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

EVALUACIÓN DE LA PRESENCIA DE MICOTOXINAS EN ALIMENTOS Y PIENSOS Y SU MITIGACIÓN CULINARIA

MYCOTOXIN ASSESSMENT IN FOOD AND FEED AND THEIR COOKING MITIGATION

Tesi Doctoral Internacional

València, Maig 2017

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CERTIFIQUEN QUE:

La llicenciada en Veterinària **Dña. Josefa Tolosa Chelós** ha realitzat baix la seua direcció el treball **"Avaluació de la presència de micotoxines en aliments i pinsos i la seua mitigació culinària"**, i autoritzen la seua presentació per a optar al títol de Doctor per la Universitat de València.

I, perquè així conste, expedeixen i signen el present certificat.

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Aquest treball ha sigut plasmat en 9 articles, publicats o que es publicaran en les següents revistes:

1. Nuts and dried fruits: natural occurrence of emerging *Fusarium* mycotoxins. (2013). *Food Control 33, 215-220. Impact factor: 2.819*
2. Multi-mycotoxin analysis in durum wheat pasta by liquid chromatography coupled to quadrupole orbitrap mass spectrometry. (2017). *Toxins 9, 59. Impact factor: 3.571*
3. New approach in mycotoxin determination in gluten-free pasta by ultra performance liquid chromatography coupled to quadrupole orbitrap mass spectrometry. (2017). *Food Additives and Contaminants.* Under review. *Impact factor: 1.878*
4. Emerging *Fusarium* mycotoxins in vegetal raw materials and feeds intended for different animal species. (2017). *Animal Feed Science and Technology*. Under review. *Impact factor: 1.713*
5. Natural occurrence of emerging *Fusarium* mycotoxins in feed and fish from aquaculture. (2014). *Journal of Agricultural and Food Chemistry* 62, 12462-12470. *Impact factor: 2.857*
6. Multi-mycotoxin analysis in fish products. (2017). *Food Control*. Under review. *Impact factor. 3.388*
7. Multimycotoxin analysis in water and fish plasma by liquid chromatography-tandem mass spectrometry. (2016). *Chemosphere* 145, 402-408. *Impact factor: 3.698*
8. Mitigation of enniatins in edible fish tissues by thermal processes and identification of degradation products. (2017). *Food and Chemical Toxicology* 101, 67-74. *Impact factor: 3.584*
9. Presence of mycotoxins in Atlantic salmon *(Salmo salar*) from aquaculture. (2017). *Food Chemistry.* Under revie*w. Impact factor: 4.052*

La doctoranda agraeix a la Universitat de València:

La beca d'investigació (UV-BI-12-007) amb càrrec al projecte "Qualitat i Prevenció de la Higiene Alimentària als Serveis de Restauració de la Universitat de València".

La plaça de tècnic superior de suport a la investigació amb càrrec al projecte "Presencia de micotoxinas clásicas y emergentes en materias primas y alimentos transformados" (OTR2013-11518INVES). CPI-16-143, a càrrec del Programa d'ajudes per a la contractació de personal de suport vinculat a un projecte d'investigació de la Conselleria d'Educació, Investigació, Cultura i Esport (APOTIP/2016/A/040).

La plaça d'investigador no doctor a càrrec del Projecte "Asesoramiento técnico sobre aspectos científicos formativos relacionados con farmacia, nutrición y salud" (CPI-16-415).

Aquesta Tesi Doctoral Internacional s'engloba dins dels següents projectes:

- ✓ Evaluación del riesgo de las micotoxinas emergentes de *Fusarium* (AGL2010-17024/ALI).
- ✓ Estudio de las micotoxinas y metabolitos en alimentos y muestras biológicas, de la toxicidad y de los procesos de descontaminación (AGL2013-43194-P).
- ✓ Mitigación, biomarcadores, y toxicidad de micotoxinas legisladas y emergentes (AGL2016-77610-R).
- ✓ Red Nacional sobre las micotoxinas y hongos micotoxigénicos y de sus procesos de descontaminación (MICOFOOD).

La doctoranda agraeix l'estància per a la menció internacional del títol de Doctor, la qual es va realizar a Nàpols (Italia) al departament de Quimica dels Aliments a la Facultat de Farmàcia de l'Università degli studi di Napoli Federico II.

"Lo que sabemos es una gota de agua, lo que ignoramos, el océano"

Isaac Newton (1643-1727)

A mon pare

List of abbreviations

IDA	Ingesta Diaria Admisible
AECOSAN	Agencia Española de Consumo, Seguridad Alimentaria y Nutrición
AFs	Aflatoxinas
AFB1	Aflatoxina B1
AFB2	Aflatoxina B2
AFG1	Aflatoxina G1
AFG2	Aflatoxina G2
AFM1	Aflatoxina M1
AFP1	Aflatoxina P1
AFQ1	Aflatoxina Q1
AFL	Aflatoxicol
ATA	Aleukia Tóxica Alimentaria
BEA	Beauvericina
BEN	Nefropatía Endémica de los Balcanes
C18	Octadecilsílice
C8	Octilsílice
CaCo-2	Células de adenocarcinoma de colon humano
CE	Energía de colisión
CEP	Potencial de entrada de células de colisión
CHO-K1	Células de ovario de hámster chino
CXP	Potencial de salida de la celda de colisión
DAS	Diacetoxiscirpenol
DLLME	Micro-extracción dispersiva liquid-líquido
DON	Deoxinivalenol
DP	Voltaje del cono
d-SPE	Dispersión en fase sólida
EC	Comisión Europea
EDI	Ingesta Diaria Estimada
EEB	Encefalopatía Espongiforme Bovina
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	Organización de las Naciones Unidas para la Alimentación y la Agricultura
FBs	Fumonisinas
FB1	Fumonisina B1
FB2	Fumonisina B2
FUS	Fusaproliferina
FUS-X	Fusarenona X
GC	Cromatografía de gases
HPLC	Cromatografía líquida de alta resolución
HRMS	Espectrometría de masas de alta resolución
HT-2	Toxina HT-2
IARC	Agencia Internacional para la Investigación del Cáncer
Ile	Isoleucina
JECFA	Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios
LC	Cromatografía líquida
LC-MS-LIT	Cromatografía líquida-Espectrometría de Masas con trampa de iones lineal
LC-MS/MS	Cromatografía líquida-Espectrometría de Masas en tándem
LOD	Límite de detección
LOQ	Límite de cuantificación
ME	Efecto matriz
MeCN	Acetonitrilo
MeOH	Metanol
MON	Moniliformina
MRM	Monitorización de Reacciones Múltiples
MSPD	Dispersión de matriz en fase sólida
NEO	Neosolaniol

NIV	Nivalenol
OTA	Ocratoxina A
PAT	Patulina
PCBs	Bifenilos policlorados
PNCOCA	Plan Nacional de Control Oficial de la Cadena Alimentaria
PNIR	Plan Nacional de Investigación de Residuos
PMTDI	Ingesta Diaria Tolerable Máxima Provisional
PMTWI	Ingesta Semanal Tolerable Máxima Provisional
POAs	Productos de Origen Animal
PSA	Primary and Secondary Amine
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe extraction
RASFF	Sistema de Alerta Rápida para alimentos y piensos
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
SLE	Extracción sólido-líquido
S/N	Relación señal-ruido de fondo
SPE	Extracción en fase sólida
SPME	Micro extracción en fase sólida
SSE	Alteración de la señal por supresión o aumento
T-2	Toxina T-2
TCs	Tricotecenos
TDI	Ingesta Diaria Tolerable
TOF	Tiempo de vuelo
t_R	Tiempo de Retención
UE	Unión Europea
UHPLC	Cromatografía líquida de ultra alta resolución
ZON	Zearalenona
α-ZOL	α-zearalenol
3-AcDON	3-acetil deoxinivalenol
15-AcDON	15-acetil deoxinivalenol

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RESUMEN

En la presente Tesis Doctoral se han desarrollado y validado diferentes procedimientos analíticos para determinar la presencia de micotoxinas clásicas y emergentes en alimentos de origen vegetal y animal. La metodología empleada varía según la matriz y las micotoxinas a determinar y ha sido validada de acuerdo a la normativa europea con resultados satisfactorios en cuanto a linealidad, exactitud, precisión y límites de detección y cuantificación.

Por lo que respecta a la presencia de micotoxinas en alimentos de origen vegetal, destacan los elevados contenidos de eniatinas y beauvericina detectados en frutos secos y frutas desecadas. En las materias primas empleadas en la elaboración de piensos se detectaron concentraciones elevadas en alfalfa deshidratada, salvado de trigo y arroz. Por lo que respecta a los piensos analizados, los resultados muestran la elevada prevalencia de estas micotoxinas en las muestras analizadas (92%), siendo la eniatina B (ENN B) y la eniatina B1 (ENN B1) las más abundantes. En muestras de pasta de trigo y de maíz y arroz (sin gluten) se ha detectado la presencia de micotoxinas clásicas, principalmente tricotecenos, fumonisinas y zearalenona y micotoxinas emergentes de *Fusarium*.

La contaminación con micotoxinas de piensos elaborados a partir de materias primas vegetales, como cereales y otras fuentes proteicas de origen vegetal, supone un impacto sobre los consumidores, ya que éstas pueden acumularse en los tejidos animales y entrar así en la cadena alimentaria a través de productos derivados de los animales alimentados con piensos contaminados. Por ello, se han analizado muestras de pescado de diferentes especies (lubina, dorada, salmón y trucha arcoíris) y diferentes muestras de

sushi, salmón y trucha ahumados y sucedáneo de gulas. Los resultados muestran la presencia de eniatinas en muestras de pescado, en cambio, en las muestras de pescado transformado no se detectan contenidos ni en salmón y trucha ahumados ni en los diferentes tipos de sushi analizados, sin embargo, sí se detectan contenidos de eniatina B y fusarenona-X en muestras de sucedáneo de gula. Estos resultados sugieren la mitigación de las micotoxinas por el procesado de los alimentos. Además, mediante espectrometría de masas de alta resolución (HRMS) se ha realizado la identificación de 40 micotoxinas, además de las eniatinas, en muestras de salmón (*Salmo salar*).

Los tratamientos térmicos aplicados a los alimentos muestran la mitigación de las micotoxinas emergentes de *Fusarium*. Los porcentajes de reducción obtenidos oscilaron entre 30 y 100%, dependiendo de la muestra, de la micotoxina analizada y de la especie animal. Tras la aplicación de diferentes procesos de cocción se han identificado 2 productos de degradación de la ENN A1 en muestras de pescado, caracterizados por la pérdida de subunidades de isoleucina.

El empleo del análisis mediante LC-Q-TOF de las muestras de músculo de salmón ha permitido la identificación de un metabolito del deoxinivalenol (DON), el deepoxi-deoxinivalenol (DOM-1). DOM-1 se forma por acción de las bacterias del tracto digestivo de diferentes especies mediante una reacción de de-epoxidación en el grupo epoxi de los tricotecenos (TCs) y también por reacción enzimática en los microsomas hepáticos.

SUMMARY

Different analytical methods have been validated in this Doctoral Thesis in order to determine the occurrence of classic and emerging mycotoxins in both vegetal and animal origin foodstuffs. These methods vary depending on the matrix and the mycotoxins analyzed and have been validated according to European Commission guidelines with satisfactory results in terms of linearity, precission, trueness and limits of detection and quantification.

Regarding mycotoxin occurrence in vegetal foodstuffs, ennaitin and beauvericin contents must be highlighted in nuts and dried fruits. In analyzed raw materials employed in feed manufacture, dehydrated alfalfa, wheat bran and rice showed higher contents than other raw materials. In analyzed feed samples, results showed the high prevalence of mycotoxins (92%), being enniatin B (ENN B) and enniatin B1 (ENN B1) the most prevalent. In wheat pasta and gluten-free pasta (maize and rice) mycotoxin occurrence has been determined. Both classic (mainly trichotecenes, fumonisins and zearalenone) and emerging *Fusarium* mycotoxins have been detected.

The presence of mycotoxins in feed manufactured with vegetal raw materials, such as cereals and other vegetal protein sources, pose a risk to consumers as they can be accumulated in animal tissues. In this sense, the mycotoxin occurrence in animal derived products has been assessed. Four different fish species have been analyzed (sea bass, sea bream, salmon and rainbow trout) and also different types of sushi, smoked salmon and trout and gula substitute. Results showed enniatin occurrence in fish samples, however, regarding processed fish, no contamination was detected in smoked fish and sushi samples, but enniatin B and fusarenon-X were detected in gula substitute

samples, suggesting their mitigation by processing steps. Furthermore, 40 mycotoxins among enniatins have been identified in salmon samples (*Salmo salar*) by high resolution mass spectrometry (HRMS).

Thermal treatments applied to foodstuffs have shown the mitigation of emerging *Fusarium* mycotoxins, showing reduction percentages between 30 and 100%, depending on the matrix, the mycotoxin analyzed and the animal specie. After the application of different cooking techniques to fish fillets, two degradation products from ENN A1 have been identified, characterized by the loss of isoleucine units.

Finally, one metabolite (deepoxy-deoxynivalenol) derived from deoxynivalenol (DON) metabolism has been identified by LC-Q-TOF in edible tissue samples of salmon. DON is transformed to deepoxy-deoxynivalenol (DOM-1) via deepoxidation and deacetylation by microorganisms from the digestive tract and also by enzymatic reaction in hepatic miscrosomes.

1. INTRODUCTION

1. INTRODUCCIÓN

La seguridad alimentaria es un concepto dinámico que ha variado con el tiempo, haciéndose cada vez más completo. Según la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO), existe seguridad alimentaria cuando todas las personas tienen en todo momento acceso físico y económico a alimentos inocuos y nutritivos en cantidad suficiente para satisfacer sus necesidades alimenticias y sus preferencias en cuanto a los alimentos a fin de llevar una vida activa y sana. La definición aceptada en la Cumbre Mundial sobre la Alimentación (1996) da mayor fuerza a la índole multidimensional de la seguridad alimentaria e incluye el acceso a los alimentos, la disponibilidad y el uso de los mismos y la estabilidad del suministro (FAO, 1996).

Los alimentos presentan riesgos, frente a los cuales se realizan esfuerzos para prevenirlos, eliminarlos o reducirlos a niveles aceptables para la salud humana. Con objeto de minimizar los riesgos, se debe llevar a cabo una estrategia global de seguridad alimentaria, mediante normas sobre alimentación e higiene redactadas según los conocimientos científicos más avanzados. La seguridad alimentaria empieza ya en la explotación, y las normas se aplican desde ese mismo momento hasta que el alimento llega a la mesa del consumidor, ya se haya producido en la Unión Europea (UE) o se haya importado de cualquier otra parte del mundo (EC, 2005).

Los consumidores europeos exigen que los alimentos que consumen sean seguros y saludables. Por eso, la UE se esfuerza para que todo lo que consumen los ciudadanos sea de elevada calidad. A partir de la década de 1990, en respuesta a las alarmas alimentarias provocados por la ingesta de priones en

productos cárnicos, los piensos contaminados con dioxinas o el aceite de oliva adulterado, se ha efectuado una profunda revisión con la finalidad de mejorar la seguridad alimentaria. Con ello no solo se ha intentado conseguir que las leyes de la UE estén lo más actualizadas posible, sino también que los consumidores estén informados al máximo acerca de los riesgos que pueden existir y de las estrategias que se siguen para minimizarlos.

Actualmente, con el objetivo de garantizar que los productos que se encuentran en el mercado son seguros y no presentan riesgos para la salud, la UE posee uno de los sistemas de seguridad alimentaria más exigentes del mundo, asegurando que cualquier alimento producido dentro de nuestras fronteras presenta un estándar de calidad alto. Así, a nivel nacional, en diciembre de 2015 se aprobó el tercer Plan de España, denominado "Plan Nacional de Control Oficial de la Cadena Alimentaria 2016-2020" (PNCOCA 2016-2020) (AECOSAN, 2016), que proporciona a nuestro país un marco sólido, estable y a la vez flexible, para la realización del control oficial sobre alimentos y piensos, donde administraciones públicas, industria y consumidores encuentran un referente para obligaciones y garantías.

La finalidad del PNCOCA 2016-2020 es cumplir con lo establecido en el Reglamento EC 882/2004 del Parlamento Europeo y del Consejo de 29 de abril de 2004 (EC, 2004), recientemente derogado por el Reglamento EC 2017/625 (EC, 2017), sobre los controles oficiales efectuados para así garantizar y verificar el cumplimiento de la legislación en materia de piensos y alimentos y bienestar animal. Los controles se llevan a cabo en todas y cada una de las etapas de la cadena alimentaria, tanto en la producción primaria como en las fases posteriores de procesado, hasta que el producto llega a los

puntos de venta y está disponible para ser adquirido. Para ello, el plan persigue como objetivos principales el desarrollo y mejora de los planes de control oficial en el ámbito de la producción primaria para certificar, mediante el cumplimiento de la normativa en materia de seguridad y calidad alimentaria, que los alimentos son seguros y de calidad; reducir los riesgos presentes en los alimentos que amenazan a las personas a niveles razonablemente posibles y aceptables; mantener un nivel elevado de lealtad de las transacciones comerciales y de la defensa integral de los derechos de los consumidores y contribuir a mantener la unidad de mercado y garantizar la consecución de un elevado nivel de calidad alimentaria de los productos agroalimentarios.

Sin embargo, en ocasiones podemos encontrar alimentos que suponen un riesgo para la salud de los consumidores, originándose alertas que, a nivel nacional, son gestionadas a través del Sistema Coordinado de Intercambio Rápido de Información (SCIRI) dependiente de la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN) (AECOSAN, 2015), mientras que a nivel europeo se gestionan a través del Sistema de Alerta Rápida para Alimentos y Piensos (RASFF). En la Comunidad Valenciana ha sido aprobado recientemente el Decreto 23/2017 por el que se regula el protocolo de gestión de emergencias alimentarias y se regulan sus órganos de gestión en esta comunidad.

De acuerdo con los datos publicados por AECOSAN, en 2015 se registraron un total de 184 notificaciones de alerta, siendo más numerosos los peligros químicos y biológicos en productos de origen animal. Por lo que respecta a las notificaciones, en función de la categoría del peligro detectado, la detección de microorganismos patógenos constituye un número importante de

las mismas (745), en segundo lugar destacan las notificaciones por presencia de micotoxinas (495) seguidas de las notificaciones por presencia de metales pesados (219) y la presencia de alérgenos no declarados en el etiquetado (137) (Tabla 1).

Tabla 1. Notificaciones por categoría de peligro y tipo de notificación (AECOSAN, 2015).

Categoría del peligro	Alerta	Rechazo en frontera	Información para atención	Información para seguimiento	TOTAL 2015
Alérgenos	114	3	18	2	137
Composición	51	19	22	26	118
Cuerpos extraños	43	23	14	30	110
Metales pesados	73	73	57	16	219
Micotoxinas	74	388	19	4	495
Microorganismos patógenos	261	265	136	83	745

Del total de notificaciones gestionadas en 2015, último año del que se dispone de datos, 184 correspondieron a expedientes de alerta, de las cuales, el mayor número de notificaciones correspondió al grupo de productos de origen animal. En relación con la naturaleza del producto implicado, cabe destacar que la mayoría de las notificaciones de alertas estuvieron relacionadas con pescados y derivados. Por lo que respecta a las notificaciones relativas a productos de origen vegetal, se originaron un total de 62 notificaciones, de las cuales, la mayor parte correspondió al grupo de cereales, harinas y derivados (AECOSAN, 2015). Además, se debe considerar el hecho de que las

micotoxinas representan mayor número de notificaciones de rechazo en frontera (Marín et al., 2013).

1.1. Micotoxinas

El término micotoxina hace referencia a los productos químicos tóxicos producidos por unas pocas especies de hongos o mohos con capacidad para infestar cosechas en el campo o después de la cosecha y que representan un riesgo potencial para la salud de las personas y los animales a través de la ingestión de alimentos o piensos elaborados a partir de dichas materias primas contaminadas (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2016a).

La FAO ha reportado que el 25% de los cultivos alimentarios mundiales se ven afectados por hongos productores de micotoxinas, estimándose que las pérdidas mundiales de dichos productos alimenticios alcanzan los 1000 millones de toneladas al año (Bhat et al., 2010).

La existencia de las micotoxinas, así como sus efectos nocivos sobre la salud humana y animal, se conocen desde tiempos remotos debido a los casos de micotoxicosis ocurridos tanto en humanos como en animales. Un buen ejemplo de las enfermedades que este tipo de contaminación ha originado en el ser humano es la conocida como "Fuego de San Antonio", que durante la Edad Media dio lugar a diversas epidemias que devastaron Europa occidental. Esta afección, conocida también como ergotismo, era causada por el consumo de centeno contaminado con alcaloides producidos por el hongo *Claviceps purpúrea*. Entre los síntomas que originaba destacan los ataques epilépticos y la aparición en las extremidades inferiores de picores tan intensos que parecía que

las piernas quemaban y que llevaron a muchos peregrinos a visitar San Antonio en Francia con la esperanza de ser curados, motivo por el que recibió el nombre de "Fuego de San Antonio" (Ramos et al., 2011).

Por lo que respecta a alimentación animal y ya en épocas más recientes, cabe destacar la muerte de 100.000 pavos y otras aves en el Reino Unido en 1960, cuyo origen se debió a la contaminación por aflatoxinas presentes en piensos que contenían cacahuetes en su composición (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2016a).

En la actualidad se conocen alrededor de 400 micotoxinas de muy diferentes estructuras químicas y diferentes modos de acción. Cada micotoxina es producida específicamente por una o más especies concretas de hongos o mohos, siendo los géneros *Aspergillus, Fusarium, Penicillium* y *Alternaria* los más importantes. Además, un determinado hongo puede ser capaz de producir más de una toxina (Martínez-Larrañaga & Anadón, 2006). El riesgo de intoxicación aguda por micotoxinas es bajo o moderado, sin embargo, los efectos crónicos son más comunes. Por sus efectos adversos sobre la salud humana y animal, las micotoxinas más estudiadas son aflatoxinas (AFs), ocratoxina A (OTA), fumonisinas (FBs), tricotecenos (TCs), zearalenona (ZON) y patulina (PAT) (Rubinstein & Theumer, 2011).

Los hongos productores de micotoxinas se encuentran distribuidos a lo largo de todos los continentes y en un amplio rango de sustratos. La prevalencia geográfica de los hongos micotoxigénicos depende de las condiciones climatológicas, reflejando los requerimientos que necesitan para su crecimiento. Así, los géneros *Aspergillus* y *Penicillium* son más comunes en regiones templadas y húmedas, mientras que el género *Fusarium* es más común

en zonas frías (Santini et al., 2009), aunque también ha sido identificado en zonas templadas (Covarelli et al., 2015). Por este motivo, las micotoxinas producidas por *Aspergillus* predominan en plantas de los trópicos. Las condiciones climáticas previas a la recolección determinan la presencia y la cantidad de micotoxinas presentes en los frutos y cereales. En este sentido, algunos estudios indican que las concentraciones de micotoxinas varían según el año dependiendo de las variaciones climáticas, viéndose favorecida la producción de micotoxinas en frutos recolectados tras estaciones lluviosas (Jestoi et al., 2004).

En cualquier caso, la mera presencia de un hongo conocido como productor de toxinas no implica automáticamente la presencia de éstas, ya que existen otros muchos factores implicados en su formación. Y al contrario, la mera observación o confirmación visual de ausencia de mohos no es garantía de que el producto esté libre de micotoxinas ya que el moho puede haber desaparecido y haber quedado las toxinas intactas.

Los síntomas causados por las micotoxinas suelen ser tan diferentes unos de otros como lo son las propias estructuras químicas de cada grupo de micotoxinas. Las micotoxinas pueden producir efectos cancerígenos, mutagénicos, teratogénicos, trastornos estrogénicos, gastrointestinales, vasculares, pueden dañar el sistema nervioso central, el hígado y los riñones (Khosravi et al., 2008). Algunas micotoxinas son también inmunosupresoras, por lo que pueden reducir la resistencia a enfermedades infecciosas. Debido a su toxicidad, algunas micotoxinas han sido clasificadas por la Agencia Internacional de Investigación sobre el cáncer (IARC) en función de su potencial cancerígeno.

Las micotoxinas se encuentran contaminando principalmente alimentos de origen vegetal, no obstante, también pueden contaminar alimentos de origen animal así como alimentos procesados. Los productos de origen vegetal mayormente afectados son los cereales (maíz, trigo, cebada, avena, arroz, etc.), frutos y frutas secas, el café, el cacao, las especias, las semillas de oleaginosas y algunas frutas, principalmente las manzanas y uvas. Algunas micotoxinas pueden resistir a los tratamientos físicos, químicos y biológicos aplicados durante el procesado de algunos alimentos, pudiendo persistir en el alimento procesado. Así, las micotoxinas también se pueden encontrar en la cerveza y el vino, como resultado de la utilización de cebada u otros cereales o uvas contaminadas en su elaboración. Por tanto, una vez la micotoxina ha contaminado la materia prima alimentaria, es muy difícil su eliminación de la misma (Serrano, 2015).

Las consecuencias directas del consumo de piensos contaminados con micotoxinas en los animales incluyen la reducción de la ingesta de alimento y rechazo del mismo, mala conversión alimenticia, disminución de la ganancia de peso corporal, aumento de la incidencia de enfermedades infecciosas y parasitarias, disminución de la capacidad reproductiva, entre otras, lo que conduce a elevadas pérdidas económicas (Streit et al, 2012).

Por último, las micotoxinas también pueden ingresar en la cadena alimentaria a través de la carne y otros productos de origen animal como huevos, leche y derivados lácteos, como resultado de la alimentación del ganado con piensos contaminados (Figura 1) contaminados (Figura 1) (Duarte et al., 2012; Marín et al., 2013).

Figura 1. Exposición de micotoxinas a través de la ingesta en humanos y animales.

1.1.1. Principales micotoxinas estudiadas

1.1.1.1. Aflatoxinas

Químicamente, las aflatoxinas (AFs) son cumarinas sustituidas, conteniendo anillos de bifurano y configuración tipo lactona, comunes a todas ellas. Existen cuatro aflatoxinas principales, conocidas como B1, B2, G1 y G2. Las AFs B1 (AFB1) y B2 (AFB2) difieren entre ellas por la presencia de un doble enlace más en la primera. Por su parte, la AFG1 y la AFG2 difieren entre sí en el mismo detalle estructural. Las AFs del tipo B difieren de las de tipo G porque el anillo de furano de las primeras se convierte en un anillo de lactona en las segundas (Juan et al., 2007).

Las AFs son producidas principalmente por cepas de *Aspergillus flavus* y *Aspergillus parasiticus*. Estos hongos son especialmente abundantes en zonas tropicales y subtropicales. No obstante, aunque las mayores concentraciones de estas micotoxinas se dan en productos cultivados y almacenados en dichas regiones, el comercio internacional existente hace que su presencia no sea un problema exclusivo de las regiones productoras sino también de los países importadores.

Los alimentos considerados más susceptibles a la contaminación por AFs son el maíz, frutos secos (principalmente cacahuetes, pistachos y nueces de Brasil), especias, frutas desecadas y café. La presencia de AFB1 en productos de origen animal no es muy común, ya que la tasa de transferencia desde el pienso hasta los productos comestibles de origen animal suele ser muy baja (Juan et al., 2007). Una vez ingerida, la AFB1 es metabolizada, transformándose en diferentes metabolitos (AFM1, Aflatoxicol (AFL), AFQ1 y AFP1, principalmente).

La AFB1 es uno de los más potentes hepato-carcinógenos conocidos, que pertenece al Grupo 1 (compuestos carcinógenos para el hombre) de acuerdo con la clasificación de la IARC para ella y la suma de las AFs (IARC, 2012). La exposición crónica a largo plazo a muy pequeñas cantidades de esta toxina a través de la dieta tiene importantes consecuencias para la salud humana, siendo el hígado el órgano principalmente afectado. También se han observado efectos sobre los pulmones, el miocardio y los riñones. Además, a altas dosis, las AFs pueden producir efectos teratogénicos en algunas especies y acumularse en el cerebro. La AFB1 es absorbida en el intestino delgado y transportada por los glóbulos rojos y las proteínas plasmáticas hasta el hígado.

En la figura 2 se muestra el mecanismo de acción de la AFB1 (Gross-Steinmeyer & Eaton, 2012).

Los efectos de una intoxicación aguda por aflatoxinas han sido demostrados en un amplio número de animales: peces, conejos, perros, primates y aves, siendo los patos y pavos especialmente susceptibles. El grado de intoxicación depende de diversos factores: edad, sexo, el estado nutricional, etc. Así, los animales jóvenes son particularmente más susceptibles y los machos más que las hembras.

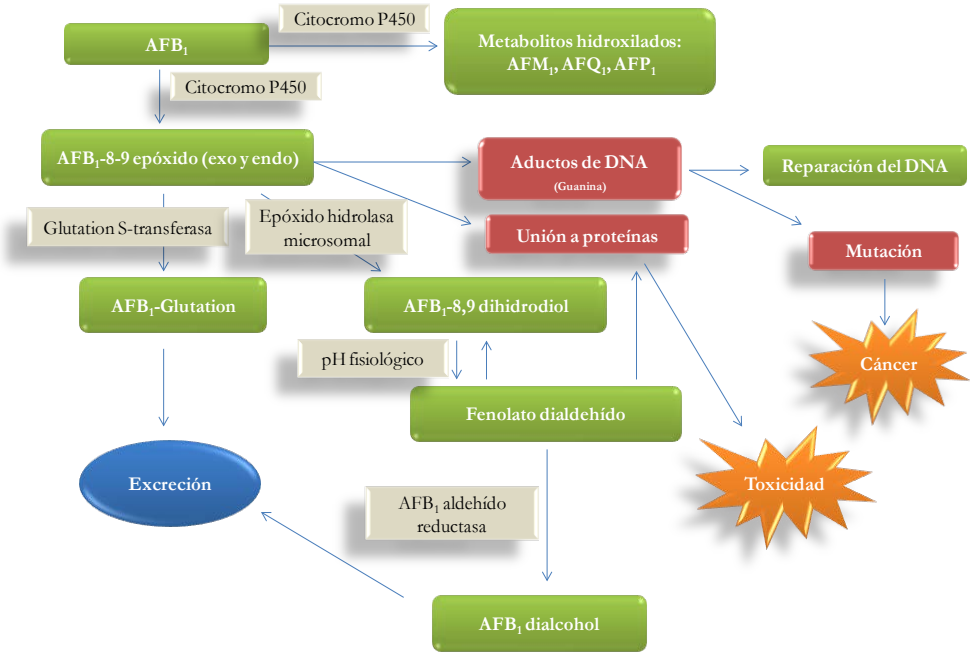

Figura 2. Mecanismo de acción de la AFB1 (Gross-Steinmeyer & Eaton, 2012).

1.1.1.2. Ocratoxina A

La ocratoxina A (OTA) es un derivado de hidrocumarinas que se encuentra unido a una unidad de L-β-Fenilalanina por un enlace amida. Está producida por determinadas especies de hongos del género *Aspergillus*, como *Aspergillus ochraceus*, principalmente en las regiones de clima tropical, y por *Penicillium verrucosum*, un hongo característico del almacenamiento, en las regiones del este y noroeste de Europa (Pfohl-Leszkowicz & Manderville, 2007).

Las principales fuentes de este contaminante alimentario son cereales como trigo, cebada, maíz o arroz y sus productos derivados, bebidas alcohólicas como cerveza y vino (especialmente vino tinto), además de otras matrices como zumo de uva, pasas, legumbres, café, cacao y nueces (Weidenbörner, 2013).

La OTA ha sido clasificada según la IARC (1993) como posible carcinógeno en humanos (Grupo 2B). Afecta principalmente a los riñones, acumulándose en los tejidos renales y produciendo daños agudos y crónicos, en función de la dosis y la duración de la exposición. Se sospecha que es el principal agente etiológico responsable de la nefropatía endémica de los Balcanes (BEN) y tumores del tracto urinario asociados. Los estudios sobre su toxicidad aguda arrojan diferentes grados de afectación según la especie animal de que se trate. Su capacidad nefrotóxica ha sido demostrada en todas las especies de mamíferos en los que se ha evaluado, siendo el perro y el cerdo especialmente susceptibles. En el caso del cerdo, se han observado similitudes sorprendentes entre la nefropatía porcina inducida por OTA y la nefropatía

inducida por OTA en seres humanos (BEN) (Pfohl-Leszkowicz & Manderville, 2007).

1.1.1.3. Micotoxinas de *Fusarium*

Fusarium es considerado el género productor de micotoxinas de mayor prevalencia en los cereales cultivados en las regiones templadas de América, Europa y Asia (EFSA, 2002a). Las diferentes especies son capaces de producir, en mayor o menor grado, dos o más micotoxinas simultáneamente (Tabla 2). Las micotoxinas de *Fusarium* se encuentran ampliamente distribuidas por la cadena alimentaria, siendo los productos elaborados con cereales, especialmente trigo y maíz, las principales fuentes de ingesta alimentaria de esas toxinas, aunque también se encuentran contaminando otros productos como cebada, avena, arroz, patata, espárragos y forrajes (Glenn, 2007). Los hongos del género *Fusarium* infectan los cereales en el campo, es decir, la producción de toxinas se produce principalmente antes de la cosecha, especialmente en zonas templadas, produciendo principalmente TCs, FBs y ZON (Stanciu et al., 2017a; Beccari et al., 2017).

Debido a su amplia distribución en la cadena alimentaria, es importante que se establezcan contenidos máximos en relación con los cereales no elaborados, a fin de evitar que entren en la cadena alimentaria cereales muy contaminados. Para ello se deben llevar a cabo diferentes actuaciones durante las fases de cultivo, recolección y almacenamiento dentro de la cadena de producción mediante la aplicación de buenas prácticas agrícolas, de recolección y de almacenamiento (Nesic et al. 2014).

Tabla 2. Micotoxinas producidas por diferentes especies de *Fusarium* (Nesic et al., 2014).

Especie	Micotoxina
F. culmorum	Aurofusarina, butenolida, clamidosporol, culmorina, ciclonerodiol, ciclonerotriol, fusarina, moniliformina, tricotecenos, zearalenona
F. graminearum	Aurofusarina, butenolida, clamidosporol, culmorin, ciclonerodiol, fusarina, tricotecenos, zearalenona
F. sporotrichioides	Aurofusarina, beauvericina, butenolida, culmorina, eniatinas, fusarina, moniliformina, tricotecenos
F. crookwellense	Aurofusarina, butenolida, culmorina, ciclonerodiol, ácido fusárico, fusarina, tricotecenos, zearalenona
F. acuminatum	Acuminatum, aurofusarina, beauvericina, clamidosporol, eniatinas, fusarina, moniliformina, tricotecenos
F. equiseti	Beauvericina, equisetina, fusarocromanona, moniliformina, tricotecenos, zearalenona
F. proliferatum	Beauvericina, eniatinas, fumonisinas, ácido fusárico, fusaproliferina, moniliformina
F. verticillioides	Fumonisinas, ácido fusárico, fusarina, naftoquinonas
F. armeniacum	beauvericina, fusarina, tricotecenos
F. pseudograminearum	tricotecenos, zearalenona

1.1.1.3.1. Tricotecenos

Los tricotecenos (TCs) son un complejo grupo de sesquiterpenoides químicamente relacionados. Se encuentran divididos en cuatro grupos (A, B, C y D) de acuerdo a los grupos sustituyentes funcionales. Se han descrito más de 45 tipos de TCs. Los principales TCs tipo A incluyen a las toxinas T-2 y HT-2, diacetoxiscirpenol (DAS), neosolaniol (NEO), etc. y poseen en posición C-8 un grupo funcional distinto a cetona, mientras que los del tipo B se caracterizan por poseer un grupo cetona en posición C-8. Los tricotecenos tipo B engloban a micotoxinas como deoxinivalenol (DON) y nivalenol (NIV) y sus

compuestos acetilados: 3-acetildeoxinivalenol (3-ADON), 15-acetildeoxinivalenol (15-ADON) y fusarenona X (FUS-X) (Krska et al., 2007).

Las principales especies productoras de TCs tipo A son *Fusarium sporotrichioidies*, *F. poae* y *F. equiseti,* mientras que los TCs tipo B son producidos por especies de *F. graminearum* y *F. culmorum*.

Los alimentos comúnmente contaminados con TCs comprenden diferentes tipos de cereales y granos, principalmente trigo, maíz, cebada, avena y arroz y los productos elaborados con estas materias primas (Marín et al., 2013).

Los TCs han sido clasificados como no carcinógenos para el ser humano (Grupo 3) según la IARC (1993). Su citotoxicidad se atribuye a su fuerte inhibición de la síntesis proteica, de RNA y DNA. Entre los efectos producidos por los TCs destaca su participación en la disrupción del transporte trans membrana. Además de su actividad citotóxica, su actividad inmunosupresora es destacable provocando una supresión de la resistencia a microorganismos patógenos (Nesic et al., 2014). Entre los síntomas que provoca debido a una toxicidad aguda, destacan las náuseas, vómitos, pérdida de apetito, pérdida de peso y diarrea. La ingestión de altas dosis puede provocar lesiones en tejidos linfoides hemorragias intestinales severas y destrucción celular en las membranas mucosas epiteliales del intestino (Brera et al., 2013).

Dentro de los TCs, las toxinas HT-2 y T-2 son conocidas por producir la Aleukia Tóxica Alimentaria (ATA), caracterizada por sepsis, hemorragias y pancitopenia general. Los efectos tóxicos sistémicos comprenden la inhibición de la hematopoyesis, mediante apoptosis de células de la médula ósea y de las células del sistema inmune (EFSA, 2011a). En los animales existen diferencias

significativas en la sensibilidad de las especies monogástricas y rumiantes, que se atribuye a la eliminación mediante de-epoxidación de estas toxinas en el rumen por la microflora ruminal (Jakic- Dimic y Nesic, 2011).

