, Ajedrea, Zarzaparrilla, Malva, Tila, Sauce,

	Romero, Eneldo, Orégano, Laurel, Siempreviva, Sauco, Llantén, Albahaca, Pulmonaria, Verbena, Sanguinaria, Escaramujos, Jengibre, Alcachofera, Harpagofito, Frambuesa, Artemisa
Muestras líquidas	
Infusiones de té "Camellia sinensis"	Té negro (12) Té rojo (14) Té verde (10) Té verde con menta (8)
Infusiones de plantas medicinales	Manzanilla (8) Manzanilla con miel (5) Manzanilla con anís (6) Tila (9) Poleo Menta (10) Tomillo (6) Valeriana (4) Cola de Caballo (4)
Infusiones de plantas medicinales (preparadas a partir de las muestras en crudo)	(224 muestras)
Zumos	Zumos de un solo tipo de fruta (40): Zumos de naranja naturales (7) Zumos a base de concentrados de fruta (33) - Naranja (3) - Pera (4) - Manzana (12) - Piña (7) - Melocotón (7) Zumos elaborados a base de mezclas de zumos, pures, ingredientes lácteos y funcionales (40): ecológicos (5) con propiedades saludables (24): - Con hierbas y especias (15) - Ricos en fibra (4)

	- Con antioxidantes (5) Mezclas de zumos con etiqueta común (11)