1.1.1.3.2. Zearalenona

La zearalenona (ZON) es una micotoxina estrogénica no esteroidea. Los principales hongos productores de ZON son *Fusarium graminearum*, *F. culmorum, F. equiseti* y *F. verticillioides*. Se encuentra principalmente en el maíz y también, aunque en menor medida, en la cebada, avena, trigo, sorgo, mijo y arroz (Nesic et al., 2014).

ZON ha sido clasificada como no carcinógena para el ser humano (Grupo 3) de acuerdo a la clasificación de la IARC (1993). La toxicidad de ZON y algunos de sus metabolitos, especialmente α-zearalenol (α-ZOL), está asociada con problemas reproductivos en algunos animales, y posiblemente en humanos, ya que tienen la capacidad de unirse de forma competitiva con los receptores estrogénicos. Compite fuertemente con el 17 β-estradiol para unirse a los receptores citosólicos de estrógeno presentes en el útero, hipotálamo, y glándulas mamaria y pituitaria.

ZON es rápidamente biotransformada y excretada en animales, observándose diferencias significativas en su perfil metabólico dependiendo de la especie. Así, se han estudiado sus efectos en ratones, ratas, cerdos, hámsters, conejos y animales domésticos, observándose una disminución de la fertilidad, resorciones embrionarias, reducciones de la camada, cambios en los niveles séricos de progesterona y estradiol y en glándula tiroides, adrenal y pituitaria (Nesic et al., 2014).

1.1.1.3.3. Fumonisinas

Las fumonisinas (FBs) son producidas por varias especies del género *Fusarium*, aunque principalmente por *Fusarium verticillioides* y *F. proliferatum*. En la actualidad se han identificado más de 12 variantes de FBs. Según los grupos químicos presentes a lo largo de la cadena lineal, se pueden clasificar en cuatro series: A, B, C y P dentro de las cuales resultan especialmente tóxicas las pertenecientes a la serie B, ya que además son las que mayoritariamente se detectan en alimentos.

Las FBs se encuentran contaminando principalmente cereales y derivados, destacando el maíz como matriz alimentaria más susceptible de presentar contaminación por estos compuestos (Weidenbörner, 2013). Otras matrices donde se ha detectado la presencia de FBs son los piensos o la cerveza, como consecuencia de la utilización de sémola o jarabe de maíz como coadyuvante tecnológico.

Las FBs han sido clasificadas como posibles carcinógenos (Grupo 2B) según la IARC (1993). La sintomatología de una intoxicación aguda por FBs incluye dolor abdominal, borborigmo y diarrea. A nivel crónico, existe una asociación probable entre el consumo de alimentos contaminados por FBs y cáncer de esófago e hígado. En los équidos se ha descrito el desarrollo de un proceso conocido como leucoencefalomalacia, y en cerdos se asocia con el síndrome de edema pulmonar, así como a procesos de pancreatitis y de daño hepático (De Girolamo et al., 2014). En roedores se han descrito efectos citotóxicos por la ingesta de FBs (Dall'Asta et al., 2008).

1.1.1.3.4. Eniatinas y Beauvericina

El género *Fusarium* es también responsable de producir otro grupo de toxinas, llamadas micotoxinas emergentes de *Fusarium*, que incluyen las eniatinas (ENNs), beauvericina (BEA), fusaproliferina (FUS) y moniliformina (MON). Este grupo debe su nombre a que fueron descubiertas con posterioridad a otras micotoxinas del género *Fusarium* y a que en la actualidad se dispone de pocos datos sobre su toxicidad (EFSA, 2014). Hoy en día estas micotoxinas son compuestos de creciente interés debido a su posible impacto negativo en la salud humana y animal.

Las ENNs son hexapéptidos cíclicos consistentes en tres residuos de ácido D-2-hidroxicarboxílico y ácido N-metilamino alternantes sintetizadas, principalmente, por los géneros *Fusarium, Alternaria*, *Halosarpheia* y *Verticillium*. En la literatura hay descritas 4 eniatinas: eniatina A (ENN A), eniatina A1 (ENN A1), eniatina B (ENN B) y eniatina B1 (ENN B1), según la naturaleza del residuo del ácido N-metilamino (Ivanova et al., 2006). Sin embargo existen otros análogos que también han sido identificados, como son las ENNs B2, B3, B4, D, E, F y G (Mahnine et al. 2011).

Actúan como ionóforos y como inhibidores del enzima acil-CoA y colesterol acil transferasa, presentan propiedades antibacterianas, antifúngicas e inmunomoduladoras. Además son capaces de transportar cationes mono y divalentes a través de la membrana celular. Los datos sobre la toxicidad crónica o a largo plazo de las micotoxinas emergentes son escasos en comparación con las micotoxinas clásicas. La mayoría de los estudios realizados hasta el momento se centran en la toxicodinamia (interacción primaria con las células diana), pero no existen suficientes datos de su toxicocinética (absorción,

distribución, metabolismo y excreción) ni de su posible interacción con otras micotoxinas, así como de los efectos aditivos o sinérgicos que pueden presentar al interaccionar con otras micotoxinas o entre ellas (Fernández-Blanco et al., 2016; Santini et al., 2009).

Los estudios toxicológicos sobre los efectos de las mezclas de micotoxinas son de gran importancia a fin de determinar si podrían interactuar entre sí y producir un efecto sinérgico, aditivo o antagonista. Los efectos tóxicos de las ENNs in vitro se han realizado previamente en diferentes tipos de células.

Teniendo en cuenta que la vía principal de las micotoxinas es a través de la ingestión oral, las células de adenocarcinoma de colon humano (Caco-2), son las comúnmente empleadas en los ensayos de toxicidad. Las células Caco-2 se consideran células humanas diana del sistema digestivo, además, esta línea celular ha demostrado ser sensible a la citotoxicidad de ENNs, como han demostrado otros autores (Meca et al., 2011; Ivanova et al., 2011). Los resultados reportados por algunos autores muestran que las ENNs del tipo A (ENN A y ENN A1) resultan ser las más citotóxicas entre las ENNs ensayadas, produciéndose efectos sinérgicos cuando su presencia tiene lugar junto a las ENNs del grupo B (Prosperini et al., 2014). Además, estos autores han demostrado la participación de especies reactivas de oxígeno (ROS) en la apoptosis y daño mitocondrial en células Caco-2 inducidas por diferentes ENNs (A, A1, B y B1).

Estos resultados demuestran que la citotoxicidad de las ENNs depende de sus concentraciones, así como de su combinación con otras micotoxinas (Lu et al., 2013). Por lo que respecta a los estudios *in vivo*, pese a que éstos son

todavía escasos, algunos de ellos han demostrado la distribución tisular tras la ingestión de ENN A y sus efectos sobre el sistema inmune en ratas Wistar (Manyes et al., 2014; Juan et al., 2014).

La Beauvericina (BEA) es un ciclohexapéptido con propiedades insecticidas capaz de inducir apoptosis en células de mamíferos y con propiedades antibióticas frente a bacterias gram-positivas y micobacterias. Fue aislada en primer lugar de cultivos de *Beauveria bassiana*, pero también es producida por otros hongos, entre ellos varias especies de *Fusarium*. Las moléculas de BEA no poseen ningún grupo iónico pero pueden formar complejos lipofílicos con cationes y transportarlos a la fase lipídica. Además, puede transportar metales alcalino-térreos e iones de metales alcalinos a través de las membranas celulares.

Los datos reportados sobre su toxicidad demuestran que la BEA induce citotoxicidad de manera dependiente de la dosis y del tiempo en las células de ovario de hámster chino CHO-K1. Por otra parte, la BEA inhibe la proliferación celular deteniendo las células en fase G0/G1 y aumentando la apoptosis (Mallebrera et al., 2016). En células Caco-2, su citotoxicidad es inducida a través de la producción de ROS y daño mitocondrial (Prosperini et al., 2013).

1.1.1.3.5. Patulina

Estructuralmente, la patulina (PAT) es una lactona heterocíclica insaturada, producida principalmente por el género *Penicillium*, mayoritariamente *P. expansum*, aunque también pueden producirla otros hongos de otros géneros, como *Aspergillus* o *Paecylomyces*.

La principal fuente de contaminación por PAT, a diferencia del resto de micotoxinas descritas anteriormente, son las frutas, concretamente la manzana y sus derivados (zumos y compotas). A pesar de que originariamente la PAT fue descubierta por sus propiedades antibióticas, el descubrimiento de sus efectos negativos sobre la salud hizo que se sometiera a un estudio intenso. Aunque no existen suficientes evidencias que demuestren la carcinogenicidad de la PAT, ésta ha sido clasificada en el grupo 3 de acuerdo a la clasificación de la IARC (IARC, 1993). Entre sus efectos tóxicos destacan su mutagenicidad, carcinogenicidad y teratogenicidad. Además, esta micotoxina induce lesiones intestinales, incluyendo la degeneración de células epiteliales, inflamación, ulceración y hemorragias (Marín et al., 2013).

1.1.2. Legislación

Desde hace años, la Unión Europea tiene como firme objetivo contribuir a la calidad higiénica y sanitaria de todos los eslabones de la cadena alimentaria y por ello, a través de la Autoridad Europea de Seguridad Alimentaria (EFSA), se han elaborado las numerosas normativas que regulan todos los sectores relacionados con la alimentación, tanto humana como animal.

Los principios básicos que rigen la legislación europea sobre contaminantes se encuentran regulados por el Reglamento EC 315/93 del Parlamento Europeo y del Consejo (EC, 1993), por el que se establecen procedimientos comunitarios en relación con los contaminantes presentes en los productos alimenticios, quedando así totalmente prohibida la puesta en el mercado de alimentos que contengan contaminantes en cantidades inaceptables.

El reconocimiento por parte de las autoridades de los distintos efectos que sobre la salud de las personas y de los animales se derivan de la contaminación de alimentos y piensos con micotoxinas, ha llevado al establecimiento de límites máximos para aflatoxinas y otras micotoxinas en diferentes países de todo el mundo. En el caso concreto de Europa, el establecimiento de límites máximos de micotoxinas por parte de algunos de los Estados miembro de la UE, llevó a la Comisión a adoptar una normativa armonizada a nivel europeo, con el principal objetivo de proteger la salud de los consumidores, pero también para evitar distorsiones en el mercado interior por causa de la existencia de diferentes legislaciones nacionales en la materia.

1.1.2.1. Legislación en alimentos de origen vegetal destinados al consumo humano

En la actualidad, el Reglamento EC 1881/2006 (EC, 2006a) sigue siendo el Reglamento marco a nivel europeo en materia de contenidos máximos de contaminantes en los productos alimenticios, si bien este Reglamento ha sido objeto de diversas modificaciones desde su publicación inicial. En base a sucesivas evaluaciones de riesgo y dictámenes de la EFSA, ha sido modificado mediante el Reglamento EC 1126/2007 (EC, 2007), en lo que respecta a los límites para las micotoxinas de *Fusarium* en maíz y derivados, mientras que, en lo que respecta a los contenidos de AFs, ha sido modificado por el Reglamento EC 165/2010 (EC, 2010a), el cual incrementó el contenido máximo permitido para AFs y el Reglamento EC 1058/2012 (EC, 2012a) en lo referente al contenido máximo de AFs en higos secos. Algunas de las modificaciones en relación a los contenidos de OTA se recogen en el Reglamento EC 105/2010 (EC, 2010b), por el que se estableció un contenido máximo de OTA en

especias y regaliz, y por los Reglamentos EC 594/2012 y 1137/2015 (EC, 2012b; EC, 2015a). Los contenidos máximos de citrinina han sido modificados por el Reglamento EC 212/2014 (EC, 2014), mientras que el contenido máximo de esclerocios de cornezuelo de centeno se ha visto modificado por el Reglamento EC 1940/2015 (EC, 2015b).

Los contenidos máximos legislados dependen de la matriz analizada y del grupo de población al que van destinados, correspondiendo los contenidos máximos más bajos a aquellos alimentos destinados a lactantes, niños de corta edad o a usos médicos especiales. El rango de contenidos máximos para las micotoxinas legisladas en alimentos destinados al consumo humano es de 0,1-8 μg/kg para la AFB1, de 0,5-10 μg/kg para la OTA, de 10-50 μg/kg para la PAT, de 20-200 μg/kg para DON, de 200-1750 μg/kg para DON y de 200-2000 μg/kg para la suma de FBs.

Paralelamente a la publicación del citado Reglamento marco 1881/2006 (EC, 2006a) se publicó el Reglamento EC 401/2006 de la Comisión, de 23 de febrero de 2006 por el que se establecen los métodos de muestreo y de análisis para el control oficial del contenido de micotoxinas en los productos alimenticios, el cual, para evitar que se produzcan diferentes interpretaciones o variaciones en la transposición por parte de los Estados miembro, armoniza todo lo referente a métodos de muestreo y análisis de micotoxinas (EC, 2006b).

1.1.2.2. Legislación en productos destinados a la alimentación animal

En el campo de la nutrición animal, a nivel europeo, la Directiva 2002/32/EC del Parlamento Europeo y del Consejo, de 7 de mayo de 2002

(EC, 2002a), cuyo anexo ha sido modificado por el Reglamento EC 574/2011 de la Comisión de 16 de junio de 2011 (EC, 2011a), contribuye a normalizar el contenido de sustancias indeseables de todos los productos relacionados con la alimentación animal. A nivel nacional, la normativa relativa al control de sustancias indeseables se recoge en el Real Decreto (RD) 465/2003. Estas normativas pretenden exigir unos límites máximos rigurosos y firmes, basándose tanto en las opiniones elaboradas por la EFSA como en los datos aportados por el sector. Las especificaciones relativas a la presencia de micotoxinas en el pienso se reflejan en la Directiva 2003/100/EC (EC, 2003) y en la Recomendación 2006/576/EC (EC, 2006c). Únicamente la AFB1, cornezuelo del centeno, DON, ZON, FBs y OTA se encuentran reguladas por la legislación europea para alimentación animal (Zachariasova et al., 2014), y por ello son las más ampliamente estudiadas. De acuerdo a lo establecido en la Directiva 2003/100/EC (EC, 2003), los contenidos máximos fijados para la AFB1 en piensos son de 20 μg/kg para cerdos y aves y para rumiantes. Sin embargo, este contenido es inferior para la alimentación destinada a terneros y corderos (10 μg/kg) y para los piensos destinados a animales productores de leche (5 μg/kg). Los niveles permitidos para la AFB1 son mucho menores que los permitidos para el resto de micotoxinas reguladas, debido a su poder carcinógeno (IARC, 1993).

Aunque la Directiva 2002/32/EC del Parlamento Europeo y del Consejo, de 7 de mayo de 2002 (EC, 2002a), sobre sustancias indeseables en la alimentación animal establece únicamente el contenido máximo de AFB1 en los piensos por las razones anteriormente expuestas, en la gestión de los posibles riesgos para la salud de los animales debidos a la presencia de otras micotoxinas, la Comisión optó por el establecimiento de valores orientativos

para OTA, ZON, DON y FBs, recogidos en la Recomendación 2006/576/EC de la Comisión Europea (EC, 2006c). Dichos contenidos máximos recomendados para DON, ZON, OTA y FBs son mayores que los permitidos para la AFB1, siendo diferentes según la especie a la que van destinados. Los niveles recomendados de DON para cerdos son de 0.9 mg/kg, y para terneros, corderos y cabritos, 2 mg/kg, ya que los rumiantes son menos sensibles que los animales monogástricos. Los niveles establecidos para ZON también diferencian entre rumiantes (0,5 mg/kg) y cerdos (0,25 mg/kg para cerdos de engorde y 0,1 mg/kg para lechones y cerdas nulíparas). Respecto a los contenidos recomendados de OTA, los niveles establecidos en piensos para aves de corral son de 0,1 mg/kg, mientras que para cerdos son de 0,05 mg/kg. Por último, los contenidos legislados para las FBs (suma de FB1 y FB2) son mayores para rumiantes (50 mg/kg para adultos y 20 mg/kg para terneros, corderos y cabritos) que para cerdos, conejos, mascotas y caballos (5 mg/kg), ya que éstos últimos son muy sensibles a los efectos de la ingesta de FBs. Los piensos destinados a la acuicultura presentan un valor orientativo para la suma de FBs de 10 mg/kg.

Por último, junto a estos valores orientativos, la Recomendación 2013/165/EC sobre la presencia de las toxinas T-2 y HT-2 en los cereales y en los productos a base de cereales (EC, 2013a), también establece valores orientativos o de referencia para estas micotoxinas en cereales y productos a base de cereales utilizados como materias primas para alimentación animal así como en piensos y piensos compuestos. Respecto a las toxinas T2 y HT-2, los datos disponibles sobre su presencia en los alimentos para animales son por ahora muy limitados. Tal y como se ha señalado anteriormente, las diferencias significativas en la sensibilidad a las micotoxinas entre especies monogástricas y

rumiantes es atribuible a la disminución de la toxicidad de las toxinas debida a la actividad enzimática de la flora microbiana del rumen. Según las investigaciones publicadas, los cerdos se encuentran entre los animales más sensibles a los efectos de la toxina T-2, con efectos sobre su sistema inmunológico y hemático que aparecen a partir de dosis de 29 μg/kg de peso al día.

1.1.2.3. Legislación en productos de origen animal destinados al consumo humano

Los productos destinados a la alimentación animal pueden contener sustancias indeseables capaces de perjudicar a la salud animal o, por su presencia en los productos de origen animal (POAs), a la salud humana y al medio ambiente. Algunas micotoxinas tienden a acumularse en distintos órganos de los animales, con el riesgo de que si éstos ingresan en la cadena alimentaria a través de los alimentos de origen animal contaminados (carne, leche, huevos), pueden llegar a afectar a la salud de las personas.

DON, ZON y FBs, son toxinas que prácticamente no se transfieren desde los piensos a la carne, leche y huevos, motivo por el cual, la ingesta de productos de origen animal contribuye de manera muy marginal a la exposición total del ser humano a estas toxinas. Por su parte, la OTA sí puede ser transferida desde los piensos a los alimentos de origen animal. Algunos autores han demostrado la presencia de esta micotoxina en productos derivados de animales alimentados con piensos contaminados (Dall'Asta et al., 2010; Duarte et al., 2012; Chen et al., 2012). Sin embargo, también en este caso los estudios han demostrado que los productos de origen animal contribuyen mínimamente a la exposición total del ser humano a la OTA a través de la dieta. Sin embargo,

dado que la ingesta de AFB1 da lugar a la presencia de AFM1 en productos derivados de los animales que la han ingerido, la presencia de esta micotoxina sí se encuentra regulada por el Reglamento EC 1881/2006 (EC, 2006a) en leche y productos lácteos, siendo la única micotoxina legislada en POAs (Tabla 3).

No obstante, la EFSA a través de las opiniones científicas emitidas y el Plan Nacional de Investigación de Residuos a nivel estatal, recogen la preocupación y necesidad de realizar la determinación de diferentes grupos de micotoxinas en POAs y otros productos con la finalidad de realizar una evaluación de la exposición de la población a estas micotoxinas a través de la ingesta de alimentos.

En nuestro país, el RD 1262/89 aprobó la regulación inicial del mencionado Plan de Investigación de Residuos en animales y carnes frescas, estableciendo la sistemática de vigilancia sobre residuos, sustancias de acción farmacológica y contaminante de origen medioambiental, en determinadas especies animales y en sus carnes. En el ámbito comunitario la regulación del Plan de Investigación de Residuos se produjo a través de la Directiva 96/23/EC (EC, 1996) (actualmente derogada por el Reglamento EC 2017/625 (EC, 2017)), que ha sido transpuesta al derecho nacional mediante el RD 1749/98, por el que se establecen las medidas de control aplicables a determinadas sustancias y sus residuos en los animales vivos y sus productos. El objetivo básico de la norma es establecer la sistemática de vigilancia de la cadena de producción, tanto de los animales como de los productos primarios de consumo humano que de ellos se obtienen, destinada a detectar las sustancias, y sus residuos, que se dividen en dos grandes grupos. En el grupo A

se encuentran las sustancias con efecto anabolizante y sustancias no autorizadas, mientras que en el grupo B se encuentran los medicamentos veterinarios y contaminantes. El grupo A se divide a su vez en 6 grupos, destacando los esteroides y los β-agonistas, entre otros. El grupo B se divide en 3 subgrupos: el primero de ellos se corresponde con sustancias antibacterianas, incluidas las sulfamidas y las quinolonas; el segundo engloba otros medicamentos veterinarios (tranquilizantes, carbamatos, piretroides, etc), y el tercer grupo incluye otras sustancias y contaminantes medioambientales, destacando los compuestos organoclorados y organofosforados y las micotoxinas.

El Plan de vigilancia se lleva a cabo sobre los animales vivos, sus excrementos y líquidos biológicos, así como en cualquier órgano y tejido animal, los productos de origen animal y los piensos, el agua de bebida y otros componentes utilizados en la alimentación de los animales.

En la Parte B, Sección III (Programas de control oficial en establecimientos alimentarios) del PNCOCA figura el "Programa de control de determinadas sustancias y sus residuos en productos de origen animal". Numerosas sustancias incluidas en este programa que pueden suponer un riesgo para la salud pública ya han sido incluidas en otros programas del PNCOCA, como por ejemplo las micotoxinas, los plaguicidas, los contaminantes ambientales y las sustancias prohibidas. Cuando estas sustancias son ingeridas por los animales, dan lugar a la aparición de residuos en los productos de origen animal, y son, por lo tanto, objeto de estudio en este Programa sobre residuos de sustancias en los productos de origen animal. En este sentido, el plan anual de actuación debe prever la detección e investigación

de los diferentes tipos de sustancias/residuos, según el tipo de animal, clasificados en animales de abasto, aves de corral, conejos de caza y animales de acuicultura y según el tipo de producto (leche, huevos y miel). Por lo que respecta a la vigilancia de las micotoxinas, su estudio se debe llevar a cabo en animales de abasto, aves de corral y animales de acuicultura, así como en leche.

El estudio sobre la presencia de micotoxinas en alimentación animal destinada a diferentes especies de animales terrestres ha sido ampliamente descrito en la literatura científica, tanto en materias primas como en los forrajes y piensos empleados en su alimentación (Binder, 2007; Binder et al., 2007; Rodrigues & Naehrer, 2012; Streit et al., 2012; Warth et al., 2012; Streit et al., 2013a; Dzuman et al., 2014; Zachariasova et al., 2014). Sin embargo, los datos referentes a peces procedentes de la acuicultura son escasos. Dado el crecimiento de este sector, en parte debido al elevado consumo de pescado, y el empleo de piensos elaborados a partir de materias primas vegetales potencialmente contaminadas por micotoxinas, el análisis de la presencia de éstas en este grupo es necesario en la actualidad.

1.1.3. Análisis de micotoxinas

El análisis de micotoxinas en alimentos y piensos es, todavía hoy, un reto debido a numerosas razones, entre ellas, la heterogeneidad de las diferentes matrices alimentarias, las distintas estructuras químicas de las micotoxinas y los bajos niveles de detección exigidos. Por ello, el empleo de una única técnica de análisis para todas las micotoxinas es un objetivo a alcanzar hoy en día, siendo un paso crítico la extracción de las mismas, que debería permitir una buena recuperación de todas las micotoxinas investigadas en cada matriz alimentaria específica.

Los avances en el análisis de micotoxinas se basan en el desarrollo de métodos más rápidos, capaces de detectar diversos analitos a la vez, respetuosos con el medio ambiente, económicos y adecuados para alcanzar los objetivos propuestos en alimentos, piensos, tejidos y fluidos biológicos (De Saeger et al., 2016). La legislación comunitaria no establece ningún método de análisis específico para la determinación del contenido de micotoxinas en los productos alimenticios, sino que establece unos criterios generales y específicos a los que deberá ajustarse el método de análisis. De esta forma cualquier método puede ser empleado, siempre que su funcionamiento se ajuste a dichos criterios generales, garantizando así unos niveles de eficacia comparables (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2016a).

Los protocolos de muestreo para el análisis de micotoxinas en algunos productos alimenticios se recogen en el Reglamento EC 401/2006 (EC, 2006b), mientras que los métodos de confirmación utilizados se ajustarán a lo dispuesto en el Reglamento EC 882/2004 (EC, 2004), derogado recientemente por el Reglamento EC 2017/625 (EC, 2017), y en particular, a lo establecido en los puntos 1 y 2 de su anexo III.

La mayoría de los métodos analíticos constan de las etapas de muestreo, homogenización, extracción seguida de un paso de purificación de la muestra para reducir o eliminar componentes de la matriz no deseados, y finalmente, las etapas de separación y detección, usualmente realizadas mediante una técnica cromatográfica en combinación con una variedad de detectores, aunque también son realizados mediante métodos basados en reacciones antígeno-anticuerpo (Pereira et al., 2014).

1.1.3.1. Métodos de extracción

En los últimos años, se ha realizado un gran esfuerzo para desarrollar metodologías analíticas para una determinación eficaz de las micotoxinas, en particular los métodos para múltiples micotoxinas. Para ello, se han utilizado varias técnicas de extracción recientemente desarrolladas tales como QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) o micro-extracción líquido-líquido dispersiva (DLLME); sin embargo, las técnicas más convencionales como la extracción en fase sólida (SPE) o la extracción sólido-líquido (SLE) siguen siendo ampliamente utilizadas.

Otras técnicas comúnmente empleadas son la Dispersión de Matriz en Fase Sólida (MSPD, Matrix Solid Phase Dispersion), y también la Micro-Extracción en Fase Sólida (SPME, Solid Phase Micro-Extraction), llevadas a cabo mediante baño de ultrasonidos, Ultra-Turrax y diferentes homogenizadores o agitadores. Estas técnicas presentan las ventajas de ser rápidas, económicas y de emplear un reducido volumen de disolvente y poder ser aplicadas en diferentes matrices alimentarias (Serrano et al., 2015).

1.1.3.1.1. DLLME

La extracción DLLME es una de las extracciones líquido-líquido con más proyección de futuro por las ventajas que presenta. Rezaee et al. (2006) describieron por primera vez el empleo del método de extracción DLLME en el análisis de contaminantes orgánicos en muestras de agua. Desde entonces, existen diferentes trabajos que emplean esta técnica para la determinación de micotoxinas (Karami-Osboo et al., 2015). Esta técnica se basa en la dispersión de un pequeño volumen de disolvente inmiscible con el agua en la muestra acuosa junto con un segundo disolvente miscible que actúa como dispersante.

Los analitos quedan retenidos en el disolvente de extracción, el cual se dispersa en la muestra en forma de microgotas, enturbiando la disolución de manera que el área superficial entre el disolvente de extracción y la muestra acuosa es infinitamente grande, por lo que el estado de equilibrio se logra rápidamente y el tiempo de extracción pasa a ser muy corto (Karami-Osboo et al., 2013).

Los dispersantes más comúnmente empleados son acetonitrilo, acetona y metanol. Por otro lado, el extractante debe tener la capacidad de solubilizar los analitos y mezclarse con el dispersante (Rezaee et al., 2006). En la mayoría de los casos se emplean extractantes con densidades mayores a la de la muestra en cuestión, siendo los disolventes más empleados el cloroformo, tetracloruro de carbono o diclorometano. Sin embargo, en la actualidad, algunos autores han descrito el empleo de extractantes con densidades inferiores a la de la muestra. De esta manera, según la densidad del disolvente de extracción utilizado, el método DLLME puede clasificarse en dos grandes categorías (Campone et al., 2011a; Taherimaslak et al., 2015).

El empleo de la DLLME para la extracción de micotoxinas es todavía escaso, aunque algunos autores la han aplicado con éxito. Sin embargo, en estos estudios, normalmente se analiza sólo una micotoxina o un grupo reducido de la misma familia. Algunos ejemplos son los trabajos publicados sobre la presencia de ZON en cerveza (Antep & Merdivan, 2012), OTA en vinos (Arroyo-Manzanares et al., 2012; Campone et al., 2011b), DON en harina de trigo (Karami-Osboo et al., 2013), PAT en jugo de manzana (Víctor-Ortega et al., 2013), AFs en pistachos (Taherimaslak et al., 2015), y AFM1 en leche (Campone et al., 2013), entre otros.

1.1.3.1.2. QuEChERS

En la actualidad, uno de los métodos de extracción sólido-líquido más empleado es el método QuEChERS, el cual permite realizar la extracción y purificación en presencia de sales inorgánicas. Este método fue empleado inicialmente para la determinación de pesticidas (Anastassiades, 2003), pero en los últimos años está siendo empleado también para la extracción y purificación de otros compuestos, como antibióticos y micotoxinas. El método de preparación de muestras QuEChERS presenta diversas ventajas sobre las técnicas tradicionales, como un alto rendimiento y el uso de pequeñas cantidades de solvente orgánico y disolventes no clorados, lo que lo convierte en un método rápido y económico para la extracción de contaminantes alimentarios (Arroyo-Manzanares et al., 2014a). Recientemente se ha empleado en la determinación de micotoxinas en métodos multirresiduo en cereales para la determinación conjunta de distintas familias de micotoxinas (Sospedra et al., 2010; Rodríguez-Carrasco et al., 2012; Azaiez et al., 2014).

El método consiste en una extracción basada en el reparto de los analitos entre una fase acuosa y un disolvente, seguido de un paso de purificación mediante dispersión en fase sólida (d-SPE). En la fase de extracción se emplea un disolvente orgánico, generalmente acetonitrilo, y posteriormente se adicionan una serie de sales, sulfato de magnesio o sulfato de sodio y cloruro sódico o acetato de sodio para eliminar la humedad de la muestra y propiciar el reparto de los analitos hacia la fase orgánica. La etapa de purificación se lleva a cabo mediante la adición de PSA (Primary and Secondary Amine) para eliminar pigmentos polares, ácidos orgánicos y otros productos presentes en la matriz

que pudieran interferir con los analitos. Generalmente, la PSA se usa combinada con C18 para eliminar lípidos, esteroles y pigmentos.

1.1.3.2. Identificación y cuantificación

Para la identificación y cuantificación de micotoxinas se requiere la separación cromatográfica de los diferentes analitos acoplada a diferentes tipos de detectores, principalmente espectrómetros de masas, dado que el análisis de alimentos y piensos requiere el empleo de métodos sensibles y selectivos.

La diferente estructura química de las micotoxinas, así como la variedad de matrices analizadas, dificulta el desarrollo de metódos analíticos multi-micotoxina. Por lo tanto, el desarrollo de métodos analíticos que empleen una única preparación de muestra y una única determinación final es, hoy en día, la meta a alcanzar (Krska et al., 2012).

1.1.3.2.1. Técnicas cromatográficas

La cromatografía líquida (LC) y la cromatografía de gases (GC), acopladas a detectores de espectrometría de masas (MS), son las más utilizadas en el análisis cuantitativo por su capacidad de separar micotoxinas de similar y diferente estructura química, mientras que los métodos inmunoquímicos son generalmente específicos para una sola micotoxina o un pequeño grupo de compuestos estructuralmente relacionados.

Durante la última década se han publicado diversos métodos basados en la LC-MS/MS para la identificación y cuantificación de micotoxinas en diferentes matrices (Zachariasova et al., 2010; Rasmussen et al., 2010; Zhao et al., 2015; Stanciu et al., 2017b).

1.1.3.2.2. Espectrometría de masas

La espectrometría de masas permite obtener la distribución de las moléculas de una sustancia en función de su relación masa/carga (m/z). Es una herramienta analítica muy potente para identificar compuestos, elucidar el peso molecular y la estructura química de moléculas y cuantificar contaminantes en matrices complejas. En la actualidad se encuentran disponibles diferentes tipos de espectrómetros de masa, empleados en función de las necesidades del método aplicado. Entre los más utilizados en el análisis de alimentos se encuentran los espectrómetros de masa con triple cuadrupolo y con trampa de iones lineal, que tienen un poder de resolución menor a 10.000 (precisión de masa > 5 ppm) mientras que una alta resolución (HRMS) puede definirse como una potencia de resolución de 10.000-100.000 con una precisión de masa de <5 ppm (Holčapek et al. 2012).

El papel de la HRMS en el análisis de contaminantes en alimentos y muestras biológicas ha sido estudiado por diferentes autores, concluyendo que se trata de una potente herramienta para la evaluación de la seguridad alimentaria (Kaufmann 2012; Senyuva et al., 2015). Esto se debe a que realizan un análisis más robusto, sensible y selectivo al permitir la exploración de los espectros de masa en modo de exploración completa, obteniendo información más completa, detallada y precisa sobre la composición de una muestra. En consecuencia, esta característica permite realizar el análisis de compuestos desconocidos de los cuales no se posee un patrón con el que comparar, así como la posibilidad de análisis de datos retrospectivos sin necesidad de volver a inyectar la muestra y la capacidad de realizar elucidaciones estructurales de compuestos desconocidos o sospechosos (Senyuva et al., 2015).

La HRMS se ha utilizado durante mucho tiempo en el análisis de alimentos, pero hasta hace relativamente poco tiempo, se asociaba con una alta complejidad instrumental. La aparición de instrumentos modernos de HRMS como el TOF y los analizadores Orbitrap ha cambiado esta situación, por lo que actualmente, estos instrumentos están siendo empleados para el análisis de micotoxinas en diferentes alimentos y piensos (Herebian et al., 2009; Kaufmann, 2012), y, en particular, para toxinas "enmascaradas" (Kluger et al., 2013; De Boevre et al. 2014; Arroyo-Manzanares et al., 2014b) y ENNs (Vaclavikova et al., 2013).

La HRMS se emplea actualmente como una herramienta de cribado en alimentos y piensos para el análisis de residuos de medicamentos veterinarios, residuos de plaguicidas y toxinas naturales. El interés en la determinación de las micotoxinas reside en que pueden estar presentes un gran número de ellas, con estructuras muy similares entre sí y sin regulación normativa establecida, en una misma muestra. A pesar del número limitado de micotoxinas reguladas, existe un gran interés en el desarrollo de métodos de cribado que comprendan el análisis de un gran número de micotoxinas simultáneamente para obtener una visión más amplia, ya que en los alimentos se puede encontrar co-presencia de una amplia gama de metabolitos fúngicos y conjugados que pueden ocurrir naturalmente. Estos métodos se llevan a cabo actualmente mediante el empleo de LC-HRMS.

1.2. Materias primas y pienso

Actualmente, la demanda de alimentos seguros por parte de los consumidores ha hecho que se revise la alimentación animal, ya que ésta constituye el primer eslabón de la cadena alimentaria y depende en gran medida

del cumplimiento de la legislación aplicable por parte de los fabricantes, así como del uso de piensos seguros por parte de los ganaderos.

Los cambios recientes en la tecnología agrícola han llevado a una práctica generalizada de recolectar los cereales aún con alto contenido de humedad, los cuales, durante el almacenamiento, permiten la proliferación de hongos que pueden dar lugar a la generación de micotoxinas (Martins et al., 2008). Además, los piensos se componen de diferentes materias primas provenientes de diversos orígenes, por lo que pueden presentar contaminación de diversas fuentes (Binder et al., 2007). Generalmente, los productos mayormente afectados por la contaminación por micotoxinas son los cereales, semillas oleaginosas y harinas derivadas de estas semillas. El movimiento de los piensos en todo el mundo, facilitado por los acuerdos de comercio internacional, requiere un control constante de los niveles de micotoxinas por parte de las autoridades y los comerciantes.

1.2.1. Ingredientes y materias primas

En los últimos años, a raíz de la crisis de la Encefalopatía Espongiforme Bovina (EEB), se ha producido un incremento del uso de materias primas de origen vegetal como fuente de proteína en sustitución de la harina de origen animal. Este hecho ha conllevado un incremento del riesgo potencial de la presencia de micotoxinas en piensos (Martins et al., 2008).

Los Reglamentos EC 178/2002 (EC, 2002b) (modificado parcialmente por el Reglamento EC 2017/625 (EC, 2017)) y 999/2001 (EC, 2001), pretenden conseguir la prevención, control y erradicación de la EEB mediante la prohibición del uso de subproductos ganaderos en la alimentación de

animales de consumo. Desde entonces el número de casos de EEB ha caído espectacularmente demostrándose la efectividad de las medidas. No obstante, la EFSA ha constatado que el uso de proteínas de animales transformadas procedentes de animales no rumiantes es completamente seguro para alimentar otros animales cuando es producido conforme a las normas europeas. Esta constatación ha permitido de forma segura el levantamiento parcial y escalonado de la prohibición de uso de algunos subproductos animales para los peces. El cambio normativo más reciente se ha producido con el Reglamento EC 56/2013 (EC, 2013b), que autoriza, entre otras cuestiones, la inclusión de algunas PATs en los piensos para peces (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2016).

Las materias primas se definen, de acuerdo al Reglamento EC 767/2009 (EC, 2009), como productos de origen vegetal o animal, cuyo principal objetivo es satisfacer las necesidades nutritivas de los animales, en estado natural, fresco o conservado, y los productos derivados de su transformación industrial, así como las sustancias orgánicas o inorgánicas, tanto si contienen aditivos para piensos como si no, destinadas a la alimentación de los animales por vía oral, directamente como tales o transformadas, o en la preparación de piensos compuestos o como soporte de premezclas.

En el Reglamento EC 575/2011 de la Comisión de 16 de junio de 2011 (EC, 2011b) se recoge el catálogo de materias primas que pueden ser empleadas para la elaboración de piensos. Diversos autores han estudiado la presencia de micotoxinas en materias primas e ingredientes de origen vegetal empleados en la elaboración de piensos destinados a la alimentación de diferentes especies animales (Streit et al., 2012; Streit et al., 2013b; Mortensen

et al., 2014) así como en silos y forrajes (Rasmussen et al., 2010; Sørensen et al., 2008; Santos et al., 2010).

1.2.2. Piensos compuestos

De acuerdo al Reglamento EC 178/2002 (EC, 2002b), se entiende como pienso cualquier sustancia o producto, incluidos los aditivos, destinado a la alimentación por vía oral de los animales, tanto si ha sido o no transformado completa o parcialmente.

Los piensos compuestos, por su propia composición, representan un excelente substrato para el crecimiento fúngico, especialmente bajo condiciones favorables de temperatura y humedad (Labuda et al., 2003). Por ello, los productos destinados a la alimentación animal deben ser sometidos a estrictos controles para verificar que son sanos, auténticos y de calidad comercial y, por tanto, su uso no debe representar peligro alguno para la salud humana, para la salud animal ni para el medio ambiente, ni puede ser perjudicial para la producción ganadera.

Las empresas de materias primas y de piensos deben asegurarse de que puede seguirse la pista de cualquier producto alimenticio, pienso o ingrediente de un pienso en cualquier punto de la cadena alimentaria, desde la misma explotación hasta el establecimiento ganadero (EC, 2005).

La alimentación de los animales de acuicultura, en particular de peces, es un elemento clave de su sostenibilidad (Anater et al., 2016). En 2012 se utilizaron en España 109.200 toneladas de pienso compuesto, sobre todo de tipo extrusionado, de las cuales, la mayor parte (83,5%) se destina a peces marinos (dorada, lubina, corvina, rodaballo, anguila y lenguado). El pienso es

fabricado en su mayor parte en instalaciones localizadas en España, completándose con importaciones desde otros Estados Miembros de la UE, principalmente desde Francia y Portugal. La localización en España de las fábricas de pienso facilita la realización de una importante actividad de investigación e innovación en el campo de la nutrición y la alimentación de los peces, promovida desde las propias empresas fabricantes de pienso y también por los centros públicos de investigación y las universidades (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2016).

1.2.3. Sustancias indeseables en la alimentación animal

El concepto de sustancia indeseable se define en la Directiva 2002/32/EC (EC, 2002a) como cualquier sustancia o producto, con excepción de los agentes patógenos, que está presente en el producto destinado a la alimentación animal, y que constituyen un peligro para la salud humana o animal o para el medio ambiente, o que pueda afectar negativamente a la ganadería de producción. Sus objetivos son unificar los anexos que describen los límites máximos de sustancias indeseables, establecer las posibilidades de intervención y de descontaminación y prohibir las mezclas con fines de dilución. Esta directiva ha sido incorporada al ordenamiento jurídico interno a través del Real Decreto 465/2003, de 25 de abril, sobre sustancias indeseables en la alimentación animal, el cual regula las sustancias indeseables en los productos destinados a la alimentación animal y los niveles máximos tolerados de sustancias indeseables en aquellos, a fin de proteger la salud humana, la sanidad animal y el medio ambiente. Entre las sustancias indeseables reguladas por esta norma destacan las micotoxinas, metales pesados, dioxinas, plaguicidas y PCBs, entre otros.

Como se ha indicado anteriormente, de acuerdo con el Plan Nacional de Investigación de Residuos, de entre los contaminantes a analizar en diferentes especies animales y productos derivados, destaca la escasez de datos sobre la presencia de micotoxinas en peces de acuicultura, pese al enorme crecimiento de este sector y el importante incremento del consumo de pescado. Por ello, el estudio de la presencia de micotoxinas en los piensos empleados en el sector de la acuicultura y la transferencia de éstas a los peces alimentados con piensos contaminados es esencial.

1.3. Acuicultura: Aspectos generales

La Acuicultura se define como el cultivo de organismos acuáticos, incluyendo peces, moluscos, crustáceos y plantas acuáticas, cuya actividad implica la intervención del hombre en la cría de los mismos con técnicas encaminadas a aumentar, por encima de las capacidades naturales del medio, la producción de estos organismos, a lo largo de toda la fase de cría o de cultivo y hasta el momento de su recogida. La FAO considera que la acuicultura contribuye a la utilización eficaz de los recursos naturales, a la seguridad alimentaria y al desarrollo económico, con un limitado y controlable impacto sobre el medio ambiente (FAO, 2016).

La primera referencia escrita sobre producción de peces continentales en España data del año 1129, cuando, a iniciativa del arzobispo Gelmírez, se construyó un criadero de peces en Galicia. No obstante, la acuicultura industrial inició su desarrollo en 1961 con la instalación de Riezu, en Navarra. En 1964 se alcanzó una producción de 25.000 kg, y desde entonces ha seguido aumentando. A mediados del siglo pasado, en los años 40, se inicia el cultivo del mejillón en bateas, impulsado por la gran demanda de productos del mar.

Este cultivo ha supuesto un éxito sin precedentes y ha abierto el camino a la acuicultura. Posteriormente, en los años 60, comienza el desarrollo industrial del cultivo de la trucha arcoíris en las aguas continentales, siguiendo el ejemplo de otros países. En la actualidad, la producción de trucha constituye un sector consolidado en el mercado español, pasando de una demanda prácticamente circunstancial a un consumo medio de más de 0,5 kg por habitante y año (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2000).

La estabilización de la pesca en unos niveles prácticamente imposibles de superar, junto con el aumento incesante de la demanda de productos acuáticos, es lo que ha impulsado el desarrollo de la acuicultura como alternativa para el abastecimiento mundial de estos productos. Es por ello que el desarrollo de esta actividad continúa su avance y consolidación en el mundo, tanto en países desarrollados como en aquellos en vías de desarrollo, contribuyendo de manera importante y en numerosos países a reducir la pobreza, incrementando los ingresos económicos de las familias, además del acceso a los alimentos, y fomentando el comercio local e internacional.

Más de la mitad del total de los productos acuáticos consumidos hoy en el mundo procede de granjas de acuicultura, estimándose que en 2030 esa proporción será superior al 65%. Como se muestra en la figura 3, en 2014 se alcanzó un hito cuando la contribución del sector acuícola al suministro de pescado para consumo humano superó por primera vez la del pescado capturado en el medio natural (FAO, 2016).

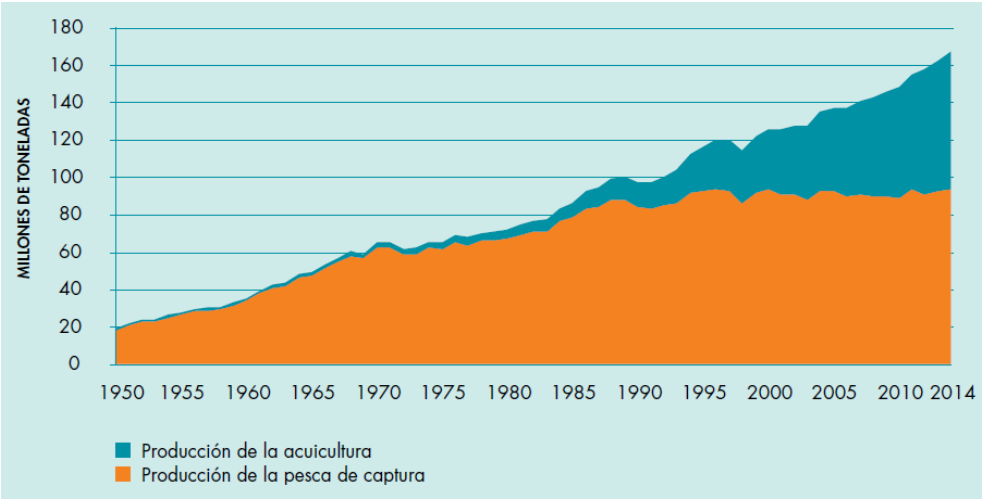

Figura 3. Estado mundial de la acuicultura y la pesca de captura 1950-2014 (FAO, 2016).

1.3.1. Producción mundial y europea

La producción mundial de pescado sigue creciendo a un ritmo más rápido que la población mundial, y la acuicultura se mantiene como el sector de producción de alimentos de más rápido crecimiento, proporcionando, actualmente, casi la mitad del total de pescado destinado a la alimentación humana. Tal y como prevé la FAO, la acuicultura supone la única posibilidad de mantener la proporción de pescado en la dieta mundial dentro de su estrategia para asegurar el suministro adecuado de alimentos, debido al estancamiento de la actividad extractiva (FAO, 2000). Así, en la tabla 3 se muestra el aumento de la producción procedente de la acuicultura frente al estancamiento de la pesca de captura en los últimos años.

Tabla 3. Producción de la pesca y la acuicultura a nivel mundial (FAO, 2016).

Producción	Millones de toneladas					
	2009	2010	2011	2012	2013	2014
Pesca de captura						
Continental	10,5	11,3	11,1	11,6	11,7	11,9
Marina	79,7	77,9	82,6	79,7	81,0	81,5
TOTAL de capturas	**90,2**	**89,1**	**93,7**	**91,3**	**92,7**	**93,4**
Acuicultura						
Continental	34,3	36,9	38,6	42,0	44,8	47,1
Marina	21,4	22,1	23,2	24,4	25,5	26,7
TOTAL acuicultura	**55,7**	**59,0**	**61,8**	**66,5**	**70,3**	**73,8**

En el entorno europeo, cabe destacar la expansión de la acuicultura en Noruega con el salmón, que pasó de una producción de 80.522 Tm en 1988 a 360.536 Tm en 1998. La principal especie de pescado de crianza producido en la Unión Europea es la trucha arco iris (*Onchorhinchus mykiss*), de la que en 2013 se produjeron 179 Tm, el 26,8% del total de pescado de acuicultura producido y cuya producción sigue en aumento (Tabla 4). La segunda especie es el salmón atlántico (*Salmo salar*), con 163.000 toneladas en 2013 y 175.000 el pasado año. La tercera especie más producida es la dorada con 85.000 toneladas, lo cual muestra un notable descenso (del 7,5%) respecto al informe del pasado año.

Tabla 4. Principales especies de peces producidas mediante acuicultura en la UE, por toneladas, en 2016 (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente , 2016b).

Especie	Nombre científico	Toneladas
Trucha arcoiris	*Onchorhinchus mykiss*	194.081
Salmón atlántico	*Salmo salar*	175.090
Dorada	*Sparus aurata*	85.483
Carpa común	*Cyprinus carpio*	70.938
Lubina	*Dicentrarchus labrax*	63.965
Rodaballo	*Psetta maxima*	11.849
Pez gato	*Clarias gariepinus*	4.986
Anguila europea	*Anguilla anguilla*	4.570

1.3.2. Producción española

España en 2013 es el Estado miembro de la UE con un mayor volumen de producción en acuicultura, con 223,09 Tm (17,5% del total de la UE), seguido por Francia con 202,10 Tm (el 15,8%) y el Reino Unido con 194,30 Tm (15,2%). Según las características de cada zona litoral, han evolucionado los distintos tipos de acuicultura, así, la acuicultura marina se desarrolla principalmente en el Cantábrico y en Galicia, donde se ha desarrollado el cultivo de especies de agua fría, principalmente moluscos, rodaballo y salmón. El engorde del salmón atlántico en España no ha llegado a desarrollarse, fundamentalmente por estar en el límite sur de la distribución natural de esta especie y por no poder competir con las industrias del norte de Europa.

Las regiones de aguas más templadas, Mediterráneo y Sur-Atlántica, se dedican fundamentalmente a la producción de lubina y dorada, generalmente en sistemas de jaulas flotantes. Esto también se produce en Canarias y Baleares, mientras que en Valencia predomina el cultivo de la anguila. En los últimos años se ha conformado un potente sector productor de acuicultura en nuestra comunidad que aprovecha las especiales condiciones de nuestras costas, liderando la producción de peces en el Mediterráneo español y en el conjunto nacional mediante la cría de diferentes especies de peces y moluscos (Ley 5/2017).

Por otro lado, el gran desarrollo de la acuicultura continental se ha basado en la producción de trucha, aunque a menor escala también se ha desarrollado el cultivo de otras especies, entre ellas la tenca, que se produce en lagos y embalses de Extremadura y Castilla y León, la carpa, producida principalmente en Baleares, el esturión y el cangrejo rojo (*Procambarus clarkii*) en la cuenca del Guadalquivir.

1.3.3. Consumo a nivel mundial, comunitario y nacional

Dentro del panorama mundial, España constituye uno de los principales países consumidores de pescado, con una de las tasas de consumo más altas del mundo (25,9 kg/per capita en 2015), sufriendo un desabastecimiento crónico de este producto. Esta tasa le sitúa como líder de consumo en Europa, a la par con Noruega y Portugal. Esta fuerte demanda procede de la tradición centenaria de consumo de pescado. Además, cabe destacar que la demanda de pescado en los mercados españoles y europeos presenta unas altas expectativas debido, fundamentalmente, a los cambios de preferencias en la dieta,

sustituyendo la carne por el pescado debido a los componentes más saludables de este último (FAO, 2000).

El gasto de los españoles en productos pesqueros mantiene un aumento anual paulatino, alcanzando en 2012 el 14% del gasto total en alimentación y bebidas, constituyendo así el segundo capítulo de gasto, inmediatamente después del grupo de carne y productos cárnicos. Tan considerable consumo responde a múltiples causas, de entre las cuales cabe destacar una tradición cultural y gastronómica favorecida por la gran diversidad ecológica y de especies capturables, así como el alto valor gastronómico y nutritivo de la gran mayoría de las especies comercializables. Además de ser una fuente rica en proteínas de alta calidad y fácil digestión que contiene todos los aminoácidos y ácidos grasos esenciales junto a vitaminas (E, A y complejo B) y minerales (como calcio, yodo, zinc, hierro y selenio).

Además, en las últimas décadas se ha producido una notable diversificación de la oferta, con la presencia en el mercado de múltiples productos derivados, caracterizados en general por su fácil conservación, rápido cocinado y precio asequible.

El consumo *per capita* mundial de productos acuáticos se ha visto incrementado gracias al incesante aumento de producción de alimentos acuáticos, las mejoras en las técnicas de conservación del pescado y unos canales de distribución más eficientes. Este crecimiento significativo del consumo de pescado ha mejorado las dietas de las personas en todo el mundo gracias a alimentos diversificados y nutritivos. En 2013, el pescado representó alrededor del 17% del consumo de proteínas animales de la población mundial y el 6,7% de las proteínas consumidas en total. Asimismo, el pescado

proporcionó a más de 3.100 millones de personas casi el 20% de la ingestión promedio de proteínas de origen animal *per capita*.

1.4. Prevención y reducción de la contaminación por micotoxinas

Como parte de las estrategias de prevención frente a las micotoxinas, los diferentes tratamientos se dividen en tres grupos: tratamientos físicos, biológicos y químicos. La aplicación de alguno de estos métodos puede reducir la presencia de micotoxinas en alimentos o bien transformarlas en metabolitos, generalmente menos tóxicos que la molécula original (Shams et al., 2011).

En los últimos años, diversos estudios se han centrado en el desarrollo de estrategias para reducir los niveles de micotoxinas durante la producción de alimentos y piensos. Según los resultados obtenidos por otros autores, el procesado de los cereales tiene efectos sobre las micotoxinas, lo que conduce a alimentos menos contaminados en comparación con las materias primas empleadas en su elaboración (Hu et al., 2014a). Sin embargo, las fracciones no destinadas a consumo humano en las que quedan retenidas y concentradas las micotoxinas son, a menudo, empleadas como materias primas en alimentación animal, como es el caso de los granos y solubles procedentes de la destilería (DDGS) (Wu & Munkvold, 2008; Manning & Abbas, 2012; Rodrigues & Chin, 2012; Oplatowska-Stachowiak et al., 2015).

Diferentes procesos industriales han demostrado ser prácticas eficaces para reducir los contenidos de micotoxinas. En este sentido, el contenido de algunas micotoxinas puede reducirse debido al procesamiento térmico aplicado a los alimentos, como asado, hervido, horneado, frito, y pasteurizado (Kabak, 2009). En la elaboración de los piensos, esta reducción puede tener lugar

durante los procesos de extrusión y/o peletización, debido a las temperaturas alcanzadas. La reducción obtenida depende de varios parámetros, algunos de ellos relacionados con la naturaleza y estructura química de las micotoxinas, así como el nivel inicial de contaminación. Otros parámetros están relacionados con el tratamiento aplicado, tales como la temperatura, el tiempo, el pH, la humedad, etc (Bretz et al., 2006; Ryu et al., 2008; Serrano et al., 2013).

En cuanto a la reducción lograda en micotoxinas tradicionales de *Fusarium*, algunos autores han reportado la degradación del NIV a altas temperaturas y tiempo de calentamiento prolongado (Bretz et al., 2005). Beyer et al. (2009) han descrito la reducción de las micotoxinas en función de la interacción con otros componentes como el azúcar, el almidón y las proteínas. En el estudio realizado por estos autores, el comportamiento de la toxina T-2 en condiciones típicas de procesamiento de los alimentos se evaluó mediante la aplicación de diferentes ensayos de calentamiento. La degradación de la T-2 se observó en todas las condiciones, viéndose acelerada por el aumento de la temperatura, y observando la mayor degradación en el modelo proteico.

Sin embargo, los estudios realizados con FBs muestran mayores porcentajes de reducción en los sistemas modelo en presencia de azúcares (Bullerman & Bianchini, 2007). La mayoría de los datos indican que los niveles de FBs se reducen durante el calentamiento, la cocción, la fritura, el tostado, la nixtamalización y la cocción por extrusión de los alimentos, concluyendo que la reducción está directamente relacionada con el tiempo y la temperatura aplicada, especialmente en los alimentos que alcanzan temperaturas superiores a 150ºC (De Girolamo et al., 2016). La reducción de los contenidos de micotoxinas en alimentos ha sido evaluada mediante diferentes estrategias de

descontaminación, como por ejemplo la aplicación de compuestos como el alil isotiocianato (Saladino et al., 2016) y también con la finalidad de prolongar la vida media de algunos alimentos (Saladino et al., 2017). Los estudios han sido llevados a cabo mediante el ensayo de diferentes AFs. Sin embargo, se dispone de datos limitados sobre los efectos en los contenidos de ENNs y BEA por el procesado de los alimentos. Como se indica en el dictamen científico de la EFSA sobre los riesgos para la salud humana y animal relacionados con la presencia de BEA y ENNs en los alimentos y piensos, se necesitan más datos para evaluar el papel de la temperatura aplicada en la reducción del contenido de estas micotoxinas (EFSA, 2014). Por un lado, algunos autores han evidenciado la reducción del contenido de ENs de las micotoxinas de *Fusarium* y beauvericina (BEA) por tratamientos térmicos (Meca et al., 2013; García-Moraleja et al., 2015). Por otra parte, estudios recientes han demostrado que los contenidos de ENNs se reducen a través de procesos industriales comunes, tales como panificación (Vaclavikova et al., 2013; Hu et al., 2014b), elaboración de cerveza (Meca et al., 2013; Hu et al., 2014a) y la elaboración de pasta (Tittlemier et al., 2013; García-Moraleja et al., 2015; Serrano et al., 2016).

Por lo que respecta a las muestras de pescado, los tratamientos térmicos se aplican con el fin de cocinarlos previamente a su consumo. Sin embargo, no hay datos disponibles relacionados con el empleo de procesos térmicos aplicados con la finalidad de reducir el contenido de micotoxinas en los filetes de pescado (Tolosa et al., 2014). Algunos estudios se han centrado en el aislamiento y caracterización de nuevos productos derivados de micotoxinas y generados después del tratamiento térmico aplicado a los alimentos, así como en la evaluación de la toxicidad de estos compuestos. En la mayoría de los casos, los productos de degradación son menos tóxicos que sus moléculas

originales (Bretz et al., 2005; Beyer et al., 2009). Shams et al. (2011) han descrito un nuevo derivado menos tóxico del DAS después del tratamiento térmico aplicado a las patatas.

1.5. Detección e identificación de metabolitos y productos de degradación

En la actualidad se están llevando a cabo análisis multirresiduo empleando la cromatografía líquida acoplada a detectores de masa "full-scan" como el tiempo de vuelo (TOF) y orbitrap. Estas técnicas pueden ser empleadas para la elucidación estructural, identificación y caracterización de compuestos por masa exacta, ya que los métodos non-target empleando analizadores TOF y Orbitrap son capaces de la determinación simultánea de cualquier compuesto en HRMS (Flores, 2015).

Los diferentes tratamientos aplicados para la reducción de micotoxinas así como ciertos mecanismos de detoxificación empleando sustancias presentes de forma natural en algunos alimentos de origen vegetal, pueden dar lugar a diferentes productos de degradación así como a las llamadas micotoxinas modificadas o enmascaradas. Además, el metabolismo de las micotoxinas ingeridas por los animales destinados al consumo humano puede generar su acumulación en diferentes órganos y tejidos, afectando potencialmente a la salud humana, ya que las micotoxinas pueden entrar así en toda la cadena alimentaria a través de una amplia gama de productos alimenticios (Brera et al., 2014). Algunos estudios indican la presencia de micotoxinas emergentes de *Fusarium* o sus metabolitos en productos de origen animal como leche, huevo y carne, entrando en la cadena alimentaria y llegando así al consumidor (Yiannikouris & Jouany, 2002; Jestoi et al., 2009; Zhao et al., 2015).

Las ENNs del tipo B son las que presentan mayor incidencia en diferentes estudios, y en concreto la ENN B. En la literatura científica se han descrito hasta un total de 13 metabolitos de esta micotoxina en estudios in vitro formados por reacciones de biotransformación en fase I en microsomas hepáticos (Rodríguez-Carrasco et al., 2016). Éstos han sido identificados gracias al empleo de equipos analíticos que permiten la detección de compuestos por masa exacta (Ivanova et al., 2011).

En la actualidad es muy difícil detectar estos compuestos en los análisis rutinarios para determinar micotoxinas. Es por ello, que son necesarios métodos analíticos específicos para la identificación de los productos de transformación, tanto de la degradación de las micotoxinas como de su conjugación con otros compuestos presentes en la matriz alimentaria, con el fin de poder evaluar la exposición real del consumidor a estos compuestos.

1.6. Evaluación del riesgo

La evaluación del riesgo se define como la evaluación de la probabilidad de que tengan lugar efectos adversos para la salud, conocidos o potenciales, resultantes de la exposición de los seres humanos a peligros trasmitidos por los alimentos, siendo la base científica para el establecimiento de límites reglamentarios (FAO, 2002). En la Unión Europea, el Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) y la EFSA son los organismos encargados del proceso de evaluación del riesgo de contaminantes alimentarios, a través de las etapas de identificación y caracterización del peligro, evaluación de la exposición y, finalmente, la caracterización del riesgo. La evaluación de la ingesta diaria estimada (EDI) es esencial para la evaluación del riesgo, por lo que los datos toxicológicos, los datos de presencia de

micotoxinas en productos alimenticios y los datos de consumo proporcionan la información necesaria para la evaluación del riesgo. Además, en el caso de algunos contaminantes, como las micotoxinas, es importante conseguir datos detallados sobre el consumo de alimentos por los grupos de población que potencialmente pueden contenerlas a fin de evaluar la exposición.

Consecuentemente, la evaluación del peligro ocasionado por las micotoxinas ha conducido a JECFA a la estimación de las dosis consideradas como seguras, como la ingesta diaria tolerable (TDI) o, en caso de escasez de datos fiables sobre las consecuencias de la exposición humana, a la estimación de la ingesta diaria tolerable máxima provisional (Provisional Maximum Tolerable Daily Intake, PMTDI) o la ingesta semanal tolerable máxima provisional (Provisional Maximum Tolerable Weekly Intake, PMTWI). Actualmente, la EFSA ha establecido ingestas tolerables para, la suma de DON y sus formas acetiladas (3-ADON y 15-ADON) (1 μg/kg pc/dia), PAT (0,4 μg/kg pc/dia), la suma de FBs (2 μg/kg pc/dia), OTA (0,017 μg/kg pc/dia), la suma de las toxinas HT-2 y T-2 (0,1 μg/kg pc/dia), ZON (0,25 μg/kg pc/dia) y NIV (1,2 μg/kg pc/dia) (EFSA, 2002ª; EFSA, 2002b; EFSA, 2003; EFSA, 2006; EFSA, 2011a; EFSA, 2011b; EFSA, 2013, respectivamente). No obstante, a día de hoy no existen datos de ingesta diaria admisible (ADI) ni de TDI para las micotoxinas emergentes de *Fusarium* (EFSA, 2014).

2. OBJECTIVES

2. OBJETIVOS

El objetivo general de la presente tesis doctoral es el estudio de la presencia de micotoxinas, sus metabolitos y productos de degradación en diferentes matrices alimentarias.

Para lograr este propósito, se han planteado los siguientes objetivos específicos:

1. Validación de métodos de análisis multi-micotoxina en diferentes matrices alimentarias.
2. Evaluación de la presencia de micotoxinas en alimentos de origen vegetal y en pescado procedente de acuicultura.
3. Estudio del procesado térmico sobre el contenido de micotoxinas.
4. Identificación de la presencia de productos de degradación originados por el procesado.
5. Análisis cualitativo de la presencia de metabolitos en tejidos de peces procedentes de acuicultura.

2. OBJECTIVES

The main objective of this research was to survey the natural occurence of mycotoxins, their metabolites and degradation products in different food matrices.

To reach this goal, the following specific objectives were proposed:

1. Method validation for multi-mycotoxin determination in different food and feed matrices.
2. Natural mycotoxin occurrence in food from vegetal origin and in fish from aquaculture.
3. Evaluation of thermal processing treatments on mycotoxin contents and their mitigation.
4. Identification of degradation products originated by food processing.
5. Mycotoxin metabolites detection in different tissues of aquaculture fish.

3. RESULTS

3.1. Nuts and dried fruits: Natural occurrence of emerging *Fusarium* mycotoxins

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Nuts and dried fruits: natural occurrence of emerging *Fusarium* mycotoxins

Josefa Tolosa, Guillermina Font, Jordi Mañes, Emilia Ferrer

ABSTRACT

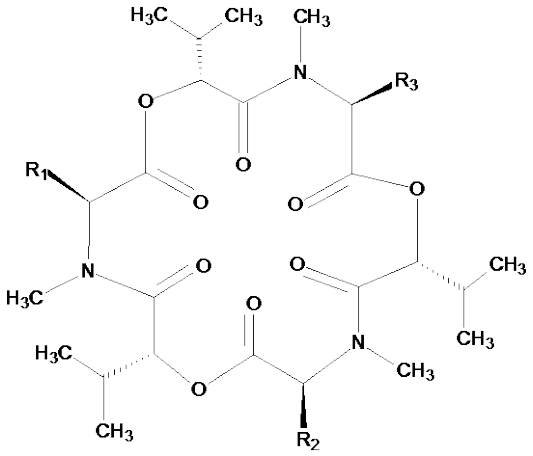
The occurrence of enniatins (ENs) and beauvericin (BEA) in nuts, dried fruits and dates available in Valencia (Spain) was surveyed in this study. To do this, seventy-four samples of nuts, dried fruits and dates were analyzed for the determination of mycotoxin contamination. Mycotoxins were identified and quantified using a ultrasonic-C18 extraction and LC–MS/MS with a triple quadrupole (QqQ) mass analyzer. The frequencies of contamination of nuts, shell, dried fruits and dates were 50%, 80%, 35.7% and 83.3%, respectively. Enniatin A (ENA) was the most predominant EN found in nuts (45.2%) while ENB was the most common EN found in dates (58.3%). The analytical results of the shell samples showed a protective effect of the shell, avoiding the contamination of the fruit with mycotoxins.

Introduction

Mycotoxins are toxic secondary metabolites produced by fungi, mainly *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. They are produced in different substrates under certain climatic conditions of temperature and humidity and in presence of large amounts of nutrients. Mycotoxins are common contaminants of different foodstuffs mainly cereals, but its presence is also found in pasta, fruits, honey, eggs, nuts and dried fruits, among others (Heperkan, 2006; Bircan, 2009; Jestoi et al., 2009).

Emerging mycotoxins include enniatins (ENs) and beauvericin (BEA) and they can be found in various foodstuffs. They were discovered after other *Fusarium* mycotoxins, such as fumonisins and trichothecenes. They are cyclic hexadepsipeptide (Figure 1) with insecticidal properties capable of inducing apoptosis in mammalian cells and have antibiotic properties against gram-positive bacteria and mycobacteria (Jestoi, 2008). These emerging mycotoxins act as enzyme inhibitors (inhibiting the enzyme acyl-CoA and cholesterol acyltransferase), antibacterial, antifungal agents and as immunomodulatory substances.

The toxicity studies have been emphasized on toxicodinamia and primary interaction with target cells, but no data is available of its toxicokinetics (absorption, distribution, metabolism and excretion) or its possible interaction with other mycotoxins (Jestoi, 2008), as well as additive or synergistic effects that may occur when interacting with other mycotoxins or between them (Santini et al., 2009).



	R_1	R_2	R_3
BEA	Phenyl-methyl	Phenyl-methyl	Phenyl-methyl
ENA	*sec*-butyl	*sec*- butyl	*sec*- butyl
ENA1	*sec*- butyl	*sec*- butyl	*iso*- propyl
ENB	*iso*-propyl	*iso*- propyl	*iso*- propyl
ENB1	*iso*- propyl	*iso*- propyl	*sec*- butyl

Figure 1. Structure of BEA and ENs.

They can cause a wide range of toxicological effects both in human and animals, ranging from the development of carcinogenic, teratogenic and mutagenic effects to the production of hormonal and immunosuppressive disorders (Köppen et al., 2010).

Some studies about emerging mycotoxins in foodstuffs are available, but most of them are focused on cereals (Manhine et al., 2011; Oueslati et al., 2011; Meca et al., 2010) and Mediterranean crops (Logrieco et al., 2003). In the

published data, the literature consulted was about aflatoxin (AF) and ochratoxin A (OTA) in nuts and dried fruits (Abarca et al., 2003; Abdulkadaret al., 2000; Bircan, 2009; Cheraghali et al., 2007; Doster and Michailides, 1994; Heperkan, 2006; Yazdanpanah, 2009). However, to our knowledge, there is no data about the occurrence of emerging *Fusarium* mycotoxins in nuts and dried fruits.

Nuts are a matrix susceptible to fungal growth because of their intrinsic characteristics of water activity, moisture and nutrient content, pH and high storage time, which favor the growth of these fungi. In addition, some are collected directly from the soil, such as figs or dates, which favors infection by fungi (Doster et al., 1996; De Mello and Scussel, 2007; Bircan, 2009).

Under the heading of nuts different seeds are included from different botanical families. It is a heterogeneous group with very similar allergenic characteristics and forms of consumption. All of them are oleaginous fruits consumed in dried form. This group include peanuts, almonds, hazelnuts, cashews, walnuts, Brazil nuts, pecan nuts, pistachios, pine nuts and sunflower seeds (AESAN, 2007). In addition, we have analyzed some legume samples (lupines, fried lima beans, chickpeas and pine nuts) because they are consumed in the same way as nuts (such a snack).

In the last few years, increased efforts have been made to develop analytical methods for the detection of very low concentrations of mycotoxins in different food samples by liquid chromatography–tandem mass spectrometry (LC–MS/MS). Furthermore, some authors have developed a multi-mycotoxin liquid chromatography/tandem mass spectrometry method for the

quantification of various mycotoxins simultaneously, including emerging mycotoxins such as ENs and BEA (Sulyok et al., 2006; Berthiller et al., 2007; Monbaliu et al., 2009; Monbaliu et al., 2010; Van Pamel et al., 2011). However, these studies dealt with cereal samples, except Sulyok et al. (2007) and Spanjer et al. (2008), that applied the method to chestnuts (and other foodstuffs) and peanuts, pistachios, raisins and figs (among cereals). Recently, in our laboratory a new rapid, sensitive and reproducible analytical strategy has been developed to determinate the emerging mycotoxins in nuts and dried fruits using a ultrasonic-C18 extraction and LC–MS/MS with a triple quadrupole (QqQ) mass analyzer.

Because of the susceptibility to mycotoxin contamination in nuts and the increased consumption of them (AESAN, 2007), currently, the aim of this study is to determine emerging mycotoxins in nuts and dried fruits by ultrasonic extraction and determination by liquid chromatography mass spectrometry (LC-MS/MS) with a triple quadrupole (QqQ).

Material and methods

Chemical and reagents

All solvents (acetonitrile and methanol) were purchased from Merck (Darmstadt, Germany). Deionized water (<8 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) was supplied by Sigma-Aldrich (Madrid, Spain). All solvents were filtered through a 0.22 μm cellulose filter from Membrane Solutions, Texas, USA, before use.

The stock standard of BEA was purchased from Sigma-Aldrich (St. Louis, USA). ENs toxin solutions were provided by Biopure (Tulln, Austria). Individual stock solutions of BEA, ENA, ENB and ENB_1 with concentration of 500 μg/ml were prepared in methanol and ENA_1 with concentration of 250 μg/ml was prepared in methanol. They were stored in glass-stoppered bottles and darkness in security conditions at -20ºC. These stock solutions were then diluted with pure methanol in order to obtain the appropriate working solutions and were stored in darkness at 4ºC until the LC-MS/MS analysis.

Sampling

Seventy-four samples of nuts, including crude peanuts (n=6), toasted peanuts (n=3), fried peanuts (n=2), crude almonds (n=8), toasted almonds (n=2), pistachios (n=3), walnuts (n=5), hazelnuts (n=4) and sunflower seeds (n=5), and dried fruits, including dried apricots (n=2), dried figs (n=2), raisins (n=3), dried plums (n=4), blueberries (n=1), Goji berries (n=2) and dates (n=12) were purchased from Valencian supermarkets and local markets. Besides, we decided to analyzed 6 samples of legumes (lupines, fried lima beans, chickpeas and pine nuts) and 4 samples of fried maize because of their treatment (roasted) and their way of consumption (as snacks) we considered interesting to include them in the study.

Its composition includes less than 50% of water and a low quantity of carbohydrates (except walnuts), but they are rich in protein (10-30%) and fat (30-60%), specially mono and polyunsaturated fatty acids (AESAN, 2004).

All samples were stored in a dark and dry place until analysis. After their packages had been opened they were put into specific glass food containers

and analyzed within 3 days. Shell samples were separated by analyzing the fruit on one side and the shell on the other. Previously, according to European Regulation 401/2006, the percentage of shell and fruit was calculated for each sample to estimate the exact content of each mycotoxin in them.

Mycotoxin extraction procedure

The method used for mycotoxins analysis (ENs and BEA) was previously optimized in our laboratory. The optimization results are showed in section 3.1. A 10 g aliquot of each nut samples was homogenized with 50 ml of acetonitrile for 30 min and 35°C using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). The extract was centrifuged at 3544.4 g for 15 min and 5°C. The supernatant was filtered and purified using C18 columns (Waters, Milford, Massachusetts) by applying a slight vacuum. The extract was transferred to a 15ml conical tube and evaporated to dryness at 35ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the solution was reconstituted with 1 ml of AcN-MeOH 50:50 v/v and placed again in the ultrasonic bath (30 min, 35°C). Then, the solution was filtered through 13 mm/0.22 μm nylon filter (Membrane Solutions, Texas, USA) prior to injection into the LC-MS/MS system. The samples were extracted in triplicate.

LC-MS/MS analysis

The method of analysis by MS/MS was optimized according to the guidelines established by the European Commission (Commission Decision, 2002; Commission Regulation, 2006). The optimization of MS/MS conditions was performed by direct injection of individual standards at 100 μg mL-1 in "full

SCAN", both positive and negative ESI mode. The most abundant mass to-charge ratio (m/z) was selected for each compound of interest. The mycotoxins exhibited precursor ions and product ions with reasonably high signal intensities in positive ESI mode (ESI^+), being found protonated molecules $[M+H]^+$, sodium adduct ions $[M+Na]^+$ and potassium adduct ions $[M+K]^+$. The adducts of $[M+Na]^+$ and $[M+K]^+$ were removed with the addition of ammonium formate at a concentration of 10 mM to the mobile phase and with the increase cone voltage at 40 eV.

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA) consisting of an autosampler, a quaternary pump, and a pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Version 4.1, were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C18 (150 mm x 4.6 mm I.D., 5 µm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a guard column C18 (4 mm x 2 mm I.D.). The analytical separation for LC-MS/MS was performed using gradient elution with 90% of methanol (with 10 mM of ammonium formate) as mobile phase A, and 10% of acetonitrile as mobile phase B, increasing linearly to 50% B for 10 min; then, decreasing linearly to 40% B for 3 min, and then gradually up to 10% B for 5 min. Finally, initial conditions were maintained for 3 min. Flow rate was maintained at 0.2 ml min. Analysis was performed in positive ion mode (ESI^+). The ESI source values were as follows: capillary voltage, 3.50 kV; source temperature, 100 ºC; desolvation temperature, 300 ºC; cone gas 50l/h; desolvation gas (nitrogen 99.99% purity) flow, 800 l/h.

The analyser settings were as follows: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, -3 and 1; multiplier, 650; collision gas (argon 99.99% purity) pressure, 3.83 x 10-3 mbar; interchanel delay, 0.02 s; total scan time, 1.0s; dwell time 0.1 ms. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode. All time measurements were carried out in triplicate. The MRM optimized parameters cone voltages and collision energies were: 35 eV, 40V for ENs and 50 eV, 40V for BEA, respectively. According to European Union criteria (Commission Decision, 2002), which establishes that a substance can be identified using LC-MS/MS in MRM mode by at least two transitions, the most abundant product ions were selected for quantification and the second one for confirmation.The precursor and product-ions selected were 681.9 $[M+H]^+$, 228.2 and 210.0 for ENA; 667.9 $[M+H]^+$, 228.2 and 210.0 for ENA1; 639.8 $[M+H]^+$, 214.2 and 196.2 for ENB; 654.9 $[M+H]^+$, 214.2 and 196.2 for ENB1; 784.4 $[M+H]^+$, 244.0 and 262.0 for BEA.

Results and discussion

Method performance

Method validation was carried out according to the guidelines established by the European Commission (Commission Decision, 2002/657/EC). The method validation included the determination of linearity, limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision) and reproducibility (inter day precision).

In order to determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in methanol and

from the standards prepared in extract of blank sample. All mycotoxins exhibited good linearity over the working range (low concentration level at LOQ), and the regression coefficient of calibration curves was higher than 0.992.

The LODs and LOQs were estimated from an extract of a blank sample, fortified with decreasing concentrations of the analytes. For 6 days additions were performed from three different blank samples (n=18), to the estimated concentrations for each mycotoxin. The LODs and LOQs were calculated using the criterion of S/N ≥ 3 and S/N ≥ 10 for LOD and LOQ, respectively. LODs for ENA, ENA_1, ENB, ENB_1 and BEA were 0.15, 0.08, 0.15, 0.15 and 0.02 μg/ kg, respectively. LOQs for ENA, ENA1, ENB, ENB1 and BEA were 0.5, 0.25, 0.5, 0.5 and 0.1 μg /kg, respectively.

The recoveries, intra-day precision and inter-day precision were evaluated by spiking different levels of standard analyte to samples at two spiked levels (LOQ and 100 times LOQ). RSD values ranged between 4 and 11% for the intra-day precision, and between 5 and 15% for the inter-day precision. Recovery ranges for the low spiked level (LOQ) and the high spiked level (100 x LOQ) were 85-110% and 86-112%, respectively. These values agree with EU criteria (Commission Decision, 2002).

For the evaluation of matrix effects, calibration curves were constructed for each studied mycotoxin from the standards prepared in methanol and from the standards prepared in extract of blank sample. For this purpose, a mixture of blank extracts of nuts was prepared as blank sample. A suppression of the signal for all mycotoxins was observed (from 40% to 70%). Therefore, we

employed LC-MS/MS with matrix-matched calibration standards prepared with different types of nuts samples to minimize these matrix effects and for a selective and reliable mycotoxin quantification.

Occurrence of ENs and BEA in analyzed samples

Results of the natural occurrence of ENs and BEA in analyzed samples of nuts (fruit and shell) and dried fruits are summarized in tables 1 and 2. Fourty-seven samples out of seventy-four (63.5%) were contaminated with ENs and BEA. The highest incidence was found in dates (91.6%), peanuts (81.8%), walnuts and sunflower seeds (80%).

Thirty-five samples out of seventy-four (26%) presented coexistence of 2 or more mycotoxins simultaneously, while in almonds and hazelnuts, one hundred percent of the samples had contents of all mycotoxins analyzed simultaneously; in walnuts and sunflower seeds it is 75%; in peanuts, pistachios and fried maize it is 66.7%; in dried fruits it is 50% and in dates it is 45.5%.

Thirty-nine samples (52.7%) are contaminated with at least one of the four ENs and fifteen samples out of seventy-four (20.3%) are contaminated with BEA.

Twelve samples of shell were contaminated with at least one of the four ENs (80%) and only one shell sample was contaminated with BEA (6.7%). The highest contents of ENA, ENA_1 and ENB were obtained in shell samples, specifically in peanuts (23.300 mg/kg, 0.523 mg/kg and 14.610 mg/kg, respectively) (Figure 2) and sunflower seeds (2.620 mg/kg, 0.151 mg/kg and 2.750 mg/kg respectively). In the remaining shell samples analyzed, levels of

mycotoxins were similar in the fruit and in the shell. In some matrices, such as pistachios, ENA_1 was found only in the shell, but in a very low range. It is noteworthy that in the case of shell samples the presence of emerging mycotoxins was found in the peel and not in the fruit. According to our results, Doster and Michailides (1994) indicate a protective effect on the fruit peel and Pitt and Hocking (2009), indicate that, although the shell represents a physical barrier and protects the developing kernels from most fungal invasion, fungi may still enter via cracks in the shell or via the pegs. On the other hand, the shell has a higher moisture content, which favors mould contamination.

ENB_1 was detected in hazelnuts, sunflower seeds, peanuts, almonds and fried maize. The highest contents were obtained in almonds (0.784 mg/kg) and shell hazelnuts (0.417 mg/kg).

The highest contents of BEA were obtained in sunflower seeds (0.042 mg/kg). Unlike other mycotoxins, BEA in sunflower seeds samples was detected in the fruit, not in the shell, maybe because they are usually slightly damage. The only shell sample positive to BEA was a hazelnut sample (0.030 mg/kg).

Table 1. Occurrence of enniatins (ENA, ENA_1, ENB, ENB_1) in analyzed samples.

Samples	Enniatins (mg/kg)											
	ENA			ENA1			ENB			ENB1		
	Mean	Minimum level	Maximum level	Mean	Minimum level	Maximum level	Mean	Minimum level	Maximum level	Mean	Minimum level	Maximum level
Peanuts fruit	0.647	0.157[a]	1.49	nd	nd	nd	0.098	0.035[a]	0.183	0.115	0.068	0.182
Peanuts shell	7.972	0.258	23.300	0.523	0.523[a]	0.523	14.610	14.610[a]	14.610	nd	nd	nd
Almonds fruit	0.205	0.053[a]	0.46	0.014	0.014[a]	0.014	0.271	0.061[a]	0.65	0.432	0.268[a]	0.784
Almonds shell	0.090	0.090	0.090	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pistachios fruit	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Pistachios shell	0.326	0.039	0.525	0.015	0.015[a]	0.015	0.209	0.022	0.397	nd	nd	nd
Walnuts fruit	0.472	0.120[a]	0.676	nd	nd	nd	0.394	0.052[a]	0.784	nd	nd	nd
Walnuts shell	0.125	0.125	0.125	nd	nd	nd	nd	nd	nd	nd	nd	nd
Fried maize	0.120	0.069[a]	0.176	0.020	0.020[a]	0.020	0.125	0.064[a]	0.186	0.219	0.219[a]	0.219
Hazelnuts fruit	0.263	0.263[a]	0.263	0.007	0.007[a]	0.007	0.146	0.146[a]	0.146	nd	nd	nd
Hazelnuts shell	0.732	0.732	0.732	nd	nd	nd	0.076	0.076	0.076	0.417	0.417	0.417
Sunflower seeds fruit	0.061	0.046[a]	0.076	nd	nd	nd	0.078	0.078[a]	0.078	nd	nd	nd
Sunflower seeds shell	2.620	2.620[a]	2.620	0.026	0.026[a]	0.151	0.047	0.047[a]	2.750	0.220	0.220[a]	0.220
Dates	0.666	0.523[a]	0.764	0.025	0.010[a]	0.034	0.490	0.054	0.72	nd	nd	nd
Dried fruits	0.242	0.180[a]	0.369	0.011	0.011[a]	0.011	0.058	0.058	0.058	0.022	0.022[a]	0.022
Other nuts	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd: not detected.

[a] This is the minimum value only in positive samples to each mycotoxin.

Table 2. Occurrence of beauvericin (BEA) in analyzed samples.

Samples	Beauvericin (mg/kg)		
	Mean	Minimum level	Maximum level
Peanuts fruit	0.015	0.003[a]	0.040
Peanuts shell	nd	nd	nd
Almonds fruit	0.013	0.004[a]	0.033
Almonds shell	nd	nd	nd
Pistachios fruit	nd	nd	nd
Pistachios shell	nd	nd	nd
Walnuts fruit	nd	nd	nd
Walnuts shell	nd	nd	nd
Fried maize	0.013	0.013[a]	0.013
Hazelnuts fruit	nd	nd	nd
Hazelnuts shell	0.030	0.030	0.030
Sunflower seeds fruit	0.023	0.004	0.042
Sunflower seeds shell	nd	nd	nd
Dates	0.006	0.001[a]	0.009
Dried fruits	0.007	0.007[a]	0.007
Other nuts	nd	nd	nd

nd: not detected.

[a] This is the minimum value only in positive samples to each mycotoxin.

In this study, we found high levels for emerging mycotoxins in the analyzed samples, however, as mentioned above, there are no similar studies available in nuts. Regarding the contents described in the scientific literature concerning mycotoxins (aflatoxins) in nuts, they include low levels of mycotoxins. However, the studies relating to emerging mycotoxins, in different matrices (mostly cereals), revealed contamination in the same order (mg/kg) as we have found (Meca et al., 2010; Oueslati, S. et al., 2011; Mahnine et al., 2011). Besides, they found mostly ENA_1 and ENB_1, but in this survey, we found the most prevalent mycotoxins were ENA and ENB.

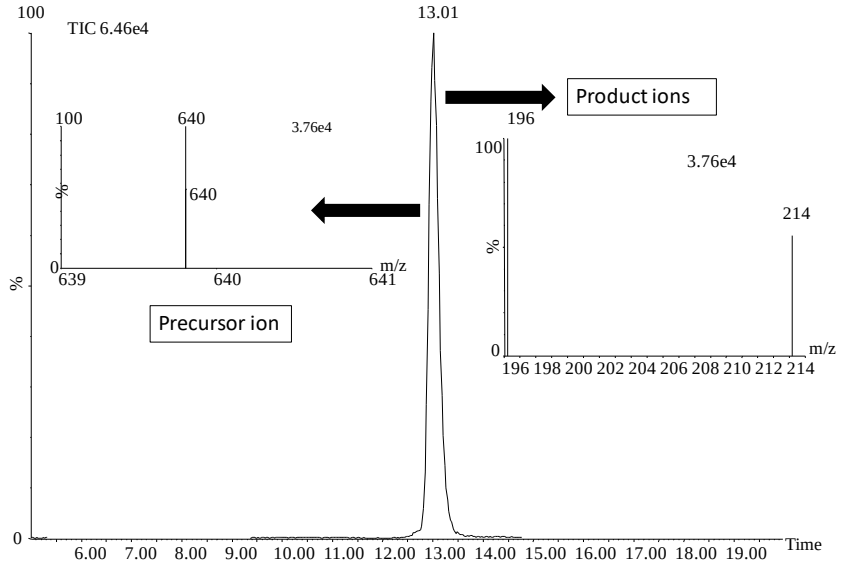


Figure 2. Chromatogram of a positive sample (of peanut shell) for ENB, showing the precursor and product ions.

Respect to mycotoxin contents found in different peanut samples subjected to different heat treatment (see section 2.2) the results are not dependent on the different processes the samples have been subjected to. However, Karathanos and Belessiotis (1997) found that if the fruits were dried by heat treatment or forced air, the toxigenic species may have died because the temperature usually used is 50-70ºC, and Wood (1992) ensured that roasting destroys at least 50% of the aflatoxin in peanuts.

The contents of mycotoxins found in almonds were similar in all samples analyzed; there was no differences between the fruit and peel and between the heat treatment of the samples (see table 1). In addition, ENA_1 was only present in one sample.

In pistachio samples analyzed, like other authors, the higher contents were found in the shell, due to the protective effect of it, as indicated by Doster and Michailides (1994). These authors suggest that most samples have relatively high levels of mycotoxins (aflatoxin) because their shell is damaged or they have "early splits" (the hulls split with the shell, exposing the kernel to molds and insects).

In this way, Yazdanpanah (2009) indicates that pistachios have a high susceptibility to AF contamination and often have high contents of them. Contamination starts when the nuts are still on the tree after ripening but before harvesting. This author suggests that climate variations play an important role, as the amount of rainfall during the period of time between the ripening and harvesting is directly related to the amount of AF in pistachios.

In the literature review, De Mello and Scussel (2007), indicate that walnuts are susceptible to hydrolysis and oxidation due to its components and internal characteristics such as moisture content. Therefore, the moisture content should be kept as low as possible during collection, handling, transportation and storage. In addition, it was found that, as in the rest of samples, the shell has a protective effect avoiding the contamination of the fruit (Freire, 2000).

In hazelnut samples with shell, mycotoxins were not detected in the fruit, results that agree with those obtained by Heperkan (2006). That author suggested that mycotoxin contents in fruits are due to shell damage, like in pistachios.

Contrary to the results described above, the fruit of sunflower seed samples were contaminated, not just shell samples. This is because, in most cases, the

shells of sunflower seeds are slightly damaged, so they can't protect the fruit from mycotoxin contamination.

Mycotoxins were not detected in raisins and lupines. If we consider the global number of dried fruit samples, the incidence of each mycotoxin is much smaller than for the rest of the samples analyzed in this study. In contrast, humidity is higher, and notably, lupines are seasoned with salt, acidulants and preservatives, which can protect it from contamination.

After drying, fruits are then collected and stored. During the drying process, the sugar is concentrated as the moisture content decreases, resulting in a selective medium for xerotolerant moulds such as *A. niger* (Zinedine and Mañes, 2009). The high concentration of sugar and low water activity during this drying process creates a very suitable and selective environment for fungus to grow in most dried fruits (Abarca et al., 2003). OTA production and accumulation in dried figs starts during drying and increases during storage and transportation when favorable environmental conditions occur (Karbancıoglu-Güler and Heperkan, 2009). The nutrition composition of figs fruits, include high levels of sugar like glucose and fructose which promote aflatoxin production, and also stimulates the production of OTA (Bircan, 2009). The presence of other mycotoxins in these matrices could be the cause of the lower incidence of emerging mycotoxins in them.

Mycotoxins were not detected in the samples of cashews (2 samples), chestnuts, fried lima beans, chickpeas and pine nuts analyzed. However, it should be noted the small number of samples analyzed. Sulyok et al. (2007) described the application of a multi-mycotoxin method for the quantification of 87 analytes in chestnuts and other foodstuffs and no traces of ENs and

BEA were found in chestnuts, but they found other mycotoxins, such as sulochrin, emodin, chaetoglobosin A, griseofulvin, alternariol, avenacein, tentoxin and alternariol methylether. Furthermore, Gürses (2006) indicated in his study that aflatoxin B_1 was present in high levels in hazelnuts, walnuts, peanuts and almonds, but no contents of ENs and BEA were detected in roasted chickpeas, as in this study.

Conclusions

The presence of emerging Fusarium mycotoxins (ENs and BEA) was determined in samples of nuts and dried fruits commercialized in Valencia. The results obtained showed high levels of contamination (mg/kg). The percentage of the total contamination of analyzed samples with ENs was 50% for fruit nuts, 80% for shell nuts, 35.7% for dried fruits and 83.3% for dates. The highest level found in positive samples was 23 mg/kg for ENA in shell peanuts.

The percentage of the total contamination of samples analyzed with BEA was 23% for fruit nuts, 6.7% for the shell of the nuts, 7.1% for dried fruits and 25% for dates. The highest level found in positive samples was 0.042 mg/kg in the fruit of sunflower seeds.

ENA and ENB were the most common mycotoxins found in analyzed samples. In fruit samples, the dates were the most contaminated with ENs. For BEA, almonds and sunflower seeds were the commodities more affected than the others. However, BEA was not detected in the shell but it was present in fruit samples. The analytical results of the shell samples showed a protective effect of the shell, avoiding the contamination of the fruit with mycotoxins.

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3.2. Multi-Mycotoxin Analysis in Durum Wheat Pasta by Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry

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Multi-Mycotoxin Analysis in Durum Wheat Pasta by Liquid Chromatography Coupled to Quadrupole Orbitrap Mass Spectrometry

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ABSTRACT

A simple and rapid multi-mycotoxin method for the determination of 17 mycotoxins simultaneously is described in the present survey on durum and soft wheat pasta samples. Mycotoxins included in the study were those mainly reported in cereal samples: ochratoxin-A (OTA), aflatoxin B_1 (AFB_1), zearalenone (ZON), deoxynivalenol (DON), 3-and 15-acetyl-deoxynivalenol (3-AcDON and 15-AcDON), nivalenol (NIV), neosolaniol (NEO), fusarenon-X, (FUS-X), T-2 toxin (T-2) and HT-2 toxin (HT-2), fumonisin B_1 and B_2 (FB_1 and FB_2), and four emerging mycotoxins: three enniatins (ENA, ENA_1, and ENB), and beauvericin (BEA). Twenty-nine samples were analyzed to provide an overview on mycotoxin presence: 27 samples of durum wheat pasta, and two samples of baby food. Analytical results concluded that trichothecenes showed the highest incidence, mainly DON, NIV, and HT-2 toxin, followed by ZON and ENB, while NEO, FUS-X, OTA, AFB_1, and FUM were not detected in any sample. The highest contents corresponded to ENB and ranged from 91.15 μg/kg to 710.90 μg/kg.

1. Introduction

Cereal-based products represent one of the most important dietary items in many countries around the world, mainly those from wheat, which is the most consumed cereal worldwide [1].Usually, wheat is ground to flour for the production of bread, pasta, biscuits, and other products. Durum wheat (*Triticum durum Desf.*) is the most widespread crop in the Mediterranean area. Thus, sixty-seven per cent of the Italian durum wheat production, mainly from the southern regions, is used for producing pasta [2].

Unfortunately, infection of crops and stored cereals with fungi can result in the production of secondary toxic metabolites known as mycotoxins. These hazardous natural toxins can be transferred into the final products, which constitute a main concern for all steps involved in food safety issues due to its implication on human and animal health [3,4]. Furthermore, available data indicated that durum wheat was generally more contaminated than common wheat [5].

Mycotoxins are toxic secondary metabolites produced by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. [6]. Currently, diverse mycotoxins have been identified, but the most important ones regarding their occurrence and toxicity are aflatoxins (AFs), fumonisins (FMs), trichothecenes (TRC), ochratoxins (OTs), patulin (PAT), and zearalenone (ZON) [4].

ZON and its metabolites are estrogenic mycotoxins commonly found in maize, but barley, oats, wheat, rice, sorghum, and soy beans are also susceptible of contamination with *Fusarium* (*F. graminearum*). Those raw cereals, are often contaminated with type B-TRC particularly deoxynivalenol (DON) and nivalenol (NIV). DON is the most prevalent TRC worldwide. TRC are a

family of related sesquiterpenoids and according to their functional groups are divided into four groups (A–D). HT-2, T-2 toxins, diacetoxyscirpenol (DAS) and neosolaniol (NEO) are type A TRC. Type B TRC are represented by DON and its derivates 3-acetyl-deoxynivalenol (3-AcDON) and 15-acetyl-deoxynivalenol (15-AcDON) and also fusarenon-X (FUS-X) and NIV. The most common TRC found in cereal and cereal products include: NIV, DON, FUS-X, 15-AcDON, 3-AcDON, DAS, NEO, HT-2, and T-2 toxins [7]. In this sense, *Fusarium* mycotoxins have been reported as the most prevalent in cereal foodstuffs, mainly type B TRC.

Legislation on the maximum permitted limits in many raw and processed products, mainly for cereals and cereal-based products, has been established by the Commission Regulation (EC) No. 1881/2006 [8] and its amendments. These limits have been set for DON, the key representative of group B TRC, the sum of fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2), ZON, AFs, ochratoxin A (OTA), and PAT. For two other *Fusarium* mycotoxins, HT-2 and T-2 toxin, indicative levels have been recommended in cereals and cereal based products [9].

In addition to those regulated *Fusarium* mycotoxins, other "emerging mycotoxins", such as enniatins (ENs) and beauvericin (BEA), have been reported in cereals mainly in Northern Europe (Norway and Finland) [10], but also in Central and Southern Europe, especially in the Mediterranean area [11]. ENs and BEA are produced by different *Fusarium* species, such as *Fusarium verticillioides*, *Fusarium proliferatum*, *Fusarium subglutinans*, *Fusarium oxysporum*, *Fusarium poae*, and *Fusarium avenaceum*. These molds are common in areas of Central and Southeastern Europe and can affect many important food commodities [12]. The level of contamination reported in literature varies

considerably worldwide according to geographical area, region and year, which ranges from a few ng/g to several μg/g. These mycotoxins have been reported in cereal based products [3] and in pasta samples in higher contents, up to μg/g [13].

Up to now, despite the wide presence of these mycotoxins in different foodstuffs, limited knowledge is available on the transfer of ENs across the food chain and no maximum limits (MLs) are currently available for emerging *Fusarium* mycotoxins. Thus, monitoring studies for them are necessary for legislative purposes, in order to establish an appropriate maximum contamination level by the authorities [14].

With the aim of detecting as many mycotoxins as possible in a single run, multi-mycotoxin methods have been commonly employed in last years [15–18]. These multi-mycotoxin methods often include the analysis of TRC, FUM, and/or ZON, as well as OTA, AFs, and other toxins. All these mycotoxins show very different chemical and physicochemical properties and behaviors and can be present in various matrix–toxin combinations.

Thus, multi-mycotoxin method development employing the same sample preparation and a single final determination is a challenge. In this sense, the most critical step is the extraction, which should allow good recoveries for all mycotoxins under investigation in a specific food matrix. Therefore, it is necessary to develop multi-toxin methods for the simultaneous analysis of emerging and traditional mycotoxins. To our knowledge, this is the first report on multi-mycotoxin analysis in pasta samples by orbitrap mass spectrometry.

Within this context, the aim of the study was to determine the incidence and levels of common mycotoxins (AFB_1, OTA, NIV, NEO, FUS-X, FB_1, FB_2,

DON, 15-AcDON, 3-AcDON, HT-2, T-2, ZON, ENB, ENA, ENA_1, and BEA) found in pasta samples available on the Italian market, by using one single extraction method and comparing the results with the ML established by the EU. Determination has been carried out by UHPLC-orbitrap mass spectrometry.

2. Results

2.1. Method Implementation: Optimization of the UHPLC-Q-Orbitrap Conditions

First, in order to evaluate chromatographic conditions, several experiments were performed on different gradients of mobile phases. Better results were obtained when initializing with 60% phase B. The optimum mass spectrometric parameters for the identification and quantitation of the seventeen analytes were first obtained after analyzing the standard work solution at 100 μg/L. Sensitivity was checked by recording full scan chromatograms in both positive and negative ionization modes. Due to adduct formation with formic acid/ammonium formate buffer, some analytes exhibit strong formic or ammonium adduct species ($[M+FAc]^-$ or $[M+NH_4]^+$, respectively) which appear to be the most predominant ions in the mass spectrum (Table 1).

2.2. Validation Parameters

Recoveries were performed by adding fortification standards to samples at three concentration levels (125 μg/kg, 62.5 μg/kg, and 12.5 μg/kg) using two grain samples (durum wheat, and soft wheat). This procedure was carried out in triplicate.

Table 1. Ultra high performance liquid chromatography **(**UHPLC)/Q-Orbitrap parameters for the 17 mycotoxins analyzed.

Mycotoxin	Retention Time (min)	Elemental Composition	Adduct Ion	Theoretical Mass (m/z)	Measured (m/z)	Accuracy (Δppm)
Neosolaniol	0.49	C19H26O8	$[M + NH_4]^+$	400.19659	400.19638	−0.52
Fusarenon-X	0.47	C17H22O8	$[M + H]^+$	355.13874	355.13846	−0.79
Nivalenol	0.52	C15H20O7	$[M + FAc]^-$	357.11911	357.11771	−3.92
Deoxynivalenol	0.56	C15H20O6	$[M + FAc]^-$	341.12419	341.12378	−1.20
Sum of 3- and 15-AcDon	0.65	C17H22O7	$[M + Na]^+$	361.12577	361.12589	0.33
HT-2 toxin	0.93	C22H32O8	$[M + Na]^+$	447.19894	447.19916	0.49
T-2 toxin	1.22	C24H34O9	$[M + Na]^+$	489.20950	489.20978	0.57
Zearalenone	1.55	C18H22O5	$[M + H]^+$	319.15400	319.15375	−0.78
Aflatoxin B_1	0.71	C17H12O6	$[M + H]^+$	313.07066	313.07014	−1.66
Fumonisin B_1	1.00	C34H59NO15	$[M + H]^+$	722.39574	722.39396	−2.46
Fumonisin B_2	2.47	C34H59NO14	$[M + H]^+$	706.40083	706.40215	1.87
Enniatin A	7.71	C36H63N3O9	$[M + NH_4]^+$	699.49026	699.49048	0.31
Enniatin A_1	7.32	C35H61N3O9	$[M + NH_4]^+$	685.47461	685.47449	−0.18
Enniatin B	6.37	C33H57N3O9	$[M + H]^+$	640.41676	640.41930	3.97
Beauvericin	7.18	C45H57N3O9	$[M + Na]^+$	806.39870	806.39697	−2.14
Ochratoxin A	1.53	C20H18NO6Cl	$[M + H]^+$	404.08954	404.08925	−0.72

As reported in scientific literature, spiked matrices are not affected by the conditions occurring in naturally-contaminated samples, where the mycotoxins are fixed in the matrix due to the fungi production in the substrate. Thus, the procedure of spiking blank matrices may not simulate the real extraction efficiency, due to the fact that mycotoxins are only applied to the surface of the matrix [19].

For this reason, recovery assays were performed in naturally-contaminated samples, taking into account the mycotoxin amount in samples. In this sense, to the final observed concentration after addition, the mycotoxin amount naturally present in the samples had been subtracted. Then, the recovery quantitation was performed using calibration curves constructed in solvent and then corrected by the matrix effect. The mean recoveries calculated for all mycotoxins and for both matrices at the three fortification levels are provided in Table 2.

The accuracy was estimated by calculating the recovery for each compound. Recovery experiments were conducted at three different levels for each matrix as described in Section 5.7.1. The mean recovery values ranged between 67%–206%, 67%–213%, and 69%–210%, respectively. In Table 2, the recovery values represent the average obtained from each spiked level performed in triplicate (n=9). Intraday precision was determined by calculating the relative standard deviation (RSDr), obtained from results generated under repeatability conditions of six determinations for each spiked sample in the same day. RSDr for the validated procedures at each spiked level were lower than 8%, 6%, and 5%, respectively. The same approach was applied for the determination of the interday precision, differing in three days instead of one day. The interday precision was calculated by the relative standard deviation (RSDr) from spiked

samples under reproducibility conditions by one determination per concentration on three consecutive days. RSDr for the validated procedures at each spiked level were lower than 13%, 11%, and 8%, respectively.

2.3. Matrix Effect Studies

Matrix effects for mycotoxins analyzed in the study are showed in Table 2. As it can be observed, except for BEA and some TRC, ME were close to 100%. The matrix effect (signal enhancement or suppression) was investigated by calculating the ratio percentage between the slopes of the matrix-matched calibration curve and the curve in solvent. To correct the matrix effects, matrix-matched calibration curves were constructed initially in the matrix for quantification purposes. However, as it was not possible to find a blank sample for all analyzed mycotoxins, calibration standards were dissolved in solvent, in order to avoid undesirable interactions between naturally occurring mycotoxins and spiked ones. For this reason, the final amount was corrected by the calculated matrix effect for each mycotoxin.

2.4. LOD, LOQ, and Linearity

Sensitivity was evaluated by limit of detection (LOD) and limit of quantitation (LOQ) values. The LODs and LOQs were determined as described in Section 5.7.3. Calculated LOD and LOQ are shown in Table 2. As can be observed, LOD and LOQ for legislated mycotoxins were lower than the ML established. Furthermore, according to Commission Decision EC/2002/657 [20], all mycotoxins exhibited a good linearity in the working range as it is shown in Table 2 expressed as "r^2".

Table 2. Validation results in terms of recovery, matrix effect (ME, expressed in %), limits of detection and quantitation (LOD and LOQ, respectively), linearity (expressed as "r^2") and calibration curve for each mycotoxin.

Mycotoxin	Recovery (%)	ME (%)	LOD (μg/kg)	LOQ (μg/kg)	r^2	Calibration Curve
Neosolaniol	68	36	0.03	0.11	0.9988	Y = −1.091370+644,247X
Fusarenon-X	99	55	2.80	8.42	0.9982	Y = −35,936.3+14,855.6X
Nivalenol	74	35	0.29	0.87	0.9996	Y = −75,591.9+12,7604 X
Deoxynivalenol	89	57	0.03	0.09	0.9989	Y = −199,036+76,100.7 X
Sum of 3- and 15-AcDon	135	102	0.45	1.37	0.9956	Y = 2.296670+629,614 X
HT-2 toxin	118	68	0.15	0.45	0.9993	Y = −569,704+407,261 X
T-2 toxin	149	57	0.23	0.70	0.9997	Y = −1.715200+1.581560 X
Zearalenone	148	86	0.21	0.65	0.9996	Y = −575,778+340,107 X
Aflatoxin B_1	128	64	0.03	0.11	0.9998	Y = 668,377+6.271150 X
Fumonisin B_1	105	72	1.65	4.95	0.9939	Y = −298,323+62,417 X
Fumonisin B_2	132	85	8.21	24.6	0.9864	Y = −655,884+70,612.3X
Enniatin A	165	105	0.39	1.19	0.9999	Y = −186,911+1.928930X
Enniatin A_1	148	90	0.28	0.85	0.9999	Y = −486,269+1.682150X
Enniatin B	123	89	1.08	3.26	0.9971	Y = −255,826+47,880X
Beauvericin	210	17	0.89	2.68	0.9992	Y = −483,543+157,248X
Ochratoxin A	139	91	0.07	0.22	0.9986	Y =−717,566 + 224,556X

2.5. Mycotoxin Occurrence

A multianalyte method employing ultra high performance liquid chromatography coupled with orbitrap high-resolution mass spectrometry has been applied for monitoring cereal-based products. Thus, the proposed method has shown to be suitable to investigate the occurrence of seventeen mycotoxins in a total of twenty-nine commercial pasta (durum wheat and soft wheat baby food). To our knowledge, this is the first report on multi-mycotoxin analysis in pasta samples by orbitrap mass spectrometry.

The mycotoxin incidence and contents in pasta samples, as well as MLs established, are presented in Table 3. As it can be observed, DON was the prevalent toxin (100%), followed by NIV, ZON, ENA_1 (93%), HT-2, and ENB (90%). In the present survey, DON also showed higher contents, which ranged from 20.89 μg/kg to 247.27 μg/kg. Data reported in the present study about DON contamination levels were all very far from the maximum limit (1750 μg/kg) set for unprocessed durum wheat [8]. For NIV, 93% of pasta samples analyzed were positive with an average amount of 15.33 μg/kg. Referent to 3-AcDON and 15-AcDON, scarce literature has been reported regarding wheat contamination. In our study, 87% of wheat samples were positive for the sum of 3-AcDON and 15-AcDON with a mean value of 2.47 μg/kg. Regarding HT-2 and T-2 toxin, mean contents of 36.95 μg/kg and 12.46 μg/kg for T-2 and HT-2, respectively, were found.

Although ZON occurrence has been widely described mainly in maize samples, wheat and other cereals can be also contaminated by this estrogenic mycotoxin. In the present study, ZON was detected in 93% of samples and contents ranged from 16.84 μg/kg to 19.94 μg/kg. The levels found did not

exceed the ML established (20–75 μg/kg). Regarding emerging *Fusarium* mycotoxins, our results showed that ENs were present in 72% of pasta samples analyzed. The percentages for each EN are reported in Table 3, as well as mean values. As it can be observed, ENB showed the highest incidence (90%) and also the highest content reported for all mycotoxins included in the study (710.90μg/kg). The mycotoxins OTA, NEO, FUS-X, AFB_1, and FUM, were not found in the present study, although their presence has been reported by other researchers in cereal samples [21,22].

2.5.1. Mycotoxin Occurrence in Baby Food

Regarding results obtained for baby food samples analyzed, although only two samples were included in the survey, mean contents for some mycotoxins were higher in baby food than in durum wheat pasta samples. In this sense, DON presented a mean value of 103.82 μg/kg and the highest content was 124.55 μg/kg. DON was also one of the mycotoxin with highest incidence together with 3-AcDON, 15-AcDON, ZON and ENA_1. HT-2 and T-2 toxin were only detected in one out of two samples of baby food analyzed, with 7.60 μg/kg and 27.54 μg/kg, respectively. 3-AcDON and 15-AcDON were present in baby food samples analyzed in our survey, but showed lower contents (2.46 μg/kg), similar to those reported for these mycotoxins in durum wheat samples analyzed in our study (2.12 μg/kg). For ZON, contents reported in baby food were the same than contents in durum wheat pasta, 17.52 μg/kg and 17.54 μg/kg, respectively. However, although NIV was present in durum wheat pasta samples, no sample of baby food analyzed were positive for this TRC.

Table 3. Incidence and mycotoxin contents in samples analyzed, and MLs established for cereal foodstuffs.

Mycotoxin	Incidence (%)	Range(Mean) (µg/kg)	IARC Classification	MLs (EC) No. 1126/2007 (µg/kg)
Neosolaniol	ND	ND	NC	No limits established
Fusarenon-X	ND	ND	3	No limits established
Nivalenol	93.33	11.54–16.90(13.87)	3	No limits established
Deoxynivalenol	100.00	20.89–247.27 (96.93)	3	<750
Sum of 3- and 15-AcDon	86.67	1.46–4.18 (2.47)	NC	No limits established
HT-2 toxin	90.00	10.92–14.60 (12.46)	NC	No limits established
T-2 toxin	76.67	36.45–38.02 (36.95)	NC	No limits established
Zearalenone	93.30	16.84–19.94 (17.54)	3	20 *–75
Aflatoxin B_1	ND	ND	1	0.1 *–2
Fumonisin B_1	ND	ND	2B	200–1000**
Fumonisin B_2	ND	ND	2B	
Enniatin A	33.30	4.25–5.09 (4.46)	NC	No limits established
Enniatin A_1	93.30	4.47–18.83 (9.83)	NC	No limits established
Enniatin B	90.00	91.15–710.90 (326.17)	NC	No limits established
Beauvericin	10.00	53.66–73.67 (63.66)	NC	No limits established
Ochratoxin A	ND	ND	2B	0.5*–3

*: values reported for infants; **: limits referred to maize products; ND: not detected; NC: not classified.

2.5.2. Mycotoxin co-occurrence

The simultaneous presence in the same commodity of mycotoxins produced by fungi belonging to different genera is not uncommon [23]. In this sense, given the simultaneous presence of emerging *Fusarium* mycotoxins commonly reported in literature and combinations with the so called traditional *Fusarium* mycotoxins (TRC, FMs, etc.) found by many authors in foodstuffs, special focus should be paid to the simultaneous presence of various mycotoxins in a sample, as synergistic effects have been reported in literature [24].

The co-occurrence of more than two mycotoxins in a single sample has been evidenced, representing a possible health risk than the intake of only one mycotoxin alone due to the combined intake of mycotoxins. In this sense, more than 80% of each sample analyzed were contaminated with 6–10 mycotoxins.

3. Discussion

In the last years, increasing *Fusarium* incidence and a higher DON accumulation in durum wheat has been reported in Italy, especially in northern regions. Its occurrence in durum wheat increases from southern to northern areas in Italy, with a heavy influence of some factors, such as year and area of cultivation [2,7].

Results found in our survey (Table 3) are in accordance with those reported by other authors, who found DON as the most prevalent mycotoxin in wheat and wheat-based foodstuffs [22, 25–30] and pasta samples [31, 32]. These results were also reported by Rodríguez-Carrasco et al. [21] in semolina samples; however, these authors reported lower DON contents than those found in our

study. Regarding NIV occurrence, the values obtained are similar to those found by other researchers, although they reported lower incidences, but contents were in the same range. In this sense, according to Rodríguez-Carrasco et al. [21], the 20% of semolina samples were positive for NIV with an average amount of 10.9 μg/kg. Malachova et al. [3] reported a mean value of 30 μg/kg for NIV. However, Jestoi et al. [10] and Lattanzio et al. [33] reported higher mean contents in wheat samples, 150 μg/kg, and 63.5 μg/kg, respectively, which were higher than those found in our survey.

Regarding 3-AcDON and 15-AcDON contents, similar results were reported by Jestoi et al. [10] and Rodríguez-Carrasco et al. [21], who found an average of 17 μg/kg in Finnish grain samples, and 4.4 μg/kg in semolina, respectively. On the other hand, higher contents for the sum of 3-AcDON and 15-AcDON were reported by Bryła et al. [30].

For HT-2 toxin, similar contents to those obtained in our study were reported by other authors [21, 33–36]; however, in the study conducted by Cano-Sancho et al. [34], contents up to 46 μg/kg were reported. Regardin ZON contamination, lower incidence was reported by Juan et al. [37], who found ZON in 9% of wheat samples. Levels were well below the ML established. Nevertheless, in the study reported by Bryła et al. [30], level was exceeded in a single sample of grain from Osiny (Poland) which presented 100 μg/kg, although the mean average reported was 43 μg/kg.

Regarding emerging *Fusarium* mycotoxins, ENs and BEA, the occurrence of relatively high levels (up to mg/kg) of these mycotoxins in cereal grains, has been reported in some studies conducted in Europe [10,38]. In this sense, Uhlig et al. [39] reported an incidence of 100% for ENB in grain samples. ENB was also the most abundant mycotoxin found pasta samples analyzed by

Serrano et al. [38]. However, in the study reported by Malachova et al. [3], ENA was detected in 97% of samples (concentration range of 20–2532 μg/kg) followed by ENB and ENB_1 (91% and 80%, respectively), while ENA_1 was found only in 44% of samples. Similar results were reported by Juan et al. [40] who reported an ENB prevalence of 44% and levels up to 106 μg/kg. Concerning BEA, lower incidence was reported by different authors [38, 40].

Special attention should be paid in contents reported in baby food samples because children are considered a vulnerable population group more susceptible to mycotoxin exposure than adults, since they have a restricted diet and they consume more food on a body weight basis than adults. Additionally, they could reach the totally daily intake (TDI) established even with very low levels of Besides, they could reach the totally daily intake (TDI) established even with very low levels of contamination in baby foods [41, 42]. This fact must be taken into account especially in legislated mycotoxins because lower limits than those for adults have been set for baby food. Furthermore, another problem to deal with is that related to durum wheat pasta with small size, not specifically defined as baby food, but usually consumed by children. The most detected mycotoxin in baby food was DON [41, 43]. Regarding emerging mycotoxins, in our study the only EN detected in baby food samples was ENA_1 with an average amount of 1.54 μg/kg, contrary to durum wheat pasta where all ENs were detected and ENB showed higher contents (Table 3). In the study conducted by Juan et al. [41], ENB was also the EN with higher incidence in baby food (70%) with a maximum value of 1100.00 μg/kg. Serrano et al. [38] analyzed ENs and BEA in 45 cereal-based baby food and ENA, ENA_1, ENB, and ENB_1 were detected in 2.2%, 13.3%, 2.2%, and 40% samples at levels below 149.6, 101.7, 39.4, and 35.8 mg/kg, respectively.

According to available data, the co-occurrence of different mycotoxins in one sample at the same time has been widely described. Special attention should be paid to mycotoxin co-occurrence as different mixtures of TRC have been described to cause additive, antagonistic, or synergistic effects [24, 44].

4. Conclusions

A multi-mycotoxin method has been applied in routine for mycotoxin screening and quantitation in pasta samples. In summary, by combining a validated QuEChERS extraction procedure with an UHPLC/ESI Q-orbitrap, an accurate and highly sensitive method has been developed to propose a useful approach to multi-residual analysis of mycotoxins in pasta samples.

5. Materials and Methods

5.1. Chemical and Reagents

Mycotoxin standards, OTA, AFB_1, NIV, NEO, DON, FUS-X, 15-AcDON, 3-AcDON, NEO, HT-2, T2, ZON, BEA, ENs (ENA, ENA_1, and ENB), FB_1, and FB_2 were purchased from Sigma Aldrich (Milan, Italy).

Acetonitrile (MeCN), methanol (MeOH), and water for LC mobile phase and organic solvents were HPLC grade from Merck (Darmstadt, Germany), while formic acid and ammonium formate were obtained from Fluka (Milan, Italy). Sodium chloride and magnesium sulphate were obtained from Sigma Aldrich (Milan, Italy).

Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 mm) were provided by Phenomenex (Castel Maggiore, Italy). Corning PQ centrifuge polypropylene tubes of 50 and 15 mL (Corning Cable Systems SRL, Turin, Italy) were used.

5.2. Analytical Standards

The individual stock solutions of mycotoxins (AFB_1, OTA, NEO, HT-2, T-2, DON, 3-AcDON, 15-AcDON, NIV, ZON, BEA, FUS-X, and T-2, ENs (ENA, ENA_1, ENB) were prepared by diluting 1 mg of each mycotoxin in 1 mL of MeCN. However, for FB_1 and FB_2 the standards were prepared in MeCN/H_2O 50:50 *v/v* solution at 1000 mg/L. On the other hand, for preparing intermediate solutions (working standard solutions) the individual stock standards were diluted in the same mixture solvent at different concentration levels. All these solutions were kept in safe conditionsat −20°C.

5.3. Samples

Occurrence of mycotoxins were analyzed in a total of 58 samples of different conventional pasta products collected from several local markets of Campania region (Italy) and analyzed in order to investigate the presence of 17 mycotoxins. In accordance to the Commission Regulation EC/401/2006 [45], before the analysis performance, all samples were milled with a knife mill (Grindomix GM 200, Retsch, Haan, Germany) and then, samples were grounded employing a high speed food blender (Ika, mod. A11 basic, Germany) in order to obtain a fine ground. Ground samples were mixed by hand and a 100 g portion was removed by manual scooping. The subsamples were stored in a dark and dry place at 4ºC until analysis. Finally, portions of 4 g were placed into a 50 mL PTFE centrifugal tube for extraction purposes.

5.4. Extraction Procedure

QuEChERS extraction has been widely used by different authors in cereal based foodstuffs, showing good results. The procedure followed in the present

survey was based in that reported by Zachariasova et al. [19]. Briefly: 4 g of homogenous representative sample were weighted into the PTFE cuvette and 7.5mL of 0.1% (*v*/*v*) formic acid and 10mL of MeCN were added. The suspension was shaken vigorously for 3min. After addition of 1 g of NaCl and 4 g of $MgSO_4$, the mixture was shaken again. To separate aqueous and organic phase, the sample was centrifuged (5 min, 5000 rpm (1960g)). The 0.5 mL aliquot of upper organic phase was diluted with deionized water in 1:1 (*v*/*v*) ratio. The sample solution was filtered through the 0.2 μm filter prior to instrumental analysis.

5.5. Instruments and Analytical conditions

5.5.1. UHPLC Chromatographic Analysis

Qualitative and quantitative profile of mycotoxins has been obtained using ultra-high-performance liquid chromatography (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000 a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated (T=50°C) Accucore aQ C18 column (100 × 2.1 mm 2.6 μm particle size), (Thermo Fisher Scientific, Bellefonte, PA, USA). Injection volume was 5 μL. Eluent phase was formed as follows: phase A (H_2O in 0.1% formic acid and 5mM ammonium formate), phase B (methanol in 0.1% formic acid and 5mM ammonium formate). Analytes have been eluted using a 0.5 mL/min flow rate with the following programmed gradient: 0 min—60% of phase B, 9 min—100% of phase B, 12 min—100% of phase B, 12.1 min—60% of phase B, 15 min—60% of phase B.

5.5.2. High-Resolution Mass Spectrometry Analysis: Q Exactive Orbitrap Mass Spectrometry Analysis

For the mass spectrometry analysis a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) has been used. An electrospray ionization ESI source (HESI II, Thermo Fischer Scientific, Waltham, MA, USA) operating in negative (ESI^-) and in positive (ESI^+) ion modes for all the analyzed compounds. Ion source parameters in both ESI− and ESI+ mode were: spray voltage 3.50 kV, sheath gas (N_2> 95%) 30, auxiliary gas (N_2> 95%) 10, capillary temperature 320°C, S-lens RF level 50, auxiliary gas heater temperature 300°C.

Value for automatic gain control (AGC) target was set at 1 $\times 10^6$, with a resolution of 70,000 FWHM (full width at half maximum), isolation window to 5.0 *m/z*, and a scan rate in the range between 90 and 1000 *m/z* in full MS/scan mode.

The accuracy and calibration of the Q Exactive Orbitrap LC-MS/MS was checked weekly using a reference standard mixture obtained from Thermo Fisher Scientific. Data processing has been performed using the Xcalibur software, v. 3.0.63 (Xcalibur, Thermo Fisher Scientific, Waltham, MA, USA).

5.6. Data Analysis

Mycotoxins were identified by their retention time from the extracted ion chromatogram (XIC) of target ion *m/z* for each mycotoxin and the exact mass set to five decimal places. The mass accuracy (δM) for a measured ion has been calculated by dividing the difference between the theoretical and measured *m/z* by the theoretical m/z and expressed as part-per-million (ppm):

$$\delta M\ (ppm)\ = 1 \times 10^6 \frac{(m/z_{measured}\ - m/z_{theoretical}\ )}{m/z_{theoretical}}$$

5.7. Analytical Parameters

5.7.1. Recovery Studies

The accuracy of the extraction method was evaluated with the recovery test as the ratio of the mean observed concentration and the known spiked concentration in both durum wheat pasta and baby food, and was expressed as [(mean observed concentration)/(added concentration)] × 100 and the results were corrected by the diluting factor. Method recovery from two different homogenized samples was performed at three spiking levels (125µg/kg, 62.5µg/kg and 12.5µg/kg), except for FB_2, which was calculated at two spiking levels (125µg/kg, and 62.5µg/kg). To achieve these fortification levels, different aliquots from the 10 mg/kg standards were added to 4 g of grounded samples. After the standard addition, samples were placed overnight to allow solvent evaporation and to establish equilibration between the analytes and the matrix, and then were extracted as described in Section 5.4. Extraction Procedure.

5.7.2. Matrix Effect Studies

To assess the possible matrix effect on the chromatographic response, the matrix effect (signal enhancement or suppression) was investigated by calculating the ratio percentage between the slopes of the matrix-matched calibration curve and the curve in solvent as follows:

$$\%\mathrm{ME} = \left(1 - \frac{\mathrm{Sm}}{\mathrm{Ss}}\right) \times 100$$

Where *Sm* is the slope of calibration curve in matrix-matched calibration solution and *Ss* is the slope of calibration curve with solvent. Solvent employed to calculate ME was MeCN, except for FUMs, which solvent employed was MeCN/H2O 50:50 *v/v*. Negative results were obtained when signal suppression occurs, while positive results corresponded to signal enhancement due to matrix effects.

5.7.3. LOD, LOQ, and Linearity

Sensitivity was evaluated by limit of detection (LOD) and limit of quantitation (LOQ) values. The LODs and LOQs were determined by analyzing spiked samples and expressed as the ratio between standard deviation of three replicates measurements at low concentrations and the slope of the linear calibration curve generated at those low levels. LOQ was calculated as three times the LOD. Thus, LOD and LOQ were calculated according to the following formula:

$$\mathrm{LOD}\ \left(\frac{\mu g}{kg}\right) = \frac{\text{STD areas lowest detection level}}{\text{slope}} \times 3$$

$$\mathrm{LOQ}\ \left(\frac{\mu g}{kg}\right) = \mathrm{LOD} \times 3$$

Linearity was evaluated for each mycotoxin using the calibration curve of each standard at different concentration levels, which ranged from 0.25 μg/kg to 1000 μg/kg for all analyzed mycotoxins.

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3.3. New approach in mycotoxin determination in gluten-free pasta by ultra performance liquid chromatography coupled to quadrupole-Orbitrap mass spectrometry

Food Additives and Contaminants (2017) Under review

New approach in mycotoxin determination in gluten-free pasta by ultra performance liquid chromatography coupled to quadrupole-Orbitrap mass spectrometry

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ABSTRACT

Maize is commonly contaminated with mycotoxins, mainly fumonisins (FBs) and aflatoxins (AFs). Thus, diet is the main mycotoxin source of exposure, especially for high gluten-free foodstuffs consumers. A multi-mycotoxin method for the simultaneous determination of 17 mycotoxins is described in the present survey on gluten-free pasta samples. Mycotoxins included in the study were those whose occurrence has been widely reported in cereal samples: ochratoxin-A (OTA), aflatoxin B1 (AFB1), zearalenone (ZON), deoxynivalenol (DON), 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol (3-AcDON and 15-AcDON, respectively), nivalenol (NIV), neosolaniol (NEO), fusarenon-X, (FUS-X), T-2 toxin (T-2) and HT-2 toxin (HT-2), fumonisin B1 and B2 (FB1 and FB2, respectively), three enniatins (ENN A, ENN A1 and ENN B), and beauvericin (BEA). To provide an overview on mycotoxin presence in this kind of samples, fourty-two samples of gluten-free pasta were analyzed. As our knowledge, this is the first multi-mycotoxin study carried out in gluten-free pasta employing Orbitrap mass spectrometry. Results showed that 95% of samples were contaminated, being FB1, ZON and DON the most predominant mycotoxins in gluten-free pasta samples.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by certain strains of filamentous fungi, mainly *Aspergillus, Penicillium* and *Fusarium*, which affect crops in the field and also during storage. Foods that mainly contribute to the mycotoxin intake with diet are cereals, accounting for 50% of mycotoxin ingestion (Brera et al. 2014). Among cereals, maize is the most risky commodity, especially for high consumers of maize-based products due to regional diet habits or to specific pathologies like celiac disease (Esposito et al. 2016).

Celiac disease (CD), also called gluten enteropathy and celiac sprue, is a genetic-based autoimmune disorder characterized by inflammation in the small intestinal mucosa, caused by gluten ingestion due to the intolerance to wheat gluten and also to similar proteins of barley and rye (Guandalini & Assiri, 2014). Currently, CD is one of the most common food induced disease. In fact, recent data verify that 1 in 100 people worldwide suffer from this disorder (Rizzello et al. 2016). The only treatment for CD is a strict gluten-free diet (Foschia et al., 2016). Patients throughout their lifetime cannot eat any food that contains even little amount of wheat, oat, rye, barley and products thereof. Maize and rice as well as potatoes and other few cereals and pseudo-cereals can be safely employed as carbohydrate source, since these products do not contain gluten (Saturni et al., 2010).

Maize and also rice are reported as good substrates for fungal growth and mycotoxin production, mainly aflatoxins (AFs) (Shephard 2008; Reddy et al. 2009) and fumonisins (FBs) (Nikiema et al. 2004; Domijan et al. 2005). Among the known mycotoxins, AFs, deoxynivalenol (DON) and FBs pose the greatest threat to human health worldwide (Reddy et al. 2009).

FBs are *Fusarium* mycotoxins produced mainly by *F. verticilloides* and *F. proliferatum*. The major FBs produced in maize and the most prevalent in naturally contaminated foods are FB1, FB2, and FB3, although, to date, 15 FBs analogues have been characterized. Because of their occurrence in maize-based food, diet is the main source of exposure to these mycotoxins, especially among people affected by CD (Esposito et al. 2016).

The most prevalent mycotoxins such as zearalenone (ZON), AFs, ochratoxin A (OTA), trichothecenes (TCs), and DON have been frequently studied. However, there is limited data on the occurrence and toxicity of the so-called "emerging" mycotoxins. The most frequently reported in literarure are enniatins (ENNs) and beauvericin (BEA) (Decleer et al. 2016), which have been reported in cereals from different countries and also in human biological fluids (Serrano et al. 2015). Recently, EFSA published an opinion on the presence of ENNs and BEA in food and feed, however, no limits could be established due to the lack of relevant toxicity data to carry out a risk assessment (EFSA 2014).

The most exposed to those mycotoxins present in maize and rice foodstuffs (gluten-free products) are ethnic groups and also celiac people. However, the higher exposures, mainly for AFs and FBs, are for children age group. This fact has to be taken into account as CD is mostly diagnosed in childhood period, however, the major part of gluten-free products are intended mainly for adults. Thus, maximum levels established for this type of foodstuffs are not related with the limits established for children.

The International Agency for Research on Cancer (IARC) has classified AFB1 and AFs mixtures as group 1 carcinogens. IARC has further classified mycotoxins produced by *F. verticillioides* which are mainly the FBs and fusarin C

in the group 2B as carcinogenic in animals and possibly also in humans (IARC 1993).

As described above, because of their recognized harmful effects, maximum amounts of FBs and AFs in maize food are regulated by Commission Regulation 1881/2006/EC (EC 2006) and its amendments, setting maximum levels for these mycotoxins in maize and maize-based products, among other mycotoxins (DON, OTA, PAT and ZON).

Within this context, the aim of the study was to establish a multi-mycotoxin method for the analysis of 17 different mycotoxins in gluten-free pasta (maize and rice) by ultra high performance liquid chromatography (UHPLC) coupled to HRMS-Orbitrap.

Although AFs and FBs occurrence has been studied in maize products intended for ethnic groups and celiac people, this is the first study reporting the analysis of 17 mycotoxins in gluten-free pasta by UHPLC coupled to HRMS-Orbitrap.

2. Materials and Methods

2.1. Chemical and reagents

Mycotoxin standards, ENNs (ENN A, ENN A1, and ENN B), NIV, NEO, DON, 15-AcDON, 3-AcDON, FUS-X, NEO, ZON, HT-2, T2, BEA, AFB1, OTA, FB1 and FB2 were purchased from Sigma Aldrich (Milan, Italy). Acetonitrile (MeCN), methanol, and water were LC-MS grade and purchased from Merck (Darmstadt, Germany). Formic acid and ammonium formate were purchased from Fluka (Milan, Italy), while magnesium sulphate and sodium chloride were supplied by Sigma Aldrich (Milan, Italy).

Plastic materials, mainly syringe filters with politetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 mm) were provided by Phenomenex (Castel Maggiore, Italy). Centrifuge polypropylene tubes Corning PQ of 50 and 15 mL (Corning Cable Systems SRL, Turin, Italy).

2.2. Analytical standards

The individual mycotoxin standard solutions (ENNs (ENN A, ENN A1, ENN B), NEO, HT-2, T-2, ZON, DON, 3- ADON, 15- ADON, NIV, AFB1, OTA, BEA and FUS-X) were prepared weighing accurately solid portions of each mycotoxin to prepare 1 mg/mlstock solution in MeCN, except for FB1 and FB2, which were prepared in MeCN/H_2O 50:50 v/v solution. For working standard solutions preparation, mycotoxin standard solution was diluted in a blank matrix extract at different addition levels. All these solutions were kept in safety conditions at -20°C.

2.3. Samples

In order to evaluate the natural mycotoxin occurrence in gluten-free pasta, the method has been applied to 42 samples of different samples collected from several local markets of Campania region (Italy). In particular, considering the difficulty in finding a significant number of different samples, 2 different lots for each sample, were analyzed and results were expressed as the average value of both. Pasta samples were acquired taking into account that all of them were gluten-free. Regarding composition, all of them were elaborated with corn and rice flour in different proportions (mainly 30-70%, respectively), except one 100% rice sample, one 100% buckwheat sample and two samples made with corn flour (100%). First, according to Commission Regulation EC/401/2006

(EC 2006), all samples were grounded in a high speed food blender (Ika, mod. A11 basic, Germany), mixed and divided in subsamples of 100 g, which were stored in a dark and dry place at 4°C until analysis.

2.4. Extraction procedure

A QuEChERS like procedure has been employed for the mycotoxin extraction step. The procedure was as follows: 4 g of sample previously grounded were homogenized with 7.5 mL of 0.1% (v/v) formic acid and 10mL of MeCN. The mixture was vigorously shaken for 3min. Then, 1 g of NaCl and 4 g of $MgSO_4$ were added, and shaken again. After that, the sample was centrifuged at 5000 rpm (1960g) during 5 min and a 0.5 mL of supernatant was diluted with deionized water in 1:1 (v/v) ratio. Sample solution was filtered through the 0.2 μm filter prior to instrumental analysis.

2.5. Instruments and analytical conditions.

2.5.1. UHPLC chromatographic analysis

Qualitative and quantitative profile of mycotoxins has been obtained using Ultra High Performance Liquid Chromatography (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000 a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated (50°C) Luna Omega C18 (50 x 2.1 mm 1.6 μm particle size) column (Phenomenex). Injection volume was 1 μL. Eluent phase was formed as follow: phase A (H_2O in 0.1% formic acid and 5mM ammonium formate), phase B (methanol in 0.1% formic acid and 5mM ammonium formate).

All the metabolites have been eluted using a 0.4 mL/min flow rate with a gradient programmed as follows: 0 min -20% of phase B, 0.5 min-20% of phase B,1 min -40 % of phase B, 6 min - 100% of phase B, 8 min - 20% of phase B, 10 min - 20% of phase B.

2.5.2. High resolution mass spectrometry analysis:Orbitrap Q Exactive mass spectrometry analysis

For the mass spectrometry analysis a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) has been used. An ESI source (HESI II, Thermo Fischer Scientific, Waltham, MA, USA) operating in negative (ESI-) and in positive (ESI+) ion mode for all the analyzed compounds. Ion source parameters in both ESI- and ESI+ mode were: spray voltage 3.50 kV, sheath gas (N_2> 95%) 30, auxiliary gas (N_2> 95%) 10, capillary temperature 320°C, S-lens RF level 50, auxiliary gas heater temperature 300°C. Value for automatic gain control (AGC) target was set at 1 x 10^6, with a resolution of 70000 FWHM (full width at half maximum), isolation window to 5.0 m/z, and a scan rate in the range between 90 and 1000 m/z in Full MS/Scan mode.

The accuracy and calibration of the Q Exactive Orbitrap LC-MS/MS was checked weekly using a reference standard mixture obtained from Thermo Fisher Scientific. Data processing has been performed using the Xcalibur software, v. 3.0.63 (Xcalibur, Thermo Fisher Scientific).

2.6. Data analysis

Both chromatographic and mass spectrometry data have been used for confirmation. In this sense, mycotoxin identification was performed by 3

identification points: mycotoxins were identified by their retention time from extracted ion chromatogram (XIC) of target ion m/z for each mycotoxin; then by the exact mass set to five decimal places and finally, by one fragmentation ion. The mass accuracy (δM) for a measured ion has been calculated according to the following formula and expressed as part-per-million (ppm):

$$\delta M\ (ppm) = 1 * 10^6 \frac{(m/z_{measured} - m/z_{theoretical})}{m/z_{theoretical}}$$

2.7. Analytical parameters

2.7.1. Recovery studies

The evaluation of method accuracy was evaluated by the recovery test and was calculated as the ratio of the mean observed concentration and the known spiked concentration in gluten free pasta [(mean observed concentration/added concentration) × 100] and results were corrected by the diluting factor. Recovery assay from two different homogenized samples was performed at three spiking levels (125, 62.5 and 12.5 μg/kg) and DON and FBs at 500 μg/kg. Different aliquots from the mycotoxin standard were added grounded samples to carry out recovery experiments. Finally, samples were placed overnight to allow solvent evaporation and to establish equilibration between the analytes and the matrix, and then were extracted as described in section "2.4. Extraction procedure".

2.7.2. Matrix effect studies

To assess the possible matrix effect on the chromatographic response,

expressed as SSE, the matrix effect for mycotoxins included in the study was calculated as follows:

$$\%\text{ME} = \left(1 - \frac{Sm}{Ss}\right) * 100$$

Where *Sm* is the slope of calibration curve in matrix-matched calibration solution and *Ss* is the slope of calibration curve with solvent. The ME was calculated employing MeCN as solvent, except for FBs, where solvent employed was MeCN/H_2O 50:50 v/v. Negative results were obtained when signal suppression occurs, while positive results corresponded to signal enhancement due to matrix effects.

2.7.3. LOD and LOQ

LOD and LOQ values were determined by analyzing spiked samples and expressed as the ratio between deviation standard of areas corresponding to the lowest level of calibration curve and the slope of calibration curve for each mycotoxin. Thus, LOD and LOQ were calculated according to the following formula:

$$\text{LOD}\left(\frac{\mu g}{kg}\right) = \frac{\text{STD areas lowest detection level}}{\text{slope}} * 3$$

$$\text{LOQ}\left(\frac{\mu g}{kg}\right) = \text{LOD} * 3$$

2.7.4. Linearity

Linearity (expressed as "r^2") was evaluated by constructing calibration curves for all analyzed mycotoxins at different concentration levels, which ranged from 0.25 to1000 μg/kg.

3. Results

3.1. Method implementation. Optimization of the UHPLC-Q-Orbitrap conditions

For mycotoxin screening and quantitation on gluten-free pasta samples, a multi-mycotoxin method has been applied in routine by an accurate and highly sensitive UHPLC/HRMS-Orbitrap, based on a multi-mycotoxin method for pasta analyses by UHPLC-Orbitrap mass spectrometry (Tolosa et al. 2017). However, due to different composition of pasta samples included in the present survey (maize and rice pasta) and those analyzed in the past (durum wheat pasta), the analytical parameters were validated for gluten-free pasta samples. To achieve the best compound separation, chromatographic conditions for mycotoxin identification and quantitation were studied in order to obtain optimum mass spectrometric parameters. Chromatograms in full scan method in both positive and negative ionization mode were examined in order to determine the method for sensitivity. For some analytes, formic or ammonium adduct species ($[M+FAc]^-$ or $[M+NH_4]^+$) have been selected as precursor ions, which appear to be the most predominant ions in the mass spectrum (Table 1).

3.2. Validation parameters

3.2.1. Recovery and matrix effect

Recoveries were performed by standard addition to blank samples at three different levels (125, 62.5 and 12.5 μg/kg) using a blank gluten-free sample and then were calculated using calibration curves constructed in a blank gluten-free pasta sample. For DON and FBs another addition level was assayed (500 μg/kg). In Table 2 are provided the mean recoveries at the different fortification levels.

Table 1. UHPLC/ESI Q-Orbitrap parameters of the 17 mycotoxins analyzed.

Mycotoxin	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (m/z)	Measured mass (m/z)	Accuracy (Δ ppm)
Neosolaniol	3.03	C19H26O8	[M + NH4]+	400.19659	400.19638	-1.12
Fusarenon-X	2.30	C17H22O8	[M + H]+	355.13874	355.13846	-1.13
Nivalenol	1.97	C15H20O7	[M + FAc]-	357.11911	357.11771	1.96
Deoxynivalenol	1.13	C15H20O6	[M + FAc]-	341.12419	341.12378	-0.59
Sum of 3- and 15-AcDON	1.42	C17H22O7	[M + Na]+	361.12577	361.12634	0.06
HT-2	5.68	C22H32O8	[M + Na]+	447.19894	447.19937	0.07
T-2 toxin	6.11	C24H34O9	[M + Na]+	489.20950	489.20993	0.27
Zearalenone	6.53	C18H22O5	[M + H]+	319.15400	319.15375	-0.22
Aflatoxin B1	5.00	C17H12O6	[M + H]+	313.07066	313.07014	-0.51
Fumonisin B1	6.00	C34H59NO15	[M + H]+	722.39574	722.39396	-0.75
Fumonisin B2	6.67	C34H59NO14	[M + H]+	706.40083	706.40215	-0.45
Enniatin A	8.24	C36H63N3O9	[M + NH4]+	699.49026	699.49068	-0.73
Enniatin A1	8.11	C35H61N3O9	[M + NH4]+	685.47461	685.47563	-0.54
Enniatin B	7.81	C33H57N3O9	[M + H]+	640.41676	640.41930	-2.87
Beauvericin	7.96	C45H57N3O9	[M + Na]+	806.39870	806.39697	-0.86
Ochratoxin A	6.50	C20H18NO6Cl	[M + H]+	404.08954	404.08925	-0.22

Thus, recovery values are expressed as the mean value obtained in triplicate from each spiked level (n=9).

The matrix effect, expressed as signal enhancement or suppression (SSE), was calculated as described in section 2.7.2. To correct the matrix effects, matrix matched calibration curves were constructed in a blank matrix and used for quantification purposes in samples analyzed. Thus, the final mycotoxin amount was calculated employing those matrix-matched calibration curves.

Intraday precision, expressed as the relative standard deviation (RSDr), was obtained under repeatability conditions in the same day. The calculated RSDr of repeatability were lower than 7, 5 and 3%, respectively. The same approach was applied for the determination of the interday precision, but in 3 consecutive days. Thus, the RSDr for reproducibility were lower than 10, 9 and 6 %, respectively.

3.2.2. Sensitivity and linearity

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated as described in section 2.7.3 and reported in Table 2. For legislated mycotoxins all LOD and LOQ were lower than the ML established and for non regulated mycotoxins, results were acceptable. According to Commission Decision EC/2002/657 (EC 2002), all mycotoxins exhibited a good linearity, evaluated by the "r^2" calculated from the calibration curves As shown from the data, satisfactory results were obtained ($r^2 > 0.99$), except for ZON.

Table 2. Analytical parameters (Recovery, signal suppression enhancement (SSE), limits of detection and quantitation (LOD and LOQ, respectively), and linearity (r^2)) for each mycotoxin in analyzed samples.

Mycotoxin	Recovery (%)	SSE (%)	LOD (μg/kg)	LOQ (μg/kg)	r^2
Neosolaniol	128	39	2.31	6.94	0.9971
Fusarenon-X	99	48	7.84	23.52	0.9971
Nivalenol	74	75	5.37	16.12	0.9967
Deoxynivalenol	89	94	2.14	6.41	0.9973
Sum of 3- and 15-AcDON	82	73	1.09	3.27	0.9920
HT-2	127	87	1.19	3.58	0.9972
T-2 toxin	135	66	0.83	2.5	0.9997
Zearalenone	132	87	0.13	0.38	0.9870
Aflatoxin B1	121	83	0.06	0.17	0.9939
Fumonisin B1	111	72	1.88	5.63	0.9978
Fumonisin B2	113	85	0.26	0.76	0.9979
Enniatin A	122	99	0.05	0.14	0.9985
Enniatin A1	127	86	0.1	0.31	0.9963
Enniatin B	117	41	0.05	0.16	0.9904
Beauvericin	102	88	3.62	10.87	0.9955
Ochratoxin A	112	91	0.08	0.25	0.9978

3.3. Method application to real samples

Nowadays, a lot of gluten-free products, containing variable amounts of maize flour (up to 100%) are available on the market. Because of the mycotoxin occurrence in maize-based food, mainly FBs, ZON and TCs, it is important to

determine the natural mycotoxin occurrence in these products due to the highest intake for some groups (ethnic and celiac). The occurrence and mycotoxin contents obtained in the present survey are shown in Table 3. Results showed that ninety-five percent of samples analyzed were contaminated with at least one mycotoxin. Only one sample showed no mycotoxin contamination, corresponding to that made with parboiled Italian rice and water. Thus, this sample was employed for recovery and matrix effect assays. In contaminated samples, the most prevalent mycotoxins were FB1, ZON and DON, with an incidence of 90.5, 71.4 and 66.7%, respectively, followed by FB2 and NIV (33.3%).

Regarding contents, the highest content corresponded to DON (377.4 μg/kg), followed by NIV. The highest NIV content corresponded to a 100% buckwheat pasta sample, in which NIV contents co-occurred with DON, HT-2 toxin, and ZON. Special attention should be paid to those samples contaminated with more than one mycotoxin, as synergistic effects can occur.

Table 3. Incidence and mycotoxin contents in samples analyzed, and MLs established for cereal foodstuffs.

Mycotoxin	Incidence (%)	Range (mean) (μg/kg)	IARC Classification	MLs (EC) No. 1881/2006 (μg/kg)
Neosolaniol	ND	ND	NC	No limits established
Fusarenon-X	ND	ND	3	No limits established
Nivalenol	33.3	209.2-367.6 (241.3)	3	No limits established
Deoxynivalenol	66.7	182.2-377.4 (239.8)	3	750
Sum of 3- and 15-AcDON	ND	ND	NC	No limits established
HT-2	9.5	18.2-26.3 (22.2)	NC	No limits established
T-2 toxin	ND	ND	NC	No limits established
Zearalenone	71.4	9.2-26.9 (13.5)	3	20-200***
Aflatoxin B1	ND	ND	1	0.1**-2
Fumonisin B1	90.5	39.9-246.9 (116.2)	2B	200**-400
Fumonisin B2	33.3	44.0-53.4 (48.0)	2B	
Enniatin A	ND	ND	NC	No limits established
Enniatin A1	4.8	1.7*	NC	No limits established
Enniatin B	ND	ND	NC	No limits established
Beauvericin	9.5	17.3-21.9 (19.6)	NC	No limits established
Ochratoxin A	ND	ND	2B	0.5**-3

*: only one positive sample
**: products intended for children.
***: maize-based products; ND: not detected; NC: not classificated.

4. Discussion

Multiresidual methods for mycotoxin analysis in gluten-free products are usually focused on FBs, AFs and ZON determination. However, in the present survey, a method for the determination of multiple mycotoxins simultaneously in gluten-free pasta has been developed, supposing a novelty in the mycotoxin determination on gluten-free products. Furthermore, as our knowledge, until now have not been reported methods on multi-mycotoxin analysis in gluten-free pasta by Orbitrap mass spectrometry.

On one hand, regarding method validation, different sensitivity was observed in terms of LOD and LOQ between durum wheat pasta and maize or maize and rice mixed samples. In this sense, LOD and LOQ were higher in the case of gluten-free pasta analyses, probably because maize matrixes present a high matrix effect. Other authors reported this pattern both in wheat and corn samples analyzed (Liao et al. 2015), showing different matrix-dependent instrument limits and also different recoveries and SSE, obtaining better results mainly for wheat samples (Zachariasova et al. 2010). On the other hand, regarding mycotoxin occurrence reported for pasta samples, different mycotoxin contamination pattern was observed between durum wheat and gluten-free pasta. In this sense, in maize based samples, FBs were the most detected mycotoxins in the present study, mainly FB1, while no FBs contents were detected in wheat based samples analyzed in the first house-validated method (Tolosa et al. 2017). TRC and ZON were predominant in durum wheat pasta; however, although higher incidence was reported for those samples, higher contents were reported for gluten-free pasta. Finally, ENs showed high incidence and contents for durum wheat pasta.

Natural occurrence of mycotoxins in gluten-free pasta analyzed in this study was consistent with surveyed literature data. In this sense, detected mycotoxins have been described in gluten-free foodstuffs by other authors (Magro et al. 2011; Esposito et al. 2016), mainly FBs, which have been the most analyzed mycotoxins in gluten-free foodstuffs. Thus, many authors have reported FBs contents in this kind of products. Esposito et al. (2016) analyzed 154 gluten-free products from Italy (biscuits, breakfast cereals, canned corn, bread, pasta, cookies, cornmeal, rice, sweet snacks and savoury snacks) intended for people affected by CD. Results reported showed an occurrence of 85% for the sum of FB1, FB2 and FB3 in gluten-free pasta, with a maximum content of 272 μg/kg.

In the study conducted by Magro et al. (2011), higher FBs levels were found in maize, corn-meal, and maize-flour samples, where 7% of tested samples showed a value higher than the maximum level fixed by the EU Regulation 1126/2007/EC (EC 2007). Free FBs were also found by Dall'Asta et al. (2009) in 90% of the gluten-free food samples analyzed in their study; FB1 and FB2 were found simultaneously in most of the gluten-free samples analyzed by Cano-Sancho et al. (2012), but also in combination with DON, AFs, and ZON. FBs detection reported by these authors was in accordance with our results, where FB1 was detected in higher proportion than FB2. Higher contents for the sum of FB1 and FB2 have been also reported by Brera et al. (2014) and Bryla et al. (2016), showing a maximum value of 421 and 759 μg/kg, respectively, in contrast with FBs contents detected in our study. Besides, in the study conducted by Huong et al. (2016) 24% of maize samples and 8% of rice were contaminated by FBs.

Although AFs have been widely described in maize foodstuffs, mainly AFB1, in the present survey AFB1 was not detected, as in the survey reported by Brera et al. (2014), where none of the analyzed samples were contaminated by AFB1. Nevertheless, these authors also found contents of OTA and T-2 toxin, despite OTA and T-2 toxin were not detected in our study. Furthermore, some of the positive samples reported by these authors exceeded the maximum level set by the European legislation for OTA (Commission Regulation 1881/2006). Regarding DON contents, only two samples out of 18 gluten-free pasta (spaghetti, macaroni, etc.) analyzed by Cano Sancho et al. (2012) showed DON contamination, with respective levels of 163 and 270 μg/kg, while AFs and ZON were not detected in any gluten-free sample but were found in 6 ethnic food samples.ZON presented a high incidence in gluten-free pasta analyzed in our study, however, no ZON contamination was found in gluten-free pasta analyzed by Brera et al. (2014). Emerging *Fusarium* mycotoxins showed lower incidence than in other studies where cereal based foodstuffs have been analyzed. Regarding gluten-free samples, only ENN A1 was detected in one sample and BEA was detected in two samples. Our results are in accordance with that reported by Decleer et al. (2016), where no ENN A was detected in the samples analyzed and only traces of ENN A1 were found. However, regarding ENNs type B, while in our survey were not detected, according to these authors, ENN B and ENN B1 were the most found mycotoxins. Regarding BEA contents, levels reported by these authors were higher (up to 209.0 ± 39.7 μg/kg) than those obtained in the present study.

Gluten-free foodstuffs are mainly intended for adults; however, CD is diagnosed in early ages. For this reason, special attention should be paid to mycotoxin limits established for children regarding maize-based products, as

these limits are lower than those established for adults (see table 3) although gluten-free products mainly intended for adults can be eaten by children, being a risk for them.

5. Conclusions

The results obtained showed that FBs, ZON and DON were widely found in maize-based foodstuffs, highlighting that these products can be contaminated with mycotoxins at levels that could represent a health hazard for the higher consumers of these products, especially celiac people, due to the continuous ingestion of these foodstuffs. Thus, research on mycotoxin contamination in gluten-free products and their mitigation should be carried out in order to reduce the risk associated to the ingestion of mycotoxins commonly present in these foodstuffs.

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3.4. Emerging *Fusarium* mycotoxins in vegetal raw materials and feeds intended for different animal species

Animal Feed Science and Technology (2017) Under review

Emerging *Fusarium* mycotoxins in vegetal raw materials and feeds intended for different animal species

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ABSTRACT

The aim of this study was the determination of emerging *Fusarium* mycotoxins in different raw materials (n=39) and feed manufactured with them (n=48) by liquid chromatography coupled with mass spectrometry linear ion trap (LC-MS/MS LIT). The extraction was carried out by a QuEChERS method. Results showed that both raw materials and finish feedstuffs were contaminated by enniatins and beauvericin. Different concentrations were obtained depending on the raw material and the feedstuff analyzed. Dehydrated alfalfa as raw material showed the highest contents (194.8 μg/kg) for the sum of enniatins and beauvericin, followed by wheat bran (86.5 μg/kg) and rice (64.8 μg/kg). Regarding feed samples, feed intended for rabbits showed the highest content of enniatins and beauvericin (96.2 μg/kg), followed by feed intended for sheeps (91.9 μg/kg). These results suggest that, although feed processing is thought to mitigate mycotoxin contamination, post contamination during storage could occur and contaminate finish feedstuffs.

1. Introduction

Mycotoxins are secondary fungi metabolites that widely contaminate plant origin products such as crops, fruits, and also foods and feeds. Most common mycotoxins can be categorized into *Aspergillus* mycotoxins (aflatoxins, ochratoxins, etc.), *Fusarium* mycotoxins (trichothecenes, zearalenone, enniatins, etc.) and *Penicillium* mycotoxins (citrinin, verruculogen, etc.). Neverthless, the major mycotoxins occurring in cereals, at levels of potential concern for human and animal health, are *Fusarium* mycotoxins (Cheli et al., 2013).

Mycotoxins can enter human and animal food chains through the ingestion of contaminated food and feed, respectively. In addition, mycotoxins have the potential to enter food chain through milk, meat and eggs from livestock and poultry animals fed with contaminated feed. Cereals and cereal by-products represent the main components of industrial feedstuffs. Thus, corn and wheat are commonly used for both human and animal consumption, but also for other industrial purposes, such as ethanol and flour production. By-products of these processes are available to supplement or replace expensive grains in feed formulations (Manning and Abbas, 2012). Ethanol production from maize is considered as a potentially cost-effective renewable energy source. Some recent studies reported high mycotoxin levels in dried distillers grain with solubles (DDGS) from ethanol production plants (Oplatowska-Stachowiak et al., 2015). During the production of ethanol from maize, mainly the endosperm (82% of the grain) undergoes fermentation and distillation processes. For dry-milled products, the most highly mycotoxin-contaminated fractions are those that contain the whole or the outer portions of the grain (Rodrigues and Chin, 2011), which are more contaminated (Tolosa et al., 2013). However, the co-

products formed, mainly DDGS, concentrate the initial mycotoxin levels up to three times (Wu et al., 2008; Manning & Abbas, 2012). The presence of mycotoxins in these by-products has been a matter of concern raised by their ubiquitous use. However, the inclusion of DDGS in animals' diets must be carefully calculated since mycotoxins may be present supposing a serious threat to animal health (Rodrigues & Chin, 2011).

Furthermore, the use of high levels of vegetable sources such as cereals and other raw materials increases the risk of contamination by mycotoxins (Nizza & Piccolo, 2009). Moreover, fungi can produce mycotoxins in all segments of the animal feed supply chain (Bryden, 2012). In these sense, many studies have reported mycotoxin occurrence on a wide range of feed ingredients and finished feeds intended for terrestrial animals (Binder, 2007; Binder et al., 2007; Rodrigues and Naehrer, 2012; Streit et al., 2012, 2013). Thus, mycotoxins constitute a significant problem to the animal feed industry and an ongoing risk to feed supply security.

Regarding feedstuffs, aflatoxin B1 (AFB1) is the only mycotoxin under EU feed regulation (20 μg/kg in raw materials) (EU, 2003), while guidance values have been set for animal feed ingredients and animal feed for several mycotoxins, including deoxynivalenol (DON), zearalenone (ZON), ochratoxin A (OTA), and fumonisin B1+B2 (FB1 + FB2) (EC, 2006). For T-2 and HT-2 toxins, indicative levels for cereal products, including those intended for animal feed have been set (EC, 2013; Cheli et al., 2014).

However, limits for emerging *Fusarium* mycotoxins have not been set, although their presence has been assessed by EFSA (2014) in food and feed. Emerging mycotoxins include a number of mycotoxins produced by the genus *Fusarium*.

The most prevalent in food and feed are enniatins (ENNs) and beauvericin (BEA), which are primarily contaminants of cereals. Thus, as cereal grains and by-products resulting from cereal processing are widely used as feed for livestock, their presence in feedstuffs and raw materials has been reported in different studies (Sørensen et al., 2008; Warth et al., 2012; Zachariasova et al., 2014).

Considering the levels indicated by the European legislation, results from literature data indicate that sometimes the limits proposed for cereal derived products may be not warranted by the limit for unprocessed cereals. Published data confirm that milling can minimize mycotoxin concentration in fraction used for human consumption, but concentrate mycotoxins up to eight-fold compared to original grain into fractions commonly used as animal feed (Cheli et al., 2013). Recommendations for continuous monitoring and analysis of raw materials and feed mixtures were issued under specific projects, as well as under the National residue monitoring plan and the National Plan of feed inspection and monitoring. The analysis of specific animal feeds should be focused on mycotoxins to which these animals are vulnerable to (Markov et al., 2013).

The aim of the present study was to realize a survey on emerging mycotoxins occurrence in different raw materials commonly used in animal diets and feed intended for different animal species.

2. Material and methods

2.1. Sampling

To carry out the present survey, 39 samples of raw materials commonly used as ingredients in feed manufacture and 48 samples of feed were purchased from a

feed factory (Spain). The raw materials analyzed were as follows: sunflower meal (n=1), rapeseed meal (n =1), soybean meal (n=5), wheat (n=3), wheat gluten (n=1), corn gluten (n =3), rice bran (n=4), barley (n=10), gluten feed (n=1), maize (n=2), corn pulp (n=4), sugar beet pulp (n=1), alfalfa (n=2), dehydrated alfalfa (n=1). These raw materials are usually included in feedstuffs intended for different animal species in different proportion, thus, the purchased samples have been selected taking into account those with a high inclusion percentage in feed elaboration. The percentage of inclusion depends on the specie the feed is intended for and the feed formulation.

Different feedstuffs from the same factory in order to determine emerging *Fusarium* mycotoxins occurrence. Feed samples analyzed were as follows: 8 samples of feed intended for bovine, 13 samples for ovine, 1 for caprine, 11 intended for poultry, 3 for horses, 2 for rabbit, 4 for swine, and 6 for domestic animals (dog and cat). Samples were homogenized in a food blender within the same day and then were kept at 4ºC until analysis.

2.2. Mycotoxin determination

2.2.1. Chemicals and reagents

All solvents (acetonitrile (MeCN) and methanol (MeOH)) were purchased from Merck (Darmstadt, Germany). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate ($HCOONH_4$, 97%) and formic acid (HCOOH) were supplied by Sigma-Aldrich (Madrid, Spain). All solvents were passed through a 0.22 μm cellulose filter from Membrane Solutions, Texas, USA, before use. The stock standards of ENs and BEA were purchased from Sigma-Aldrich (St. Louis, USA).

Individual stock solutions of BEA and ENs with concentration of 1000 μg/mL were prepared in MeCN. They were stored in glass-stoppered bottles and darkness conditions at -20ºC. Working standard solutions were then prepared by serial dilution from the respective stock solution.

2.2.2. Sample preparation

For mycotoxin extraction from samples analyzed, a QuEChERS method ("Quick Easy Cheap Effective Rugged and Safe") was employed. The method extraction was as follows: Two grams of finely homogenized matrix were weighed into a 50 mL polypropylene (PP) centrifugation tube, followed by the addition of 10 mL of water containing 2% formic acid. The tube was closed and the matrix was allowed to soak for 30 min. Further, a volume of 10 mL of MeCN was added into the tube containing the soaked sample and vigorously shaken on a laboratory shaker (IKA, Staufen, Germany) for 30 min. In the next step, 4 g of $MgSO_4$ and 1 g NaCl were added and shaken immediately to enable uniform distribution of $MgSO_4$. The tube was mixed again for 30s and then centrifuged (eppendorf, Germany) for 5 min (10,000 rpm). Further, 2 mL of MeCN extract were purified by dispersive solid phase extraction (dSPE, 15-mL PP tube containing 0.1 g of C18 silica sorbent and 0.3 g of $MgSO_4$) and centrifuged (10,000 rpm) for 5 min. The purified extract was transferred into a vial for the instrumental analysis.

2.2.3. LC-MS/MS equipment and conditions

Agilent 1200 chromatographic system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionisation (ESI) interface was used in this study. The QTRAP® analyser combines a fully functional triple

quadrupole and a linear ion trap mass spectrometer within the same instrument. The separation was achieved by a Gemini-NX C18 (150 mm x 2 mm I.D., 3µm particle size) analytical column supplied by Phenomenex (Barcelona, Spain). The analytical separation for LC-MS/MS was performed using gradient elution with 95% of water (with 5 mM of ammonium formate and 0.1% formic acid) as mobile phase A, and 5% of MeOH as mobile phase B (with 5 mM of ammonium formate and 0.1% formic acid), increasing linearly to 95% B for 10 min; then, decreasing linearly to 80% B for 5 min, and then gradually up to 70% B for 6 min. Finally, initial conditions were maintained for 3 min. Flow rate was maintained at 0.2 mL/min. Analysis was performed in positive ion mode (ESI+). The ESI source values were as follows: capillary voltage, 3.50 kV; source temperature, 120ºC; desolvation temperature, 400ºC; cone gas 50l/h; desolvation gas (nitrogen 99.99% purity) flow, 800 l/h.

The analyser settings were as follows: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, 5 and 3, respectively; multiplier, 650; collision gas (argon 99.99% purity) pressure, 3.83 x 10-3 mbar; interchanel delay, 0.02s; total scan time, 1.0s; dwell time 0.1 ms. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode. All time measurements were carried out in triplicate. The MRM optimized parameters, such as cone voltages, collision energies and precursor and product-ions selected are shown in Table 1.

2.2.5. Method characterization and validation

Method performance parameters were determined for method validation purposes. To carry out recovery assays and calibrate curves, a representative raw material and feed sample were first analyzed to check that no

contamination with mycotoxins was present, and these samples were used as blank matrices.

In order to determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in MeOH and from the standards prepared in extract of blank sample. The concentration range for analyzed mycotoxins ranged from 0.1 μg/L to 200 μg/L.

The limits of detection (LODs) and limits of quantitation (LOQs) were estimated from an extract of a blank sample, fortified with decreasing concentrations of the analytes. LOD was estimated as the lowest matrix-matched calibration standard corresponding to a signal to noise ratio at least 3:1 and LOQ to at least 10:1.

The recoveries, intra-day precisions and inter-day precisions were evaluated by spiking standard solution to samples at two concentration levels (10LOQ and 100LOQ). Then, samples were left to stand overnight at room temperature before the extraction to allow the evaporation of the solvent.Extraction recovery was determined by analyzing the samples spiked before and after extraction and calculating the ratio of the peak areas for each analyte.

The presence of matrix components in the extract can affect the ionization of selected analytes, producing the so-called matrix effects (ME). To avoid ME, matrix-matched calibration curves have been used for effective quantitative determination of mycotoxins in samples analyzed. ME were evaluated by comparing the slope between a calibration curve constructed in solvent and a calibration curve constructed in blank sample. Then, the matrix-matched calibration curve was used for quantification purposes. A value of 100%

indicates that there is no ME. There is signal enhancement if the value is > 100% and signal suppression if the value is < 100%.

3. Results and discussion

3.1. *Method validation*

Before optimizing the MS/MS conditions, full scan and daughter scan under positive and negative mode has been performed. In addition, each compound was also characterized by its retention time. The criteria adopted for accepting the analysis was a retention time deviation lower than 2.5% compared to the standard in solvent. Results of method validation are shown in Table 1.

The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) was performed by flow injection analysis for each standard mycotoxin. The optimized parameters are shown in Table 1. Entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively, for all analytes. The MS was operated in multiple reaction monitoring (MRM) mode.

Table 1. Validation parameters

Mycotoxin	RT (min)	Precursor ion	Product ions	DP[c] (V)	CE[d] (V)	CXP[e] (V)
ENN A	16.4	699.400	228q[a]/210Q[b]	76/76	59/35	16/14
ENN A1	15.7	685.400	214q[a]/210Q[b]	66/66	59/37	10/8
ENN B	13.3	657.300	214q[a]/196Q[b]	51/51	59/39	10/8
ENN B1	14.8	671.200	228q[a]/214Q[b]	66/66	57/61	12/10
BEA	15.3	801.200	244q[a]/784Q[b]	116/116	39/27	6/10

[a] q, confirmation ; [b] Q, quantification ; [c] DP, declustering potential ; [d] CE, collision energy; [e] CXP, entrance cell potential.

Good sensitivity was obtained for selected mycotoxins when the ESI+ mode was applied. Table 1 shows the optimum parameter values for each analyte. All mycotoxins exhibited good linearity over the working range (0.1-200 μg/L), and the regression coefficient of calibration curves was higher than 0.992 for all the mycotoxins analyzed in the study. For ENN A and BEA, the LOD and LOQ corresponded to 1 and 5 μg/L, respectively. For ENN A1, ENN B and ENN B1, the LOD and the LOQ corresponded to 0.2 and 1 μg/kg, respectively. The ME observed was: 13, 19, 11, 16 and 29% of signal suppression for ENN A, ENN A1, ENN B, ENN B1 and BEA, respectively. Recoveries were between 112-136% and 89-117%, respectively for the two addition levels (10LOQ and 100LOQ). Intra-day and inter-day precision were between 4 and 11% and between 5 and 15% for two addition levels, respectively.

3.2. Natural occurrence of mycotoxins in raw materials

The presence of emerging *Fusarium* mycotoxins has been assessed in raw materials commonly used in feed manufacture. The results revealed that the most prevalent mycotoxin was ENN B, followed by ENN B1, and BEA (Table 2). These results were in accordance with that reported by other authors. As reported by Mortensen et al. (2014), ENN B was present in all DDGS samples in by-products for animal feed collected from official control in Denmark during 1998-2009. Furthermore, Sørensen et al. (2008) reported ENN B as the predominant ENN in maize silage and appeared to be stable during ensiling over the period examined by these authors. Thus, ENNs type B are the major mycotoxins occurring in published surveys in the following decreasing order: ENN B > ENN B1 > ENN A1 > ENN A. This distribution of contamination has been also reported by Uhlig et al (2006), highlighting the

100% incidence for the ENN B in barley and wheat from Norway. Van Pamel et al. (2011) also found ENNs and BEA as the most frequently detected mycotoxins in maize silage samples.

ENNs type A (ENN A and ENN A1) were not detected in any sample of raw materials included in the study. This fact has been also reported by Sørensen et al. (2008), where ENN A and ENN A1 were detected neither in grain samples analyzed nor in 3-month-old silage stacks from whole maize.

However, in other surveys ENNs type A have been detected in cereal samples and raw materials. As reported by Streit et al., (2013), ENN A1 was the most prevalent mycotoxin in raw materials analyzed (95%) and ENN A showed an 87% of incidence. ENN B and ENN B1 showed an incidence of 95% and minor enniatins, ENN B2 and ENN B3, were also included in the survey with an incidence of 10% and 8%, respectively). BEA was found in 81 out of 83 feed and raw material samples analyzed.

Table 2. Contents obtained in analyzed raw material samples.

Raw material	ENNs and BEA contents (µg/kg)				
	ENN A1	**ENN B**	**ENN B1**	**BEA**	**Σ mean values of ENNs+BEA**
Wheat bran	nd	50.2	36.3	nd	86.5
Dehydrated alfalfa	nd	75.6	113.2	6.0	194.8
Sweet beet pulp	nd	nd	nd	3.0	3.0
Barley	nd	1.3	nd	nd	1.3
Rice	nd	nd	nd	64.8	64.8
Corn pulp	nd	1.8 (1.3-2.2)	nd	29 (20.4-37.8)	30.8

Regarding contents, ENN B levels were in a range between 1.3 and 75.6 μg/kg; ENN B1 contents ranged between 36.3 and 113.2 μg/kg, while levels of BEA were in a range from 3.0 to 64.8 μg/kg. These results are in accordance with those described by Shimshoni et al (2013) in corn and wheat silage from Israel and also with Warth et al. (2012) in grain-based processed foods containing wheat. However, the contents found in this study differ from those obtained in other studies performed in northern European regions where these raw materials showed higher contamination by these mycotoxins, as reported by Zachariasova et al (2014) in feedingstuffs and complex compound feeds intended for different animal groups, where ENNs showed high contents, mainly ENN A and ENN B with mean values of 615 μg/kg in hay samples and 748 μg/kg in wheat-based DDGS samples, respectively. This pattern of contamination was also observed by Habler and Rychlik (2016) in cereals and also by Jestoi et al (2004), who described the presence of ENN B and ENN B1 in 100% of the analyzed raw material (mainly wheat and barley) from Finland, reaching levels in the order of mg/kg. These high levels have been also reported by Uhlig et al. (2006), who found a maximum concentration of 5800 μg/kg for ENN B in wheat samples and by Sørensen et al. (2008), who reported an ENN B amount of 2600 μg/kg in whole fresh maize.

For BEA the highest contents were found in rice and corn pulp samples (Table 2). According to Streit et al. (2013), BEA was found in 98% of feed and raw material samples analyzed with a maximum content of 2326 μg/kg. In the survey reported by Lee et al. (2010) in Korea, 27% of feed ingredients were contaminated with BEA, with a mean content of 0.48 μg/g.

A high co-occurrence of BEA and ENNs was found in the present survey, as well as co-occurrence of the four ENNs, showing that emerging *Fusarium*

mycotoxins can be present in feedstuffs commodities simultaneously. The presence of other *Fusarium* mycotoxins in raw materials has been reported by different authors, mainly fumonisins (FBs), trichothecenes (TCs) and zearalenone (ZON) in the study reported by Oplatowska-Stachowiac et al. (2015), in DDGS and grain samples produced from wheat, maize and barley. In the study conducted by Nácher-Mestre et al. (2015), FBs and DON were found in plant feed ingredients commonly used in commercial aquafeeds for Atlantic salmon (*Salmo salar*) and gilthead sea bream (*Sparus aurata*). Thus, special attention should be paid to co-occurrence of *Fusarium* mycotoxins, as synergistic effects can occur (Tola et al., 2015).

3.4. Natural occurrence of mycotoxins in feed

The results of feed analyzed showed that 92% of the samples were contaminated with mycotoxins analyzed in the study. Regarding the incidence for each mycotoxin analyzed, the most prevalent mycotoxin was ENN B (89%), followed by ENN B1, BEA and ENN A1 (64, 62 and 41.5%, respectively). ENN A was not detected in any of the analyzed samples. The results obtained are shown in Table 3. The highest mean contents were found for ENB in rabbit feed, which could be explained because rabbits are usually fed a pelleted diet of dried forages, cereals and vegetable proteins, which are raw materials showing higher levels of contamination.

The concentration range for ENN A1 was between 8.1 and 13.1 μg/kg; For ENN B the concentration range ranged from 2.0 to 89.5 μg/kg; For ENN B1 the concentration range was between 7.4 and 28.8 μg/kg and for BEA, the concentration range was comprised between 4.6 and 129.6 μg/kg. In figure 1 it can be observed a chromatogram showing ENN B contamination in a feed

sample. The contents found in this study were in accordance with those reported by Warth et al (2012) in feed from Burkina Faso and Mozambique, where ENN B and ENN B1 showed contents in a range between 2.2 and 114.0 μg/kg for ENN B and 0.1 to 94.4 μg/kg, for ENN B1.

BEA was detected in 100% of the analyzed samples in a range from 3.3 to 418.4 μg/kg. The lowest contents corresponded to ENN A (0.6 to 7.9 μg/kg) and ENN A1 (3.4 to 43.9 μg/kg). These contents were similar to those detected in the present study, possibly because the weather is warmer (as in Spain) and *Fusarium* species proliferate in a different way from those prevailing in colder climates.

Table 3. Emerging *Fusarium* mycotoxin contents in feed samples analyzed.

Animal specie	Mycotoxins (average and range) (μg/kg)				
	ENN A1	**ENN B**	**ENN B1**	**BEA**	**Σ mean values of ENNs+BEA**
Bovine	9.7 (8.5-10.7)	24.1 (2.4-41.6)	15.2 (10.8-20.2)	27.4 (20.7-51.4)	76.4
Ovine	10.2 (8.1-13.1)	32.4 (2.0-89.5)	16.7 (9.4-28.8)	32.6 (8.1-129.6)	91.9
Caprine	8.4 (8.2-8.5)	16.8 (8.3-23.9)	12.7 (10.8-15.0)	13.9 (4.6-23.2)	51.8
Horses	9.4 (8.7-10.1)	21.8 (6.0-43.8)	13.6 (10.0-15.5)	19.0 (8.2-29.8)	63.8
Porcine	10.5 (9.1-11.9)	32.2 (22.1-55.1)	17.0 (14.1-24.0)	10.2 (5.7-14.6)	69.9
Poultry	9.7 (8.1-11.9)	18.4 (3.0-51.1)	15.3 (7.4-23.1)	15.8 (8.1-23.8)	59.2
Rabbits	11.8 (11.8)	47.4 (44.5-50.3)	23.5 (23.3-23.6)	13.5 (13.5)	96.2
Dogs	nd	15.4 (7.5-24.8)	10.1 (10.1)	30.9 (21.3-40.5)	56.4
Cats	nd	6.7 (6.7)	8.9 (8.9)	nd	15.6

nd: not detected

The highest contents have been reported in feed intended for rabbits, sheeps, beef, dairy cattle and swine. ENN A1 showed similar contents in all feedstuffs analyzed, except for pet feed, where this mycotoxin was not detected. Dog

feed showed higher contents of ENN B, ENN B1 and BEA than feed for cats, probably because usually, dog feedstuffs tend to contain more cereals than cat feed (EFSA, 2014). ENN B and ENN B1 showed higher mean contents in feed intended for rabbits, while BEA contamination contents were higher in feed for sheeps and dogs (Table 3). These results suggest that raw materials included in feed manufacture intended for those species showed higher contamination or were included in higher proportion than feed intended for other species (Nácher-Mestre et al., 2015).

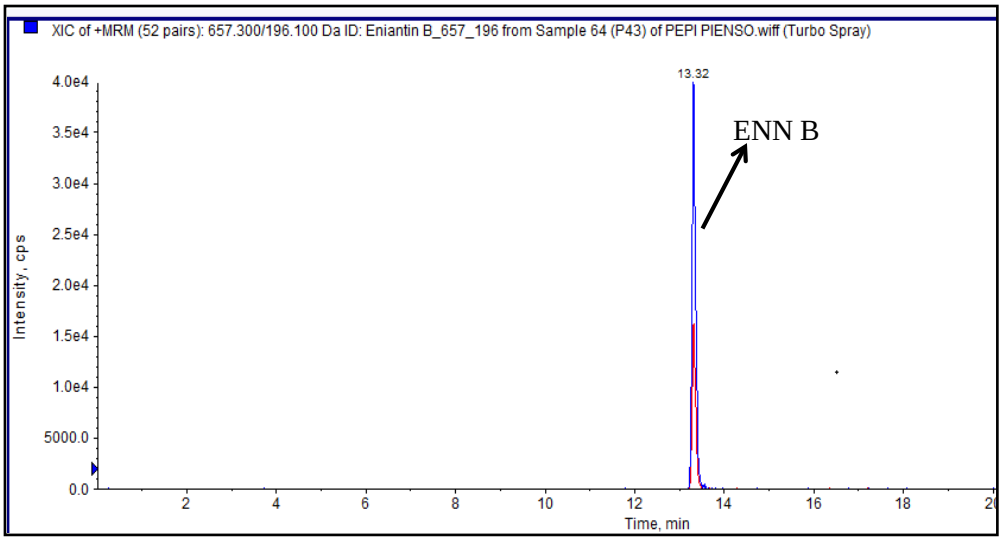

Figure 1. Chromatogram showing natural contamination of ENN B in a poultry feed sample.

Both raw materials and feed showed contamination of ENNs and BEA. The incidence was higher in feed samples; however, concentration levels were similar, except for dehydrated alfalfa, which showed the highest mean value for the sum of ENNs and BEA. ENN A1 was not detected in raw materials; however, all feed samples except those intended for dogs and cats showed contents for this ENN. This contamination pattern in feed samples can be

explained by contamination during elaboration and/or storage of finish feedstuffs.

Cereal grains intended for use as livestock feeds may undergo a number of processes, including pelletization and/or extrusion before being fed to livestock. During compound feed manufacture, raw materials are subjected to different processes, which include the application of heat and pressure. Extrusion and pelletization processes are supposed to reduce the initial mycotoxin concentration present in raw materials because of very high temperatures reached (Humpf and Voss, 2004), resulting in finish feedstuffs with lower contents than raw materials (Warth et al., 2012; Marín et al., 2013). However, inappropriate conditions during feed storage or manufacture can result in a fungal contamination and, consequently, mycotoxin production (Bryden, 2012).

4. Conclusions

In this study, a method has been validated for emerging *Fusarium* mycotoxins evaluation in raw materials and feeds based in a QuEChERS extraction and analysis by LC-MS/MS LIT. Results obtained showed that enniatin and beauvericin were present in both raw materials and feeds, showing different concentration range depending on the raw material and the feed sample analyzed. For raw materials, dehydrated alfalfa showed the highest content, while regarding feedstuffs, feed intended for rabbits and sheeps showed higher concentrations than those for other species. Thus, the control of raw materials and feedstuffs throughout the manufacture chain and the storage is essential in order to control mycotoxin contamination in these products.

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3.5. Natural occurrence of emerging *Fusarium* mycotoxins in feed and fish from aquaculture

Journal of Agricultural and Food Chemistry 62 (2014) 12462-12470

Natural occurrence of emerging *Fusarium* mycotoxins in feed and fish from aquaculture

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ABSTRACT

A new analytical method for the simultaneous determination of enniatins (ENs) and beauvericin (BEA) in fish feed and fish tissues by liquid chromatography coupled to mass spectrometry with linear ion trap (LC/MS-MS/LIT)was developed. Results showed that the developed method is precise and sensitive. The presence of emerging Fusarium mycotoxins, ENs and BEA, was determined in samples of aquaculture fish and feed for farmed fish, showing that all feed samples analyzed were contaminated with mycotoxins, with 100% of coexistence. In aquacultured fish samples, the highest incidence was found in edible muscle and liver. As for the exposure assessment calculated, it was found that average consumer intake was lower than Tolerable Daily Intake (TDI) values for other *Fusarium* mycotoxins.

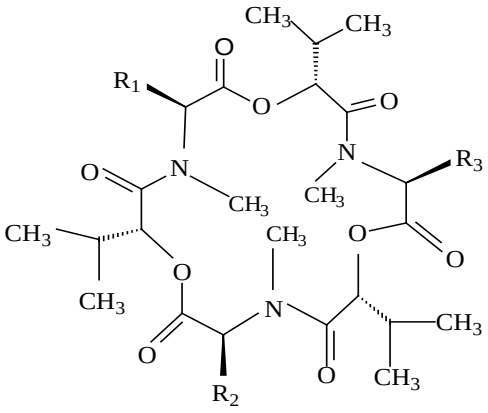
INTRODUCTION

Mycotoxins are toxic compounds produced by filamentous fungi, such as *Fusarium spp., Aspergillus spp.* and *Penicillium spp.* Trichothecenes (TC), zearalenone (ZEN) and fumonisins (FUM) are the major *Fusarium* mycotoxins occurring on a worldwide basis in cereal grains, pasta, nuts, animal feeds and forages.[1-3]However, this genera also produce other mycotoxins called emerging *Fusarium* mycotoxins because they were discovered after other *Fusarium* mycotoxins. The most studied emerging *Fusarium* mycotoxins in food (mainly cereal based food) are enniatins (ENs) and beauvericin (BEA).[4](Fig. 1).

In vitro assays have demonstrated that ENs produce reactive oxygen species (ROS) generation that induces lipid peroxidation oxidative (LPO) damage, apoptosis and necrosis via the mitochondrial pathway.[5]Further studies demonstrated that ENs and BEA induced cell death by apoptosis and DNA damage.[6] However, in scientific literature there are few *in vivo*studies related to the biological activity of ENs and BEA.[7]Recently, two studies carried out in our laboratory collected data on tissue distribution of ENA and its immunologic effects in Wistar rats.[8,9]Furthermore, Devreese et al.[10] and Ivanova et al.[11] (2014) reported the presence of ENs in pig and poultry plasma, respectively.

Research concerning the adverse effects produced by mycotoxins on the performance and animal health has been focused on terrestrial livestock species.[12,13] Direct consequences of mycotoxin feed intake for terrestrial animals include reduced feed intake, feed refusal, poor feed conversion, diminished body weight gain, increased disease incidence, reduced

reproductive capacities, which leads to economic losses.[14,15]Moreover, mycotoxin contamination of animal feeds represents a hazard to human and animal health due to potential transmission to meat, milk and by-products.[16-20]



	R_1	R_2	R_3
BEA	Phenyl-methyl	Phenyl-methyl	Phenyl-methyl
ENA	*sec*-butyl	*sec*- butyl	*sec*- butyl
ENA1	*sec*- butyl	*sec*- butyl	*iso*- propyl
ENB	*iso*-propyl	*iso*- propyl	*iso*- propyl
ENB1	*iso*- propyl	*iso*- propyl	*sec*- butyl

Figure 1. Structure of BEA and ENs. ENA, enniatin A; ENA_1, enniatin A_1; ENB, enniatin B; ENB_1, enniatin B_1; BEA, beauvericin.

In this way, the occurrence of *Fusarium* toxins in aquaculture remains mostly unknown and research is needed.[21] In fish organism, mycotoxins induce several disorders[22] such as changes in nutrient resorption, induce to cell and organ alterations, they produce functional and morphological effects and in more

severe cases mortality. Thereby, losses in aquaculture caused by mycotoxins in feed can be significant. Direct loss is a consequence of increased mortality and indirect loss is a result of production decrease and occurring of secondary diseases.[23]

Today, aquaculture produces more than a quarter of total world fisheries.[24]In 2010, world production of cultivated edible aquatic species was 60 million tonnes, which represented an increase of 7.5% over 2009. The average annual per capita consumption (in the world) of cultivated species increased reaching 8.7 kg in 2010. Moreover, sea bream (*Sparus aurata*) and sea bass (*Dicentrarchus labrax*) have a high production, as over 100 million tons in 2010.[25]

Cereals as energy and leguminose as protein feedstuffs are the main part of feed (up to 90%) for all kinds and categories of fish.[23] Especially, wheat is often used for fish feed production due to its high protein content and its benefits to preserve the pellet shape during the feed processing. Thus, the amount of wheat in fish feed varies considerably, ranging from approximately 15 to 27% for carnivorous fish whereas feed for cyprinids usually contain 20 to 70%.[21,25] In addition, in feeds for farmed fish, fish oil and fish meal is being replaced by oil seeds and vegetable oils, including palm oils,[26]which are described by some authors as raw materials contaminated with mycotoxins such as aflatoxins (AFs) and FUM.[27]

In this way, the use of such ingredients inevitably leads to the contamination of the final mixed feed with molds.[28] Furthermore, in feed manufacturing processes, different raw materials from different origin are mixed together, producing a totally new matrix with a new risk profile.[29]Thus, fish in

aquaculture is commonly exposed to feed-borne mycotoxins, such as AFs, OTA, ZEN and TC, the most important mycotoxins described in fish.[23,30]

Because of the susceptibility to mycotoxin contamination in mixed feeds, and the presence of these mycotoxins in animal tissues, the aim of this work was to develop a new rapid, sensitive, and reproducible analytical strategy forthe determination of ENs and BEA in feeds for farmed fish and fish tissuesby LC–MS/MS/LIT.

MATERIAL AND METHODS

Chemical and reagents

All solvents (acetonitrile and methanol) were purchased from Merck (Darmstadt, Germany). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) was supplied by Sigma-Aldrich (St. Louis, USA). All solvents were passed through a 0.22 μm cellulose filter from Membrane Solutions (Texas, USA).

The stock standard of BEA was purchased from Sigma-Aldrich. ENs toxin solutions were provided by Biopure (Tulln, Austria). Individual stock solutions of BEA, ENA, ENA_1, ENB and ENB_1 with concentration of 1000 mg/L were prepared in methanol. They were stored in glass-stoppered bottles and darkness in security conditions at -20ºC. These stock solutions were then diluted with pure methanol in order to obtain the appropriate working solutions (ranged from 0.01 to 100 μg/kg) and were stored in darkness at 4 ºC.

Sampling

Feed for farmed fish

Twenty samples of fish feed were purchased from valencian hatcheries. Feed samples analyzed included, as nutrient composition, crude protein (36-49%), crude fat (18-26%), ashes (7-10%), crude fiber (1-4% as cellulose) and total phosphorus (1-2%). Their composition included vegetable products (21%), such as roasted soybeans, decorticated sunflower meal, soy protein concentrate, colza oil and vegetable protein extract; cereal products (28%), such as corn and wheat gluten and wheat flour; fish and fish products (14%) such as fishmeal, fish oil, fish protein concentrate (14%), molluscs and crustaceans (6%); algae (19%), eggs and egg products (1%).

Fish samples

Twenty fishes (*Dicentrarchus labrax* (n=10) and *Sparus aurata* (n=10)) were purchased from different supermarkets located in Valencia (Spain), all of them from hatcheries. These species were selected for the study since they are two of the most important farmed Mediterranean fish species.[31] The origin of the sea bass and sea bream samples was Spain (Cartagena, Murcia) and Greece (Argolis). Nutrient composition of both is similar but fat content (concentrated mainly in liver) differs (1.3 g per 100 g of edible portion in sea bass and 2.7 g per 100 g of edible portion in sea bream).[32] These contents can differ between wild and farmed species and can also differ between seasons in wild species. Thus, cultured sea bass and sea bream contain higher lipid content than their wild counterparts (three and two times higher in cultured fishes respectively).[33-34]

For the analysis, all samples were stored in a dark and dry place at -20ºCuntil analysis. After their packages had been opened they were put into specific glass food containers and fish samples were analyzed the same day.

Mycotoxin extraction

Extraction with Ultra-Turrax

For feed samples, 5 g aliquot was homogenized with 50 mL of acetonitrile during 5 min using an Ika T18 basic Ultra-Turrax (Staufen, Germany).The extract was centrifuged at 3540 g for 15 min at 5°C. The supernatant was purified using C_{18}cartridges (Waters, Milford, Massachusetts) by applying a slight vacuum.Cartridges were previously conditioned with 5 mL of MeOH and 5 mL of deonized water, washed with 5 mL of water and then vacuum-dried for 5 min. Finally, mycotoxins were eluted with 5 mL AcN-MeOH50/50 v/v (MeOH with 20 mM ammonium formate). The extract was evaporated to dryness at 30ºC using a Büchi Rotavapor R-200 (Flawil, Switzerland). Then, the solution was reconstituted with 10 mL of AcN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate) and evaporated to dryness at 30ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). The solution is reconstituted in 1 mL of AcN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate)and filtered through 13 mm/0.22 μm nylon filter prior injection into the LC-MS/MS/LIT system. The samples were extracted in triplicate.

Extraction with ultrasonic bath

For fish samples, a 10 g aliquot was homogenized with 50 mL of acetonitrile for 30 min and 30°C using a Branson 5200 ultrasonic bath (Branson Ultrasonic

Corp., CT, USA). The extract was centrifuged at 3540 g for 15 min and 5°C. The supernatant was purified using C_{18}cartridges (Waters, Milford, Massachusetts) by applying a slight vacuum.Cartridges were previously conditioned with 5 mL of MeOH and 5 mL of deonized water, washed with 5 mL of water and then vacuum-dried for 5 min. Finally, mycotoxins were eluted with 5 mL AcN-MeOH 50/50 v/v (MeOH with 20 mM ammonium formate). The extract was evaporated to dryness at 30ºC using a Büchi Rotavapor R-200 (Flawil, Switzerland). The solution is reconstituted in 10 mL of AcN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate) and transferred to a 15 mL conical tubeto be evaporated to dryness at 30ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the solution was reconstituted with 1 mL of AcN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate)and placed again in the ultrasonic bath (30 min, 30°C). Then, the solution is washed with hexane to eliminate fat particles in liver and viscera samples and filtered through 13 mm/0.22 μm nylon filter (Membrane Solutions, Texas, USA) prior to injection into the LC-MS/MS/LIT system. The samples were extracted in triplicate.

Extraction with microwave

Fish samples (5 g) were extracted with 50 mL of acetonitrile during 3 min at 250 W using a microwave Saivod WP700P17-2 (Manisa, Turkey).The extract was centrifuged at 3540 g for 15 min and 5°C. The supernatant was purified using C_{18}cartridges (Waters, Milford, Massachusetts) by applying a slight vacuum. Cartridges were previously conditioned with 5 mL of MeOH and 5 mL of deonized water, washed with 5 mL of water and then vacuum-dried for 5 min. Finally, mycotoxins were eluted with 5 mL AcN-MeOH50/50 v/v

(MeOH with 20 mM ammonium formate). The extract was evaporated to dryness at 30ºC using a Büchi Rotavapor R-200 (Flawil, Switzerland). Then, the solution was reconstituted with 10 mL of AcN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate)and evaporated to dryness at 30ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). The solution is reconstituted in 1 mL of AcN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate) and filtered through 13 mm/0.22 μm nylon filter (Membrane Solutions, Texas, USA) prior to injection into the LC-MS/MS/LIT system. The samples were extracted in triplicate.

Instrumental and chromatographic conditions

LC–MS/MS-LIT analyses were conducted on a system consisting of an Agilent 1200 chromatographic system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionisation (ESI) interface. The QTRAP® analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer within the same instrument. Separation of analytes was performed using a Gemini-NXLC-column (Phenomenex, Aschaffenburg, Germany) (150 mm × 4.6 mm, 5 μm of particle size) preceded by a guard column utilizing the same packing material. The flow rate was set to 0.8 mL min^{-1}, and the oven temperature was 40°C, with eluent A water (mobile phase A) slightly acidified with 0.1% formic acid and 5 mM ammonium formate and eluent B (mobile phase B) methanol with 5 mM ammonium formate and 0.1% formic acid. The elution gradient started with 0% of eluent B, increased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 70% in 2 min. During the subsequent 6 min, the column

was cleaned, readjusted to the initial conditions and equilibrated for 7 min. The volume of the injections was 20 μL.

The analyses were performed using the Turbo V® ionspray in positive ionization mode (ESI+). Nitrogen served as the nebulizer and collision gas. The operating conditions for the analysis were the following: ion spray voltage, 5500 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 50 and 50 psi, respectively; probe temperature (TEM), 450°C. MRM experiments were performed to obtain the maximum sensitivity for the detection of target molecules.

RESULTS AND DISCUSSION

Method validation of the LC-MS/MS-LIT analysis

Before optimizing the MS/MS conditions, full scan and daughter scan under positive and negative mode have been used. In addition, each compound was also characterized by the retention time. The criteria adopted for accepting the analysis was a retention time deviation lower than 2.5% compared to the standard.

The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) was performed by flow injection analysis for each standard mycotoxin; entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively, for all analytes (Table 1). The MS was operated in multiple reaction monitoring (MRM) mode and with the resolution set to unit resolution for Q_1 and Q_3.

Method validation was carried out according to the guidelines established by the European Commission.[35]The method validation included the determination

of linearity, limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatabilities (intra-day precision), reproducibilities (inter-day precision) and matrix effects.

Good sensitivity was obtained for selected mycotoxins when the ESI+ mode was applied: the base peak observed was $[M+NH_4]^+$ for all the mycotoxins studied. Table 1 shows the optimum parameter values for each analyte and the two most relevant MRM transitions: the first transition was used for quantification purposes, whereas the second transition was used to confirm the presence of target compounds in the sample.

Table 1: Product-ions Obtained in Product Ion Scan Mode for Selected Mycotoxins andMRM (Multiple Reaction Monitoring) Optimized Parameters.

Mycotoxin[a]	Retention time (min)	Precursor Ion	Product Ions[b]	DP[c] (V)	CE[d] (V)	CXP[e] (V)
ENA	16.4	699.4	210^{Q}	76	35	14
			228^{q}	76	59	16
ENA_1	15.7	685.4	210^{Q}	66	37	8
			214^{q}	66	59	10
ENB	14.9	657.3	196^{Q}	51	39	8
			214^{q}	51	59	10
ENB_1	15.5	671.2	214^{Q}	66	61	10
			228^{q}	66	57	12
BEA	15.3	801.2	784^{Q}	116	27	10
			244^{q}	116	39	6

[a]ENA, enniatin A; ENA_1, enniatin A_1; ENB, enniatin B; ENB_1, enniatin B_1; BEA, beauvericin. [b]Product ions. "Q" indicates quantification transition, and "q" indicates qualification transition. [c]DP, declustering potential. [d]CE, collision energy. [e]CXP, collision cell exit potential.

To determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in methanol and from the standards prepared in extract of blank sample (fish from extractive fishing). All mycotoxins exhibited good linearity over the working range (10 LOQ and 100 LOQ), and the regression coefficient of calibration curves was higher than 0.992.For the evaluation of matrix effects, a mixture of blank extracts of different fishes was prepared as blank sample. A suppression of the signal for all mycotoxins was observed (ranged from 7% to 40%). Therefore, to minimize these matrix effects and for a selective and reliable mycotoxin quantification in different fish samples, the employment of liquid chromatography coupled to mass spectrometry with linear ion trap (LC-MS/MS/LIT) required matrix-matched calibration standards prepared with fish samples.

The LODs and LOQs were estimated from an extract of a blank sample, fortified with decreasing concentrations of the analytes. Additions were performed from three different blank samples (n=18), to the estimated concentrations for each mycotoxin. The LODs and LOQs were calculated using the criterion of S/N ≥ 3 and S/N ≥ 10 for LOD and LOQ, respectively (Table 2).

The recoveries, intra-day precisions and inter-day precisions were evaluated by spiking different levels of standard analyte to samples at two spiked levels (10 times LOQ and 100 times LOQ) (Table 2). According to Commission Decision (2002), recoveries were within the relative standard deviation (lower than 20%).[35]

Table 2. Analytical Parameters Obtained in the Validation of the Method. Limits of Detection (LODs), Limits of Quantification(LOQs), Matrix Effects (ME) (%), Recoveries, Intraday and Interday Precision for Feed (Ultra-Turrax extraction) and Fish (Ultrasonic bath method) both with AcN.

							Intraday precision				**Interday Precision**			
							Revovery (%) ± RSD[a]				Revovery (%) ± RSD[a]			
	LOD (µg/kg)		LOQ (µg/kg)		ME (%)		Feed		Fish		Feed		Fish	
	Feed	Fish	Feed	Fish	Feed	Fish	10LOQ	100LOQ	10LOQ	100LOQ	10LOQ	100LOQ	10LOQ	100LOQ
ENA	0.15	3.00	0.50	10.00	13.00	7.00	92.00±6.00	91.00±5.30	86.00±7.20	88.00±8.90	89.00±7.30	90.00±7.00	86.00±11.50	93.60±9.00
ENA_1	0.08	0.30	0.25	1.00	19.00	23.00	80.00±10.20	85.00±6.10	85.00±6.30	91.00±7.80	82.00±13.00	87.00±9.00	84.00±12.00	82.30±13.00
ENB	0.03	0.30	0.10	1.00	11.00	7.00	87.00±9.20	88.00±11.30	82.00±5.30	84.00±7.50	85.00±11.00	90.00±7.00	81.00±10.90	89.70±11.00
ENB_1	0.03	0.30	0.10	1.00	16.00	12.00	88.00±7.20	91.00±6.80	78.00±9.60	82.00±7.40	87.00±7.80	86.00±7.00	82.00±7.00	89.60±8.00
BEA	0.02	3.00	0.10	10.00	29.00	40.00	85.00±5.30	86.00±6.50	83.00±7.20	86.00±7.70	83.00±6.70	86.00±7.00	85.00±12.10	83.00±8.90

[a] RSD: Standard deviation (calculated by injecting samples in triplicate).

ENA, enniatin A; ENA1, enniatin A1; ENB, enniatin B; ENB1, enniatin B1; BEA, beauvericin.

Extraction procedures

For the optimization procedure, different parameters depending on the extraction method were tested. All experiments were carried out in triplicate using blank sample of fish fillet (hake from extractive fishing) spiked at two levels (10LOQ and 100 LOQ) of ENs and BEA. The extraction efficiency was evaluated in terms of recovery. In Table 2are shown the results for the parameters tested.

Ultra-Turrax extraction was used for feed samples. However, this method could not be used for fish samples extraction. For this reason, two extraction methods (Ultrasonic and microwave extraction) were tested for fish samples.

Ultra-Turrax optimization

This extraction method was employed only for feed samples. Critical parameters tested were the nature of the solvent, the volume of solvent (10, 20, 50 mL) and the weight of sample (2, 5, 10 g). Solvents tested with different polarities were: AcN, a mixture of AcN/MeOH 50/50 v/v (MeOH with 20 mM ammonium formate) and ethyl acetate (EtOAc). Using the mixture of AcN/MeOH 50/50 v/v (MeOH with 20 mM ammonium formate), mean recoveries were not acceptable for ENA, ENA_1 and BEA (<70%) (data not showed). Mean recoveries for all mycotoxins when EtOAc is used as extraction solventwere lower than AcN ones. Recoveries obtained with 50 mL of AcN and 5 g of feed sample were acceptable (range from 80% to 92%) according to European Commission Decision.[35] For recovery assay a sample of feed destinated to other species (but with similar composition than feed for farmed fish) was used due to all samples analyzed were positive for all

mycotoxins analyzed in this study.The results obtained are presented in Table 2. This extraction method is efficient and quickly, however, is not possible to use it for fish samples because fish tissue filaments were trapped in the Ultra-Turrax avoiding the correct mixture and contact between the solid and liquid phases.

Microwave optimization

Critical parameters tested were the time, the potency and the nature of the extraction solvent. This study was performed with three solvents: AcN, a mixture of AcN/MeOH 50/50 v/v (MeOH with 20 mM ammonium formate), and MeOH. The EtOAc was discarded due to its high volatility. Temperature applied was as low as possible because ENA_1presents a melting point lower(66-67ºC)than the other ENs.[7] In fact, when the extraction was performed using high potency (500-1000 W), temperature was higher than the solvent boiling point, so a continuous potency of 250 W was used for the extraction procedure. The solvent volume was determined according to results previously described in Ultra-Turrax optimization paragraph, but weight of sample was 10 g. Recoveries obtained with 10 g of sample and 50 mL of AcN were acceptable (range from 70 to 89%), however, the extracts were not clean, probably due to increased solubility of several compounds during the thermal treatment.

Ultrasonic bath optimization

Critical parameters tested were the nature of the extraction solvent, the temperature and time of sonication. The best recoveries were obtained when temperature applied was 30ºC (to prevent mycotoxin melting) and sonication

time applied was 30 min. As discussed above, solvents tested were AcN, mixture of AcN/MeOH 50/50 v/v (MeOH with 20 mM ammonium formate) and EtOAc. The tested solvent with best result was AcN, the extract was clear, the amount of fat in the extract was easy to eliminate and recoveries were the highest.Results are presented in Table 2 and Fig 2. It can be observed that samples containing a large fraction of AcN are appropriate for the extraction of most of the analytes. Regarding the results, this extraction method was selected for fish samples.

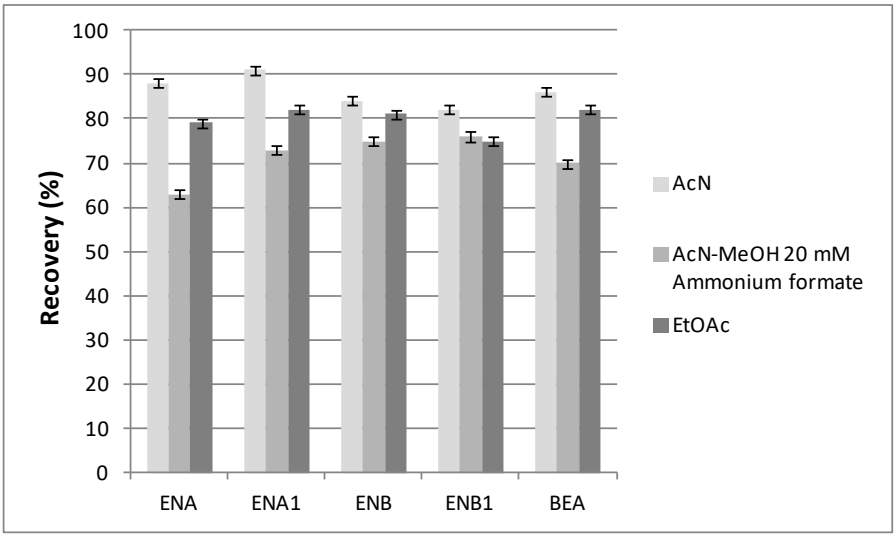


Figure 2. Recoveries with different solvents tested for fish samples by ultrasonic extraction.ENA, enniatin A; ENA_1, enniatin A_1; ENB, enniatin B; ENB_1, enniatin B_1; BEA, beauvericin. AcN, acetonitrile; MeOH, methanol with 20mM ammonium formate; EtOAc, ethyl acetate. ∓ : Standard deviation obtained by analysis performance in triplicate.

Optimization of liver and viscera preparation conditions

For animal origin samples, lipids may cause the main interference in the analysis of low polarity contaminants in biological materials. Thus, the presence of lipids in the extracts must be avoided or reduced as much as possible, in order to improve detection and quantification limits.[36] The main difference between edible muscle and liver is the lipid content, concentrated mainly in liver, as mentioned in section "Material and methods". For this reason, for live rand viscera sample preparation, some variations were necessary: before the concentration on the turbovap evaporator, the extract was reconstituted with 3 mL of n-hexane (the third part of the total volume) and 6 mL of MeOH (total volume 9 mL) and ultrasonicated for 30 min at room temperature. Two different phases appeared: the lipidic and the methanolic. The lipidic phase was discarded, and the methanolic phase was collected and evaporated.This procedure was carried out in triplicate. Finally, samples were reconstituted in 1 mL of AcN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate) and filtered through 13 mm/0.22 μm nylon filter (Membrane Solutions, Texas, USA)prior to injection. Recoveries obtained were acceptable (ranged from 72 to 88%).

Clean-up procedure

In order to obtain maximum extraction recoveries and to reduce the presence of compounds that may interfere in the determination, the extract purification was performed with cartridges with different types of dispersing agent to evaluate clean-up efficiency. Dispersing agents tested for fish samples were: C_8, silica and C_{18}. Results are showed in Fig 3.The best dispersing agent for obtaining the best performances in fish samples was C_{18}. Cartridges were

previously conditioned with 5 mL of MeOH and 5 mL of deonized water, washed with 5 mL of water and then vacuum-dried for 5 min. Finally, mycotoxins were eluted with 5 mL AcN-MeOH50/50 v/v (MeOH with 20 mM ammonium formate).

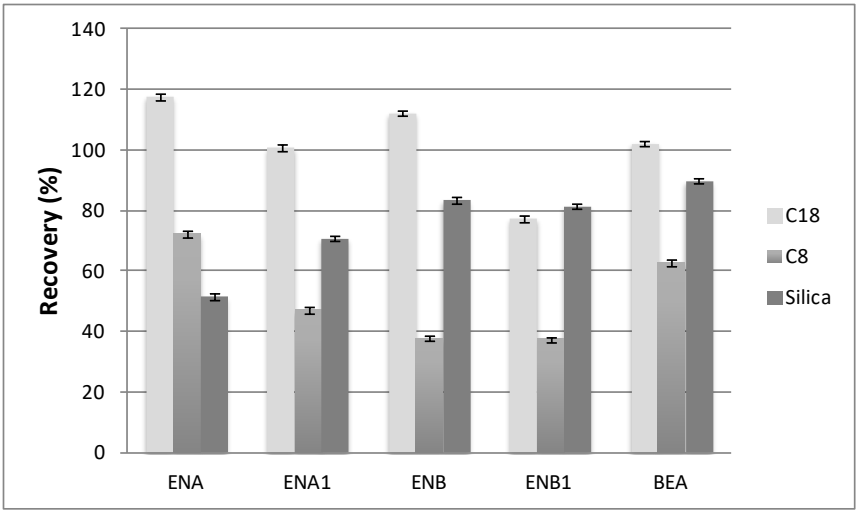


Figure 3. Recovery assays with different dispersing agents (C_{18}, C_8, Silica) tested in fish samples by ultrasonic extraction with acetonitrile.ENA, enniatin A; ENA_1, enniatin A_1; ENB, enniatin B; ENB_1, enniatin B_1; BEA, beauvericin.

$\pm$: Standard deviation obtained by analysis performance in triplicate.

Occurrence of ENs and BEA in analyzed samples

Feed for farmed fish

All the samples analyzed were contaminated with emerging *Fusarium* mycotoxins. Results are shown in Table 3. Concentrations in positive samples ranged from 0.19 to 3.40 μg/kg for ENA, from 0.24 to 6.50 μg/kg for ENA_1,

from 0.11 to 3.20 μg/kg for ENB, from 0.15 to 10 μg/kg for ENB_1 and from 0.13 to 6.60 μg/kg for BEA.

The results obtained in this work are according to contents reported in different feed studies: in a study conducted by Warth et al.[37] in 10 feed samples from Burkina Faso and Mozambique, ENB and ENB_1 showed the highest contents (2.2-114 and 0.1-94.4 μg/kg, respectively), and BEA was present in 100% of the samples analyzed (3.3-418.4 μg/kg). Contents for ENA ranged from 0.6 to 7.9 μg/kg and for ENA_1, from 3.4 to 43.9 μg/kg.

This results are also according to that reported by Jurjevic et al.,[38] in which BEAwas present in 19 out of 209 corn samples and with that reported by Lee et al.[39] about the occurrence of *Fusarium* mycotoxin BEA in animal feeds from Korea: the 27% of feed ingredients were contaminated with BEA at levels of 10-1800 μg/kg (mean 480 μg/kg). The mean concentration of BEA was higher in bran feeds, followed by vegetable protein feeds and grain feeds. In compound feeds, 33% of samples were contaminated with BEA at levels of 10-4660 μg/kg. BEA was not found in any of the 19 animal by-product feed samples (fat meal, fish meal and fish soluble) analyzed.

Mycotoxins can be present in processed feed due to the their resistance to treatments applied in feed processing (heat, chemical and mechanical extraction and dehydration). These processes eliminate most of the original contaminating mycoflora, however, fungi sporesand heat-stable toxins present in spores and mycelium can remain until more favorable conditions.[40]

Table 3. Occurrence of ENs and BEA in Feed for Farmed Fish and in Fish Samples (μg/kg).

Samples analyzed		Incidence (positives/total)					Concentration range (mean of positives)				
		ENA	**ENA_1**	**ENB**	**ENB_1**	**BEA**	**ENA**	**ENA_1**	**ENB**	**ENB_1**	**BEA**
Feed		20/20	20/20	20/20	20/20	19/20	0.6-3.4 (0.9)	0.3-6.5 (1.1)	0.1-3.2 (0.89)	0.15-10 (1.77)	0.1-6.6 (1.4)
Total fish	Muscle	0/20	8/20	13/20	10/20	0/20	nd	1.7-7.5 (4.2)	1.3-44.6 (13.5)	1.4-31.5 (10.9)	nd
	Liver	1/20	4/20	3/20	3/20	0/20	84.0 (84.0)	1.01-9.9 (5.1)	6.6-17.7 (12.6)	2.7-6.8 (5.3)	nd
	Head	0/20	4/20	5/20	5/20	0/20	nd	1.7-30.5 (11.1)	2.0-29.7 (13.0)	1.1-75.4 (19)	nd
	Viscera	0/20	2/20	3/20	3/20	0/20	nd	9.9-45.0 (27.4)	1.9-72.3 (30.6)	2.8-119.0 (42.7)	nd
Sea bass	Muscle	0/10	5/10	9/10	7/10	0/10	nd	1.7-6.9 (4.3)	1.3-44.6 (12.8)	1.4-31.5 (10.2)	nd
	Liver	1/10	3/10	2/10	2/10	0/10	84.0 (84.0)	1.0-9.9 (5.6)	6.6-17.7 (12.1)	2.7-6.4 (4.5)	nd
	Head	0/10	2/10	3/10	3/10	0/10	nd	9.3-30.5 (19.9)	2.0-29.7 (13.5)	1.1-75.4 (28.9)	nd
	Viscera	0/10	2/10	2/10	3/10	0/10	nd	9.9-45.0 (27.4)	17.7-72.3 (45.0)	2.8-119.0 (42.7)	nd
Sea bream	Muscle	0/10	3/10	4/10	3/10	0/10	nd	2.1-7.5 (4.0)	1.3-21.6 (14.9)	7.1-19.0 (12.7)	nd
	Liver	0/10	1/10	1/10	1/10	0/10	nd	3.2	13.4	6.8	nd
	Head	0/10	2/10	2/10	2/10	0/10	nd	1.7-3.0 (2.3)	8.4-15.9 (12.1)	2.8-5.2 (4.0)	nd
	Viscera	0/10	0/10	1/10	0/10	0/10	nd	nd	1.9	nd	nd

nd, not detectable.

ENA, enniatin A; ENA_1, enniatin A_1; ENB, enniatin B; ENB_1, enniatin B_1; BEA, beauvericin.

Farmed fish

To determine the differences in mycotoxin contents in different fish tissues, samples were separated into four parts (liver, viscera, tissue and head) and analyzed separately. Results are shown in Table 3.

The incidence of positive samples was lower than the incidence in feed, but the contents obtained were higher. These results suggest that contents depend on the time of withdrawal of the toxins from the diet. In this way, in a study conducted by Wu,[41] mycotoxin concentration in tissues decreased by 95 to 99% within 3 days after withdrawal of AFB_1 and OTA. In a pilot toxicokinetic study of ENB_1 in pigs, Devreese et al.[42] observed that ENB_1 is rapidly absorbed after oral administration and rapidly distributed and eliminated. The absolute oral bioavailability is high.

For the total fish samples analyzed, the highest incidence was obtained in muscle samples: 65% of samples (9 sea bass and 4 sea bream) were positive to ENB, 50% of samples (7 sea bass and 3 sea bream) were positive to ENB_1 and 40% of samples (5 sea bass and 3 sea bream) were positive to ENA_1. Thus, the highest incidence was found for ENB. The results obtained showed that incidence for ENA_1, ENB and ENB_1 in muscle samples was higher for sea bass samples than for sea bream samples. Figure 4 shows a chromatogram of a positive sea bass sample (tissue).

For ENA_1, the highest incidence was found in muscle and liver samples (40% and 20% of positive sample, respectively) while for ENB and ENB_1, the highest incidence was found in muscle (65% and 50%, respectively) and head samples (25%).

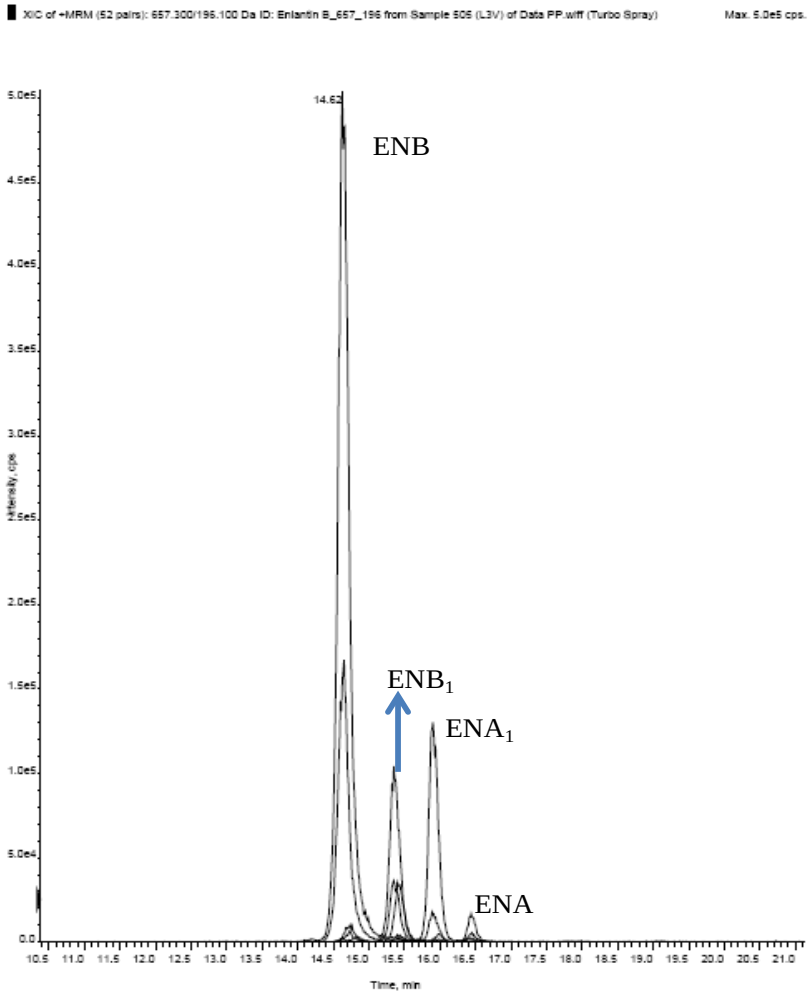


Figure 4. Chromatogram obtained from a sea bass tissue sample analyzed (contents were as follows: ENA, trace levels; ENA_1, 7μg/kg; ENB, 7 μg/kg and ENB_1, 18 μg/kg). BEA retention time: 15.3 min.

In head samples analyzed, the percentage of positive samples were 20% for ENA_1 and 25% for ENB and ENB_1. In liver, 20% of samples analyzed were positive to ENA_1, while 15% of samples were positive to ENB and ENB_1. In

viscera, 15% of samples were positive to ENB and ENB_1, while only 10% of samples were positive to ENA_1.

The mean contents obtained in muscle samples were higher in sea bream than in sea bass. This suggests differences in the mycotoxin intake and/or in mycotoxin metabolism. Contents obtained for sea bass were similar in the four parts analyzed. However, for sea bream, higher contents were found in muscle and lower contents were obtained in viscera (Table 3).

With respect to different parts analyzed in sea bass and sea bream, in head samples analyzedof sea bass,the percentage of positive samples were 20% for $ENA_1$and 30% for ENB and ENB_1, while for sea bream samples, the percentages were 20% for ENA_1, ENB and ENB_1. In sea bream samples, the highest content was found for ENB (15.9 μg/kg). However, for sea bass, ENB_1showed the highest content (75.4 μg/kg).

In liver samples of sea bass, 30% of analyzed samples were positive to ENA_1, while 20% of samples were positive to ENB and ENB_1.Regarding sea bream samples, only 10% of them were positive to ENA_1, ENB and ENB_1. Only one sample of sea bass liver was positive to ENA, with 84.0 μg/kg. The highest content in both species analyzed was detected for ENB (17.7 μg/kg and 13.4 μg/kg for sea bass and sea bream, respectively).

In viscera samples of sea bass, 30% of samples were positive to ENB_1 and 20% of samples were positive to ENB and ENA_1. The highest content was obtained for ENB_1 (119.0 μg/kg). In viscera samples from sea bream, only one sample was positive to ENB (1.9 μg/kg).

No more data are available for the toxicokinetic and bioavailability of the emerging mycotoxins in fish, but in literature consulted is reported the presence of other mycotoxins in fish tissues. In this way, results of this survey are according to that reported by El Sayed and Khalil,[43] that reported 4.2±0.8 μg/kg of AFB_1 in edible muscle of sea bassand Nomura et al.[44]reported AFB_1 concentrations in liver higher than in muscle, suggesting that AFs from feed were more highly accumulated in liver than in muscle. Deng et al.[45]reported that no detectable AFB_1 residues were found in muscle, whereas quite high residues were determined in liver.

Daily intake estimation for mycotoxins

To determine an approximation to exposure assessment related to the intake of ENs and BEA in Spanish adult population, the estimated daily intake (EDI) was calculated relating the presence of mycotoxins in samples analyzed with the consumption data of fish by Spanish population (26.84 kg per capita from January to December 2013).[46]

From this consumption data and the mean concentrations obtained (only from muscle samples analyzed), the estimated daily intake for Spanish consumers was calculated. Table 4 shows the mean concentration data for all samples (positives and negatives, criteria of nd=LOD)and only for positives samples and the EDI for each mycotoxin and also the sum of ENs (co-occurrence), sincethe obtained results are considered as the total exposure tovmycotoxins found in samples analyzed at the same time.

Table 4. Estimated Daily Intake (EDI) of ENA_1, ENB and ENB_1 for Spanish Fish Consumers.

Mycotoxin	Mean levels (nd=LOD) (µg/kg)	Mean levels (positive samples) (µg/kg)	EDI (µg/kg bw/day)	
			Mean levels (nd=LOD)	Mean levels (positive samples)
ENA_1	2.000	4.201	0.003	0.005
ENB	8.803	13.500	0.011	0.016
ENB_1	5.100	10.902	0.006	0.013
Co-occurrence (Sum of ENs)	15.903	28.603	0.020	0.034

ENA_1, enniatin A_1; ENB, enniatin B; ENB_1, enniatin B_1.

[a]nd, not detectable; LOD, limit of detection.

With the data evidenced is not possible to confirm that ENs represent a risk for Spanish population, since a provisional maximum tolerable daily intake (PMTDI) has been not established by the Joint Expert Committee on Food Additives (JECFA). For this reason, the approximation to the risk assessment was carried out according to the safety guidelines established for other *Fusarium* mycotoxins. In this way, results obtained were compared with the PMTDI values established for nivalenol (NIV) (1 µg/kgbw/day), DON (0.7 µg/kgbw/day), sum of HT-2/T-2 toxins (0.1µg/kg bw/day) and sum of FB_1 and FB_2(2 µg/kg bw/day).[47] It can be observed that the calculated EDIs for fish at mean levels (for positive samples and for all samples) werelower than the Tolerable Daily Intakes (TDIs)forNIV, DON, FB_1/FB_2 and HT-2/T-2.

Although more information about toxicological data is necessary, special attention should be paid to these results, since some authors reported that

DON cytotoxicity was not significantly different from ENs and BEA. These results indicated that ENs may have an underestimated toxic potential.[6]

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3.6. Multi-mycotoxin analysis in fish products

Food Control (2017) Under review

Multi-mycotoxin analysis in fish products

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ABSTRACT

The presence of mycotoxins in feed for farmed fish and edible tissues from aquaculture fish has been reported, due to vegetal raw materials included in feed and the carry-over to muscle portions. The aim of this study was to determine mycotoxin occurrence of 15 mycotoxins in processed fish products commonly consumed nowadays, such as smoked salmon and trout, different types of sushi and gula substitute. Samples were extracted by a QuEChERS method and analyzed by liquid chromatography-tandem mass spectrometry linear ion trap. Results showed that mycotoxins analyzed in the survey were not present in smoked fish and sushi samples. In contrast, small amounts of fusarenon-X, and enniatin B were detected in gula substitute samples.

1. Introduction

Aquaculture has gained considerable importance due to the high demand for aquatic products and seafood. On one hand, nowadays, there is a worldwide increase in demand for ready to eat food products which require little preparation before serving (Yucel-Gier et al., 2009). These requirements suppose a change in fish products preservation and processing. On the other hand, globalization has extended some products to different countries, as occurs with sushi and sashimi. Thus, a wide variety of fish products produced by different techniques, such as surimi, "gula substitute", and different types of sushi are available at markets (Cindy Hsin-I Feng, 2012). Gula substitute is made employing fish protein, called surimi, together with other ingredients, mainly wheat flour, soya, and vegetal protein. The use of these ingredients can be a source of mycotoxin contamination in this kind of foodstuffs. These ingredients and also others, such as rice, are used in the elaboration of different types of sushi, supposing a source of mycotoxin contamination in these products.

Regarding smoked fish products, currently, there are diverse methods to preserve them. Some traditional techniques are based on temperature control; however, other techniques are based on the control of water activity (a_w), including drying, salting, smoking and freeze-drying. Some of these techniques have been used for thousands of years. This is the case of smoked salmon and trout, which are highly consumed nowadays (FAO, 2012). Smoked salmon has gradually become an element of general consumption and it has now become an industrial product on a highly developed market, mainly due to aquaculture development of the Atlantic salmon (*Salmo salar*).

Smoking, often in combination with salting, is a traditional method of preservation that results in fish drying. It combines three different effects: drying, cooking, and the effect of the smoke, which confers a preservative effect on the end product, due to different compounds present in smoke from wood burning, mainly a large number of phenols, which have anti-bacterial and anti-fungal properties able to inhibit or destroy spoilage microorganisms, preserving the fish longer (Cardinal et al., 2001). However, despite the protective effect of wood smoking process, the resulting low moisture product obtained is favourable for the growth of fungi, which can produce mycotoxins as a result of their metabolism, especially during improper storage conditions (Edema and Agbon, 2009).

Mycotoxins are well known natural contaminants in vegetal feed material. The use of these alternative ingredients and also cereals in feed formulation, can introduce contaminants such as mycotoxins in the food chain (Nácher-Mestre et al., 2015). Mycotoxins are frequently monitored in foods and feeds. Among their occurrence in feeds, animal derived products can also be contaminated with mycotoxins by the ingestion of contaminated feed and can be present in edible tissues of these animals. Thus, animal by-products could hence be a potential source of mycotoxins (Mizáková et al., 2002; Sørensen et al., 2010; Ostry et al., 2013; Caruso et al., 2013). Although this fact is described in studies performed on terrestrial animal, only few recent studies are performed on farmed fish, although different surveys have shown mycotoxin occurrence on feed for farmed fish and edible tissues of aquaculture fish (Pietsch et al., 2013; Woźny et al., 2013; Tolosa et al., 2014; Nácher-Mestre et al., 2015).

Within this context, the aim of the study was to evaluate the presence of mycotoxins in smoked salmon (Salmo salar), "gula substitute" and different types of sushi samples commercially available by applying QuEChERS extraction procedure for the simultaneous multi-mycotoxin analysis of aflatoxins (AFB1, AFB2, AFG1, AFG2), fumonisins (FB1, FB2, FB3), enniatins (ENN A, ENN A1, ENN B, ENN B1), beauvericin (BEA), fusarenon-X (FUS-X), sterigmatocistin (STG) and ochratoxin A (OTA) and determination by liquid chromatography coupled to tandem mass spectrometry with linear ion trap (LC-MS/MS-LIT). As our knowledge, this is the first study on mycotoxin contamination in this type of foodstuffs.

2. Material and methods

2.1.Chemicals and reagents

All solvents, acetonitrile (MeCN) and methanol (MeOH)) were purchased from Merck (Darmstadt, Germany). Deionized water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) and formic acid (HCOOH) were supplied by Sigma-Aldrich (St. Louis, USA). All solvents were passed through a 0.22 μm cellulose filter from Membrane Solutions (Texas, USA). Anhydrous magnesium sulphate and sodium chloride were obtained from Sigma–Aldrich.

2.2. *Standards and solutions*

The mycotoxin stock standards of aflatoxins (AFB1, AFB2, AFG1, and AFG2), ochratoxin A (OTA), fusarenon-X (FUS-X), sterigmatocistin (STG),

fumonisins (FB1, FB2 and FB3), enniatins (ENN A, ENN A1, ENN B and ENN B1) and beauvericin (BEA) were purchased from Sigma-Aldrich.

2.2.1. Individual stock solutions

Analyte stock solutions were prepared by dissolving them in MeOH or MeCN depending on its solubility properties in order to obtain multi-compounds working standard solutions. The new multianalyte working solutions (ranging from 0.1 to 1000 μg/L of concentration for each compound) were prepared by diluting the individual stock solutions in solvent and then stored in darkness at -20ºC.

2.3. Procedures

2.3.1. Samples

To carry out this study, 72 samples were analyzed: twenty samples of smoked salmon (*Salmo salar*), eight samples of smoked salmon (*Salmo salar*) with dill, four samples of smoked trout (*Onchorhynchus mykiss*), thirty samples of different types of sushi (*Salmo salar*), and ten samples of "gula substitute" (fish protein) acquired in different markets located in Valencia (Spain). The ingredients and sample procedence are shown in Table 1. The samples were analyzed immediately after their acquisition, after being homogenized in a high-speed food blender.

Table 1. Samples included in the study.

Sample	Presentation (number of samples)	Type/Origin	Ingredients
Atlantic Salmon (*Salmo salar*)	Smoked salmon (n=20)	Smoked/Norway	Atlantic salmon (97%), water, salt, sugar, natural smoke, antioxidant E-331, E-501, E-262
	Smoked salmon with dill (n=8)	Smoked/Norway	Atlantic salmon (97%), salt, natural smoke, brandy, dill
Rainbow Trout (*Onchorhynchus mikyss*)	Smoked trout (n=4)	Smoked/Spain, France	Trout, salt, sugar, natural smoke
Sushi Atlantic Salmon (*Salmo salar*)	Sushi Nigiri (n=10)	Crude/Norway	Rice (rice, water, sugar, sunflower oil, cane molasses, trehalosa), salmon
	Sushi Maki (n=10)	Crude/Norway	Salmon, rice (water, rice vinegar, sugar, salt), nori algae, cucumber
	Sushi California Roll (n=5)	Crude/Norway	Rice (water, rice vinegar, sugar, salt), nori algae, cucumber, cheese, onion
	Salmon for sushi (n=5)	Crude/Norway	Salmon (*Salmo salar*), salt, natural flavour, sugar
Gula substitute	(n=10)	Crude/Spain	Surimi 47% (fish, cephalopods), water, sunflower oil, corn starch, modified starches (gluten free), aroma (soybean, traces of crustaceans) and fish extract, egg white, vegetable protein gluten), salt, flavor enhancer (monosodium glutamate, E-635), stabilizer (xanthan gum), sepia (mollusk) ink.

2.3.2. Extraction procedure

QuEChERS extraction was performed with acidified MeCN/H_2O. The extraction method was as follows: Two grams of finely homogenized matrix were weighed into a 50-mL polypropylene (PP) centrifugation tube, followed by the addition of 10 mL of water containing 2% of formic acid. The tube was closed and the matrix was allowed to soak for 30 min. Further, a volume of 10 mL of MeCN was added into the tube containing the soaked sample and vigorously shaken on a laboratory shaker (IKA, Staufen, Germany) for 30 min at 250 rpm. In the next step, 4 g of $MgSO_4$ and 1 g NaCl were added and shaken immediately to enable uniform distribution of $MgSO_4$, and then centrifuged (eppendorf, Germany) for 5 min (10,000 rpm). Then, 2 mL of MeCN extract were purified by adding 0.1 g of C18 silica sorbent and 0.3 g of $MgSO_4$, and centrifuged (10,000 rpm) for 5 min. The purified extract was filtered and transferred into a vial for the instrumental analysis.

2.4. Method validation

The method was validated according to the guidelines established by the EU Commission Decision 2002/657/EC. Representative matrixes of smoked salmon and gula substitute were selected for validation purposes. The performance characteristics, including recoveries, linearity, limits of detection (LOD) and quantitation (LOQ), intra-day and inter-day precision, and matrix effects were obtained.

Extractive recovery was determined by calculating the ratio between the peak areas for each mycotoxin from spiked samples before extraction and spiked after extraction at two addition levels (20-200 μg/L for all mycotoxins and

100-1000 μg/L for fumonisins (FBs)). The precision was expressed as the RSD of the calculated recovery. The method of standards addition to fish samples was based in the addition of a known amount of analytes to blank samples. Intraday precision was determined calculating the relative standard deviation (RSDr), obtained under repeatability conditions of three determinations for each spiked sample in the same day. The same approach was applied for the determination of the interday precision, differing in 3 days instead of 1 day. The interday precision was calculated by the relative standard deviation (RSDr) from spiked samples under reproducibility conditions by three determinations per concentration on three consecutive days. Linearity (expressed as "r^2") was obtained from calibration curves for each mycotoxin in a working range from 0.1-100 μg/L for all the mycotoxins included in the survey, except for FBs, which ranged between 1-1000 μg/L. Limits of detection (LOD) were estimated as the lowest-matrix-matched calibration standard corresponding to a signal to noise ratio at least 3:1 and limits of quantitation (LOQ) to at least 10:1.

Matrix effect (ME) was calculated by comparing the slope between calibration curve of fortified sample with a calibration curve constructed in solvent (MeOH or MeCN) in the same concentration range. The signal suppression/enhancement (SSE) due to matrix effects was calculated according to the following formula:

SSE (%) = (slope in matrix / slope in solvent)*100

A value close to 100% indicates that there is not a significant matrix effect, while values >100% and <100% indicate signal enhancement and signal suppression, respectively.

2.5. HPLC-MS/MS analysis

LC–MS/MS-LIT analyses were conducted on a system consisting of an Agilent 1200 chromatographic system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionisation (ESI) interface. The QTRAP® analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer within the same instrument. Separation of analytes was performed using a Gemini-NX LC-column (Phenomenex, Aschaffenburg, Germany) (150 mm × 4.6 mm, 5 μm of particle size) preceded by a guard column with the same packing material. The flow rate was set to 0.2 mL/min, and the oven temperature was 40°C, with eluent A (water) and eluent B (MeOH) both acidified with 0.1% formic acid and 5 mM ammonium formate. The elution gradient started with 0% of eluent B, increased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 70% in 2 min. During the subsequent 6 min, the column was cleaned, readjusted to the initial conditions and equilibrated for 7 min. The injection volume was 20 μL.

The analyses were performed using the Turbo V® ionspray in positive ionization mode (ESI+). Nitrogen served as the nebulizer and collision gas. The operating conditions for the analysis were the following: ion spray voltage, 5500 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 50 and 50 psi, respectively; probe temperature (T), 450°C. The analyser settings were as follows: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, 5 and 3, respectively; multiplier, 650; collision gas (argon 99.99% purity) pressure, 3.83 x 10-3 mbar; interchannel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass

spectrometer was operated in Multiple Reaction Monitoring (MRM) mode in order to obtain the maximum sensitivity for the detection of target molecules. All time measurements were carried out in triplicate.

3. Results and discussion

3.1.Optimization of LC-MS/MS-LIT procedure

To carry out recovery assays and calibrate curves for method optimization, smoked salmon and gula substitute samples were first analyzed to check that no contamination with mycotoxins was present, and these samples were used as blank samples. Then, fish samples were spiked at two levels before the extraction. The samples were left to stand at room temperature before the extraction to allow the evaporation of the solvent. Results of method validation are shown in Tables 2 and 3.

Before optimizing the MS/MS conditions, full scan and daughter scan under positive mode has been used. In addition, each compound was also characterized by the retention time. The criteria adopted for accepting the analysis was a retention time deviation lower than 2.5% compared to the standard in solvent.

The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) was performed by flow injection analysis for each standard mycotoxin. The optimized parameters are shown in Table 2. Entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively, for all analytes. The MS was operated in MRM mode with the resolution set to unit resolution for Q1 and Q3.

Table 2. Optimized parameters for mycotoxins included in the survey.

Mycotoxin	DP	PRECURSOR ION m/z	PRODUCT ION[Q]			PRODUCT ION[q]		
			CE	ION	CXP	CE	ION	CXP
AFB1	46	313.1 $[M+H]^+$	41	241.0	4	39	289.9	4
AFB2	81	315.1 $[M+H]^+$	33	286.9	6	39	259.0	6
AFG1	76	329.2 $[M+H]^+$	39	243.1	6	29	311.1	6
AFG2	61	331.1 $[M+H]^+$	27	313.1	6	39	245.1	4
FB1	101	722.2 $[M+H]^+$	51	334.2	20	45	352.2	26
FB2	131	706.2 $[M+H]^+$	50	336.3	16	50	318.3	18
FB3	100	706.5 $[M+H]^+$	50	336.3	16	50	318.4	18
FUS-X	47	355 $[M+H]^+$	45	175.0	3	45	246.7	3
ENN A	76	699.4 $[M + NH_4]^+$	59	228.2	16	35	210.1	14
ENN A1	66	685.4 $[M + NH_4]^+$	59	214.2	10	37	210.2	8
ENN B	51	657.3 $[M + NH_4]^+$	39	196.1	8	59	214.0	10
ENN B1	66	671.2 $[M + NH_4]^+$	61	214.2	10	57	228.1	12
BEA	116	801.2 $[M + NH_4]^+$	27	784.1	10	39	244.1	6
OTA	55	404.3 $[M+H]^+$	97	102.1	6	27	239.0	6
STG	106	325 $[M+H]^+$	51	281.0	18	50	310.0	3

m/z: mass/charge

[a]Product ions : Q: Quantification transition ; q : Qualification transition.

[b]Collision Energy (CE), Collision Cell Exit Potential(CXP). All expressed in voltage (V).

Good sensitivity was obtained for selected mycotoxins when the ESI+ mode was applied. Table 2 shows the optimum parameter values for each analyte and the two most relevant MRM transitions: the first transition was used for quantification purposes, whereas the second transition was used to confirm the presence of target compounds in the sample.

In order to determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in MeOH or MeCN and from the standards prepared in extract of blank sample (smoked salmon, and gula substitute). All mycotoxins exhibited good linearity over the working range, and the regression coefficient of calibration curves was higher than 0.992 for all the mycotoxins analyzed.

The LODs and LOQs were estimated from a blank sample extract fortified at decreasing analyte concentration. The LODs and LOQs were calculated using the criterion of S/N ≥ 3 and S/N ≥ 10 for LOD and LOQ, respectively (Table 3). The recoveries, intra-day precisions and inter-day precisions were evaluated by spiking the standard solution to samples at two spiked levels (Table 3). Regarding intraday precision, RSDr for the validated procedures at each spiked level were lower than 10, and 12%, respectively, while for interday precision, RSDr were lower than 13, and 15%, respectively.

The presence of matrix components in the extract can affect the ionization of selected analytes, producing the so-called matrix effects (ME). To avoid ME, matrix-matched calibration curves were used for quantitative mycotoxin determination in fish samples analyzed. ME was evaluated by comparing the slope of a curve constructed with standard and the slope of a matrix-matched curve.

Matrix-matched calibration curves were made by fortified samples with seven levels of addition and were used for quantification purposes. Regarding matrix effects studies, different percentages were observed (ranged from 56% to 95%, corresponding to FB1 and FUS-X, respectively). In the case of mycotoxins which showed higher recovery percentage (>100%) it could be explained by

higher ME of these mycotoxins. Therefore, to minimize these matrix effects, especially in the case of FBs, and for selective and reliable mycotoxin quantification in different samples, matrix- matched calibration standards prepared with blank samples are required.

Table 3. Retention time (RT), recovery, limits of detection and quantitation (LOD and LOQ, respectively) and matrix effect (ME).

MYCOTOXIN	RT (min)	% Recovery	LOD (μg/kg)	LOQ (μg/kg)	ME
AFB1	9.35	92.1	1.0	3.0	93.9
AFB2	9.23	84.0	1.0	3.0	93.5
AFG1	9.07	111.0	2.0	7.0	92.9
AFG2	8.95	108.5	2.0	7.0	89.3
OTA	10.53	85.0	1.0	4.0	96.7
FB1	9.33	80.4	10.0	33.3	56.3
FB2	9.85	97.4	10.0	33.3	58.7
FB3	9.64	107.0	10.0	33.3	76.5
STG	10.90	90.4	10.0	33.3	72.4
FUS-X	13.17	125.0	1.0	4.0	99.8
ENN A	18.35	107.7	1.0	5.0	82.8
ENN A1	18.10	119.2	1.0	4.0	88.1
ENN B	17.00	116.6	1.0	4.0	84.7
ENN B1	17.34	97.7	1.0	4.0	83.2
BEA	17.65	109.5	5.0	10.0	81.7

3.2. Extraction procedure

QuEChERS extraction has been demonstrated as a reliable method to be successfully applied to different matrices. As reported in other surveys,

compared with other extraction methods, QuEChERS method is able to extract adequately all selected mycotoxins, so it was selected in order to take its potential advantage for simultaneous extraction of selected compounds. QuEChERS method have been widely employed for pesticide residues determination in food matrices (Wilkowska and Biziuk, 2011), but also in mycotoxin determination (Rodríguez-Carrasco, 2012).

QuEChERS parameters were set based in other studies which applied this method to complex matrices, such as feedstuffs (Dzuman et al., 2014; Zachariasova et al., 2014), however, taking into account the complexity of these matrices, which contain lipids, proteins, and carbohydrates, an additional step with C18 is necessary in order to eliminate interferences in chromatographic analyses and to obtain suitable recoveries and minimal ME. After the first centrifugation, an aliquot of 2 mL was passed into a 15 mL polypropylene PP centrifuge tube and 0.3 g $MgSO_4$ and 0.1 g C18 were added. Furthermore, as reported by Dzuman et al. (2014), who tested different formic acid concentration, 2% aqueous formic acid solution instead of 0.1% formic acid was necessary before MeCN addition in order to obtain an efficient mycotoxin extraction.

3.3. Application to real samples

A multi-mycotoxin method has been applied to processed fish samples. Mycotoxin contamination in salmon for sushi and in smoked salmon could be expected, due to previous results reported by Tolosa et al. (2014), in which ENs were present in fish flesh of different species (20% of salmon and 10% of rainbow trout). However, results obtained in the present study showed that mycotoxins analyzed were not present neither in smoked fish nor in sushi

samples. In contrast, in gula substitute samples, small amounts of FUS-X were detected in one sample (4 μg/kg) and FUS-X and ENN B in another one (4 μg/kg and 7 μg/kg, respectively). This contamination could be produced due to the ingredients used during gula substitute elaboration.

The absence of mycotoxins in smoked fish could be explained due to fish processing. Thus, in smoking process, fat is eliminated from fillets, so lipophilic mycotoxins can be eliminated in this step with the fat. Fillets are then placed in water solution with higher concentration of salt. In this step, mycotoxins with water affinity can be transferred from fish fillets to water. Dry salt and sugar is then sprinkled on the fillets and placed in a chill until the salt content has reached the optimum level. The salt is then washed off with water to stop the curing process. Thus, during the salting step solute transfer occurs in the water phase between intracellular fluid and the outside medium (Cardinal et al., 2001). These processes can simulate an extraction step, so it would be possible to find mycotoxins, previously present in fillets from aquaculture fish fed with contaminated feed, in water and brine present in smoked fish packaging.

Smoking is the most employed processing method for fish. Traditional smoked salmon is made by 'cold smoking', using salt and wood smoke (Cardinal et al., 2001). Neverthless, there are different types of smoked fish depending on the country of consumption. Thus, in Europe is common to consume smoked salmon or trout, while in tropical countries the most consumed is the sun dried fish. Among all the different types of smoking, smoke drying methods are the most used, especially in countries where this product is highly consumed. Hot smoked and dried fish are widely distributed in many tropical countries as a

major source of good quality protein. The main concern in this type of fish is the presence of toxigenic fungi and their toxic metabolites because, as mention above, the resulting low moisture product obtained when sun drying is favourable for the growth of fungi, which can produce mycotoxins as a result of their metabolism. Futhremore, in some cases these products are dried under improper conditions.

As reported in literature, smoked and dried fish from the tropics are dominated by *Aspergillus* species (Mohamed et al., 2012). According to Sun et al. (2015), there are two possibilities for the mycotoxin contamination in fish. The first option involves the fact that mycotoxins were present in fresh fish tissues after contaminated feed was ingested by animals. On the other hand, the dried seafood could be invaded by mycotoxigenic fungi resulting in mycotoxin contamination.

Mycotoxin determination in fish is still scarce, nevertheless, considering the first option proposed by Sun et al. (2015), mycotoxin transfer from feed to edible portion of fish is reported in diverse surveys, mainly dietary studies in which fish were fed contaminated feed with different mycotoxin contents. Thus, Huang et al. (2011) reported AFB1 contents (2.4-11.8 μg/kg) in muscle and hepatopancreas of gibel carp (Carassius auratus gibelio) from subchronic oral administration. Nomura et al. (2011) reported AFB1 contents in edible muscle of rainbow trout and higher contents of AFB1 metabolites (aflatoxicol (AFL) and aflatoxin M1 (AFM1)) were also detected after dietary exposure in the following decreasing order: AFB1> AFL >AFM1. Despite these results, Nácher-Mestre et al. (2015), reported in their survey that no fish sample analyzed, neither gilthead sea bream nor Atlantic salmon, were contaminated

with mycotoxins after 8 months of feeding contaminated diets with trichothecenes (mainly DON from 19.4 μg/kg to 79.2 μg/kg) and FBs (6.4-754.0 μg/kg).

Considering the second option reported by Sun et al. (2015), suggesting that dried fish and seafood could be invaded by mycotoxigenic fungi under improper storage conditions, different studies have evaluated the presence of mycotoxigenic fungi and also mycotoxins in dried smoked fish. The major part of these surveys has been performed in tropical and subtropical regions, zones with high temperature and humidity conditions. As mentioned above, this type of smoking is different to that applied to samples analyzed in our study because is based in sun dried techniques, while samples analyzed in our survey were not sundried-smoked. However, no data are available on mycotoxin contamination in smoked fish while in sun dried fish and seafood some authors have reported fungi and mycotoxin contamination on those sun dried products.

Some authors have reported that these climatic conditions (temperatures ranging between 25-30ºC and higher humidity) are favourable to fungi growth in smoked-dried fish. Xerophiles are the fungi mostly associated with low aw dried foods, due to their ability to grow at low aw and high temperatures, which explain the widespread occurrence of this fungus in stored dried commodities (Mohamed et al., 2012). These authors reported high levels of ZEN and OTA in seafood samples (317.3 μg/kg and 1.9 μg/kg, respectively) and trace amount of AFB2 (1.2 μg/kg) in carp muscle which were kept for three months at room temperature. Ahmed et al. (2005) showed that xerophilic mold were predominant in salted fish (molouha) from Egyptian origin.

Aspergillus spp. was the most predominant mould (58.2%), followed by *Penicillium* spp. (32.7%). These two fungi genera were also predominant in the survey reported by Edema and Agbon (2009). They were tested for aflatoxin production, showing that 37.5% and 25% of the fungus, respectively, were positive.

The prevalence of *Aspergillus* genera has been widely assessed in smoked dried fish especially in Nigeria. *Aspergillus* species have been reported as the commonest fungi associated with smoked fish by different authors (Hashern, 2011; Yakubu et al., 2015; Samuel et al., 2015). Those fungi isolated from different species of aquaculture fish were tested in vitro for mycotoxin production, showing AFB1, AFB2, AFG1, STG, OTA and T-2 toxin as the most detected mycotoxins.

Furthermore, according to Orony et al. (2015), the mean concentrations of total AFs in the sun dried Dagaa fish from Kenya region were significantly higher than in the fresh ones, as no AFs were detected in the fresh samples. These results suggest that contamination is produced during drying steps, because the product is sun dried for a period of time in which it can be contaminated easily by toxigenic fungus and incomplete drying has been associated with production of AFs. AFs were also detected in smoked-dried fish in Nigeria (Akinyemi et al., 2012; Olajuyigbe et al., 2014), and studies carried out by Adebayo-Tayo et al. (2008) also in Nigeria on smoked dried fish revealed that AFB1 and AFG1 were present in samples at concentrations ranging between 1.5-8.1 and 1.8-4.5 μg/kg, respectively, supposing a risk due to the carcinogenic effects of AFB1. In addition, Job et al. (2015), reported strains of *Penicillium digitatum*, *Fusarium equiseti*, and *Fusarium semitectum* as the

most predominant at 61.7, 30.0, and 26.7% of incidence, respectively, in smoke-dried fish sold in Jos Metropolis (Nigeria).

4. Conclusions

A LC-MS/MS method has been optimized for multi-mycotoxin determination in ready to eat processed samples of fish, including smoked fish, sushi and gula susbstitute. Results obtained showed that mycotoxins were not present in smoked salmon and trout and in sushi samples; however, ENN B and FUS-X were detected in gula substitute, which can be explained by the use of contaminated products or ingredients used in their elaboration or due to improper elaboration and/or storage conditions. Thus, adequate elaboration procedures and storage conditions, mainly cold chain maintenance, are essential to preserve food products from contamination.

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3.7. Multi-mycotoxin analysis in water and fish plasma by liquid-chromatography-tandem mass spectrometry

Chemosphere 145 (2016) 402-408

Multimycotoxin analysis in water and fish plasma by liquid cromatography-tandem mass spectrometry

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ABSTRACT

High performance liquid chromatography-mass spectrometry was used for the determination of 15 mycotoxins in water and fish plasma samples, including aflatoxins, fumonisins, ochratoxin A, sterigmatocistin, fusarenon-X and emerging *Fusarium* mycotoxins. In this work, dispersive liquid-liquid microextraction (DLLME) was assessed as a sample treatment for the simultaneous extraction of mycotoxins. Results showed differences in recovery assays when different extraction solvents were employed. Ethyl acetate showed better recoveries for the major part of mycotoxins analyzed, except for aflatoxins B_2, G_1 andG_2, which showed better recoveries when employing chloroform as extractant solvent. Fumonisins and beauvericin exhibited low recoveries in both water and plasma. This method was validated according to guidelines established by European Commission and has shown to be suitable to be applied in dietary and/or toxicokinetic studies in fish where is necessary to check mycotoxin contents in rearing water and fish plasma.

1. Introduction

Mycotoxins are naturally occurring secondary metabolites of fungi colonizing a wide variety of food and feed. However, organic soil material and aqueous natural samples can be contaminated. In this sense, previous research has documented mycotoxins as environmental contaminants in aqueous natural samples (Kolpin et al., 2014; Maragos, 2012; Schenzel et al., 2010; Wettstein and Bucheli, 2010).

Since the first dispersive liquid-liquid microextraction (DLLME) method was reported in 2006 by Rezaee et al. to analyze organic pollutants in water samples, several different options have been reported. DLLME technique is based on a ternary component solvent system where the dispersion of the fine droplets of the extraction solvent is carried out in the aqueous phase. The principal advantage of DLLME is that the surface area between extraction solvent and aqueous sample is infinitely large; thus, the equilibrium state is achieved quickly and the extraction time is very short (Karami-Osboo et al., 2013). First DLLME methods employed a mixture of a high-density extraction solvent, a water-miscible solvent, and a polar disperser solvent. On one hand, the extraction solvent must be able to extract analytes, must be soluble in the disperser solvent and insoluble in aqueous samples, and normaly presents a density higher than that of water, while, on the other hand, the disperser solvent has to be soluble in both water and extraction solvent (Rezaee et al., 2006).However, nowadays, newly developed techniques have been described by several authors. In this way, depending on the extraction solvent used, DLLME method may be classified in two broad categories: those that use a lower-density extraction solvent (Taherimaslak et al.,2015), and those that use a higher-density extraction solvent (Campone et al., 2011a).

Although DLLME for mycotoxin extraction is still scarce, some DLLME studies about mycotoxin determination in different matrices are reported. However, in these studies, normaly, only one mycotoxin or a reduced group of them are analyzed, for example zearalenone (ZEN) in beer (Antep and Merdivan, 2012), ochratoxin A(OTA) in wines (Arroyo-Manzanares et al., 2012; Campone et al., 2011b), deoxynivalenol (DON) in wheat flour (Karami-Osboo et al., 2013), patulin (PAT) in apple juice (Víctor-Ortega et al., 2013), aflatoxins in pistachio nuts (Taherimaslak et al., 2015), aflatoxin M_1 in milk (Campone et al.,2013), among others.

Recent studies have reported the presence of mycotoxins in feed for aquacultured fish (Nácher-Mestre et al., 2013; Pietsch et al., 2013; Tolosa et al., 2014), which indicates that European aquaculture is commonly exposed to feed-borne *Fusarium* mycotoxins. As fish is fed in water, the contact between contaminated feed and water can lead to contamination of the rearing water. In this sense, results reported by Woźny et al. (2013) indicated that animal feed may be a possible source of ZEN contamination in water. In fact, some toxicity studies on fish, reported that mycotoxins were also present in the rearing water (Nomura et al., 2011).

As occurs in water samples, studies in plasma only analyze one mycotoxin or a reduced group of them mainly in terrestrial animals (Corcuera et al., 2011; Devreese et al., 2012) and also their metabolites (Ivanova et al., 2014; Saengtienchai et al., 2014; Winkler et al., 2014).

In this context, the aim of this study is to develop a method for the simultaneous detection of fifteen mycotoxins in water and fish plasma samples using a modified DLLME method and HPLC-MS/MS for the analysis. The

final method should be applicable for evaluation of rearing water and fish plasmafrom*in vivo*toxicity assays.

2. Materials and methods

2.1 *Chemicals and reagents*

All solvents (acetonitrile, methanol, chloroform and ethyl acetate) were purchased from Merck (Darmstadt, Germany). Deionized water (>18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) and formic acid were supplied by Sigma-Aldrich (St. Louis, USA). All solvents were passed through a 0.22 μm cellulose filter from Membrane Solutions (Texas, USA).

The stock standard of mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FUS-X, STG, FB_1, FB_2 and FB_3) were purchased from Sigma-Aldrich. Enniatins (ENs) and beauvericin (BEA) toxin solutions were provided by Biopure (Tulln, Austria). Individual stock solutions (1 mg) were diluted in 1 mL of methanol (concentration of 1000 mg/L). Then, intermediate solutions with decreasing concentration were prepared by dilution from the stock (from 1000 mg/L to 100 mg/L, from 100 mg/L to 10 mg/L, and from 10 mg/L to 1 mg/L). They were stored in glass-stoppered bottles and darkness in security conditions at -20ºC. Working standard solutions (ranged from 0.01 to 100 μg/L) were prepared by the suitable dilution from 1 mg/L solution and were kept in darkness at 4 ºC. Water and plasma samples were obtained from a group of jundiás (*Rhamdiaquelen*) maintained in tanks of 300 L at 18-20 ºC and 12 hours/day of light/darkness.

2.2. Procedures

2.2.1. Sample preparation

The method of addition of standards to water and plasma samples were based in theaddition of a known amount of analytes to be detected (fortified samples). These fortified samples were spiked with a working mixture of the mycotoxins (the concentration added corresponded to 10 and 100 times the limit of quantification (LOQ) for each mycotoxin analyzed). The concentration range for each analyte was as follows: For aflatoxins, recovery assays were performed with concentrations of 20 μg/L, and 200 μg/L; for emerging *Fusarium* mycotoxins, 10 μg/L, and 100 μg/L; and for fumonisins, FUS-X, STG and OTA, 100 μg/L, and 1000 μg/L.

2.2.2. Extraction procedure in water

A solution of 5 mL of water and 1g of NaCl was placed in a 15 mL tube with conical bottom and mixed in a vortex for 1 min. Then, a mixture of the disperser solvent (900 μL of MeCN) and the extraction solvent (700 μL of EtOAc) was added and mixed in a vortex for 1 min. A cloudy dispersion consisting of water, disperser and extraction solvents appeared. Then, the mixture was centrifuged at 4500 rpm for 5 min and 5 ºC for phase separation. After this procedure, two different phases appeared. Since ethyl acetate is lighter than water (density, d = 0.897 g/mL), the extraction phase floated on the surface of the aqueous solution, so the upper layer containing the mycotoxins was collected using a syringe and placed into a 15 mL tube, which was evaporated until dryness under a gentle stream of N_2. Finally, samples were reconstituted with 1 mL of MeOH:H_2O (50:50, v/v), filtered (0.22 μm) and injected into the LC-MS/MS system.

2.2.3. Extraction procedure in plasma

For plasma samples, an additional step before DLLME was carried out in order to precipitate proteins. To do this, 750 μL of MeCNwere added to 250 μL of plasma and this mixture was centrifuged at 4500 rpm 4 ºC for 5 min. The supernatant was placed in a clean eppendorf for DLLME extraction and 0.2 g NaCl were added. Then, a mixture of the disperser solvent (475 μL of MeCN) and the extraction solvent (350 μL of EtOAc) was added and mixed in a vortex for 1 min, followed by a centrifuged step at 4500 rpm for 10 min and 4 ºC for phase separation. After this procedure, two different phases appeared.The organic upper phase containing the analyteswas removed and evaporated until dryness under a gentle stream of N_2. Finally, samples were reconstituted with 1 mL of MeOH/H_2O (50:50, v/v), filtered (0.22 μm) and injected into the LC-MS/MS system.

2.3. Method validation

The method was validated according to the guidelines established by the EU Commission Decision 2002/657/EC. The performance characteristics, including linearity, specificity, precision, limits of detection (LOD) and quantification (LOQ), recovery, and matrix effect were obtained.Representative matrixes of water and plasma were selected for validation purposes. All mycotoxins exhibited good linearity over the working range (10 LOQ and 100 LOQ), and the regression coefficient of calibration curves was higher than 0.992 for all the mycotoxins analyzed.

Limits of detection (LOD) were estimated as the lowest-matrix-matched calibration standard corresponding to a signal to noise ratio at least 3:1 and limits of quantification (LOQ) to at least 10:1.

Extraction recovery was determined by analyzing the samples spiked before extraction and spiked after extraction and calculating the ratio of the peak areas for each analyte. These experiments were carried out in two addition levels (10LOQ and 100LOQ). The precision was expressed as the RSD of the calculated recovery.

Matrix effects were evaluated by comparing the slope of a curve constructed with standard and matrix-matched slope of a calibration curve. Matrix-matched calibration curves were made by fortified samples with seven levels of addition, depending on the LOQ of each mycotoxin. The matrix-matched calibration curve was used for quantification.

2.4. HPLC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA) consisting of an autosampler, a quaternary pump, and a pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Version 4.1, were used for the MS/MS analyses. The method was developed based in previous experience in laboratory (Rubert et al., 2011; Blesa et al., 2014) with some modifications. The separation was achieved by a Gemini-NX C18 (150 mm x 2 mm I.D., 5 μm particle size) analytical column supplied byPhenomenex (Barcelona, Spain). The analytical separation for LC-MS/MS was performed using gradient elution with 95% of water (with 5 mM of formate ammonium and 0.1% formic acid) as mobile phase A, and 5% of methanol as mobile phase B (with 5 mM of formate ammonium and 0.1% formic acid), increasing linearly to 95% B for 10 min; then, decreasing linearly to 80% B for 5 min, and then gradually up to 70% B for 6 min. Finally, initial

conditions were maintained for 3 min. The injection volume was 20 μL. Flow rate was maintained at 0.2 mL/ min. Analysis was performed in positive ion mode (ESI+). The ESI source values were as follows: capillary voltage, 3.50 kV; source temperature, 120 ºC; desolvation temperature, 400 ºC; cone gas 50l/h; desolvation gas (nitrogen 99.99% purity) flow, 800 l/h.

The analyser settings were as follows: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, 5 and 3, respectively; multiplier, 650; collision gas (argon 99.99% purity) pressure, 3.83 x 10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0s; dwell time 0.1 ms. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode. All time measurements were carried out in triplicate.

3. Results and discussion

3.1. Optimization of HPLC conditions

For method optimization, water samples from tap water and fish plasma were first analyzed to check that no contamination with mycotoxins was present and these samples were used as blank samples to carried out recovery assays and calibrate curves.

Before optimizing the MS/MS conditions, full scan and daughter scan under positive and negative mode has been used. In addition, each compound was also characterized by the retention time. The criteria adopted for accepting the analysis was a retention time deviation lower than 2.5% compared to the standard.

The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) was performed by flow

injection analysis for each standard mycotoxin. The optimized parameters are shown in Table 1. Entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively, for all analytes. The MS was operated in multiple reactionmonitoring (MRM) mode and with the resolution set to unit resolution for Q_1 and Q_3.

Good sensitivity was obtained for selected mycotoxins when the ESI+ mode was applied: the base peak observed was $[M+H]^+$ for all the mycotoxins studied. Table 1 shows the optimum parameter values for each analyte and the two most relevant MRM transitions: the first transition was used for quantification purposes, whereas the second transition was used to confirm the presence of target compounds in the sample.

In order to determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in MeOH and from the standards prepared in extract of blank sample (tap water and fish plasma). All mycotoxins exhibited good linearity over the working range (10 LOQ and 100 LOQ), and the regression coefficient of calibration curves was higher than 0.992 for all the mycotoxins analyzed. Respect to the evaluation of matrix effects, a suppression of the signal for all mycotoxins was observed (ranged from 8% to 37%). Therefore, to minimize these matrix effects and for a selective and reliable mycotoxin quantification in different samples, the employment of liquid chromatography coupled to mass spectrometry (LC-MS/MS) required matrix-matched calibration standards prepared with blank samples.

The LODs and LOQs were estimated from an extract of a blank sample, fortified with decreasing concentrations of the analytes. Additions were performed from three different blank samples (n=18), to the estimated

concentrations for each mycotoxin. The LODs and LOQs were calculated using the criterion of S/N ≥ 3 and S/N ≥ 10 for LOD and LOQ, respectively (Table 2).

The recoveries, intra-day precisions and inter-day precisions were evaluated by spiking different levels of standard analyte to samples at two spiked levels (10 times LOQ and 100 times LOQ) (Table 2). According to Commission Decision (2002), recoveries were within the relative standard deviation (lower than 20%), except for FBs and BEA in plasma samples, which were lower than 70%.To date, there are many reports describing lower recoveries for FBs.

According to other authors, the main difficulty in this regard is the low compatibility between the high organic percentage that is needed for the extraction of most mycotoxins and the high water content and/or acidic conditions that are needed for FBs extraction (Cao et al., 2013).In general, recoveries in plasma samples were lower than in water samples, this fact can be explained because of protein content present in plasma, although a deproteinization step was applied first.

Matrix effect (ME) was calculated by comparing the slope between calibration curvein sample extracts with a calibration curve constructed in pure solvent (MeOH) both with the same concentration.The calculation of the signal suppression/enhancement (SSE) due to matrix effects was performed as follows: SSE (%) = ($slope_{with\ matrix}$ / $slope_{in\ MeOH}$) * 100
A value close to 100% indicates that there is not a significant matrix effect, while values >100% and <100% indicate signal enhancement and signal suppression, respectively.

Table 1: Product-ions obtained in product ion scan mode for selected mycotoxins and MRM (Multiple Reaction Monitoring) optimized parameters.

Mycotoxin	Retention time (min)	Precursor ion (m/z)	Product ions[1]	DP (V)[2]	CE (V)[2]
AFB_1	13.18	313.2	269.0q/241.0Q	47	30/30
AFB_2	12.66	315.2	243.0q/259.0Q	50	30/30
AFG_1	12.46	329.2	215.0q/200.0Q	43	30/40
AFG_2	12.07	331.2	217.0q/189.0Q	46	25/45
OTA	15.16	404.1	358.1q/341.2Q	24	14/16
ENA	17.52	681.9	228.2q/210.2Q	40	35/35
ENA_1	17.28	667.9	228.0q/210.0Q	40	35/35
ENB	16.51	639.8	214.2q/196.2Q	40	35/35
ENB_1	16.79	654.9	214.2q/196.2Q	40	35/35
BEA	17.08	784.4	262.0q/244.0Q	35	25/25
FB_1	13.51	722.0	334.0q/352.0Q	50	30/30
FB_2	14.38	706.4	318.0 q/336.0 Q	50	30/30
FB_3	13.53	706.6	336.4q/318.1Q	40	40/40
FUS-X	17.92	355.3	144.8q/267.1Q	50	26/26
STG	15.40	325.3	297.0q/281.0Q	50	20/30

m/z: mass/charge; [1]Product ions : Q: Quantification transition ; q : Qualification transition.; [2] Declustering Potential (DP); Collision Energy (CE). All expressed in voltage (V).

Table 2. Analytical parameters obtained in the validation of the method employing ethyl acetate as extractant solvent. Limits of detection (LODs), limits of quantification (LOQs), matrix effects (ME) (%), recoveries, intraday and interday precision for water and plasma samples.

	LOD (µg/kg)		LOQ (µg/kg)		ME (%)		Intraday precision Revovery (%) ± RSD[1]				Interday precision Revovery (%) ± RSD[1]			
Analytes	Water	Plasma	Water	Plasma	Water	Plasma	Water		Plasma		Water		Plasma	
							10LOQ	100LOQ	10LOQ	100LOQ	10LOQ	100LOQ	10LOQ	100LOQ
AFB_1	0.8	0.7	2.0	2.0	91.9	89.7	120.0±8.1	108.3±7.8	91.0±7.2	85.2±7.6	109.2±8.1	111.0±7.8	88.6±7.1	87.3±6.8
AFB_2	0.8	2.0	2.0	4.0	88.8	83.4	82.1±7.8	82.2±7.3	82.2±6.3	81.3±7.1	76.3±7.8	80.2±8.1	83.5±7.3	80.2±7.1
AFG_1	0.8	2.0	2.0	4.0	87.5	85.8	83.3±8.2	72.3±7.6	76.3±8.6	74.5±7.2	72.4±8.2	74.3±8.3	73.6±7.6	77.6±7.2
AFG_2	0.8	2.0	2.0	4.0	83.6	82.8	86.6±8.3	86.4±7.7	81.6±8.4	82.9±7.5	85.6±7.3	82.6±7.6	80.2±8.2	83.5±7.8
OTA	3.0	7.0	10.0	12.0	83.9	78.3	82.4±7.6	85.6±7.5	85.3±7.8	88.3±8.1	81.3±7.8	76.4±7.4	80.2±7.6	85.0±7.6
ENA	0.5	0.5	1.0	2.0	91.2	84.8	81.0±7.8	75.4±8.1	105.2±5.2	102.7±8.2	70.2±8.1	72.6±7.8	102.3±7.1	102.3±7.8
ENA_1	0.2	0.2	1.5	2.0	90.7	86.3	80.7±8.1	83.3±7.4	116.7±7.4	110.6±6.7	82.5±7.1	81.7±7.1	104.5±6.4	106.3±6.8
ENB	0.1	0.1	1.5	1.0	88.5	87.4	91.6±6.7	89.2±7.1	82.6±6.2	83.2±6.5	92.6±7.6	87.3±7.6	70.4±6.2	72.8±6.7
ENB_1	0.1	0.1	1.5	1.0	86.8	85.5	92.3±7.1	85.8±7.2	111.3±7.2	115.2±7.2	90.7±7.3	84.4±7.3	102.8±6.7	105.8±6.8
BEA	0.4	0.5	1.5	2.0	80.8	78.7	83.8±7.3	76.7±7.1	72.4±8.6	71.3±7.6	72.4±8.2	71.8±8.2	60.3±8.1	60.8±7.4
FB_1	8.0	8.0	15.0	17.0	83.2	65.8	78.3±8.1	73.5±8.5	60.8±8.2	63.6±8.2	78.8±8.6	72.6±8.1	58.4±7.3	57.6±7.6
FB_2	10.0	10.0	17.0	15.0	84.3	72.4	67.4±8.2	67.4±8.1	71.5±7.1	73.4±7.8	65.4±8.1	69.3±7.8	56.4±8.2	58.6±7.8
FB_3	10.0	12.0	15.0	17.0	81.1	68.8	81.7±7.8	83.3±8.7	61.6±8.2	65.8±7.6	80.7±8.3	84.2±8.4	54.3±8.6	56.5±8.1
FUS-X	8.0	8.0	12.0	15.0	81.7	79.4	87.4±7.4	85.2±7.4	71.5±7.5	75.7±7.1	87.4±7.3	82.4±7.6	70.4±7.8	71.3±7.6
STG	8.0	5.0	15.0	17.0	87.3	84.7	83.6±6.7	80.7±7.1	86.2±7.3	84.3±6.8	81.3±7.6	86.7±7.3	83.5±7.1	82.4±8.1

There was a suppression of the signal for all mycotoxins, especially in plasma samples (between 8.1 and 19.2% in water samples, and 10.3 and 34.2% in plasma samples).

3.2. Optimization of sample extraction

The sample volume for water was set based in other studies about OTA in wines, such as the one conducted by Arroyo-Manzanares et al. (2012),Campone et al.(2011b),orpatulin in applejuices (Víctor-Ortega et al., 2013).

For plasma samples, the volume was set based in other studies about mycotoxin analysis in animal plasma, although DLLME was not the extraction performed in those studies (Devreeseet al. 2012).

Using MeCN as disperser solvent, the cloudy state was formed well, thus, MeCN was directly selected as the optimum disperser solvent based onprevious work and other studies reported in scientific literature (Antep&Merdivan, 2012; Karami-Osboo et al., 2013).

3.2.1 Selection of the extractant solvent

The type of extractant is one of the most important parameters that affect the efficiency of DLLME. In this sense, two different extraction solvent were tested, chloroform ($CHCl_3$) and ethyl acetate (EtOAc), in water and plasma samples. Results are shown in Fig. 1 and Fig. 2. EtOAc was chosen as an alternative to chloroform because EtOAc (and other ethers) are hydrogen bond acceptor molecules and therefore extract electron donor solutes more readily than chloroform. Fig. 1 shows recoveries of two extractant solvents for the mycotoxins analyzed in the study in water samples.

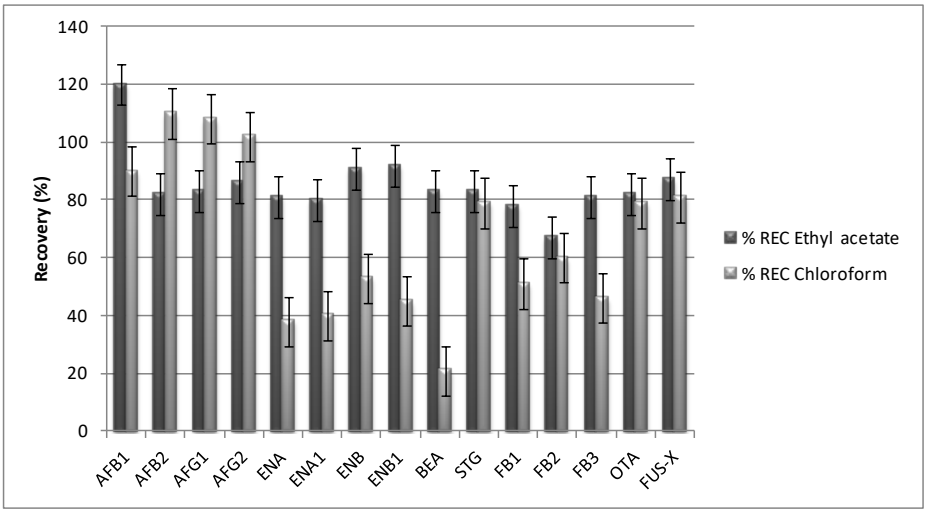

Fig. 1. Recovery assays performed comparing two different extractant solvents (ethyl acetate and chloroform) in water samples.

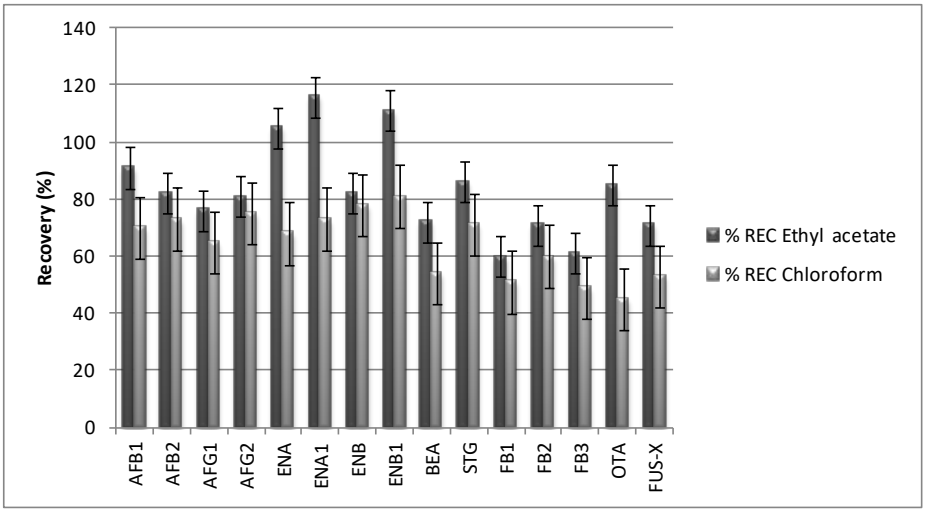

Fig. 2. Recovery assays performed comparing two different extractant solvents (ethyl acetate and chloroform) in plasma samples.

Regarding water samples, the recoveries of AFB_1 decreased from 120% to 90% when using chloroform as extraction solvent. However, recoveries of other mycotoxins remained consistent, except in the case of emerging *Fusarium* mycotoxins, which recoveries were lower when using chloroform. Thus, EtOAc was selected as the optimum extraction solvent.

Some methods reported by other authors obtained good recoveries when using chloroform as extractant solvent, possibly due to the performance of an extraction step before to apply DLLME employing other solvents, such as MeOH,or other techniques, such as quechers extraction for solid samples (Arroyo-Manzanares et al., 2013; Amelin et al., 2013) or Oasis cartridges in aqueous samples(Campone et al., 2011b; Taherimaslak et al., 2015).In this sense, aqueous mixtures of methanol were usually applied for the extraction of AFs from foods (Campone et al., 2011a).

3.2.2. Optimization of salt addition in water samples

The addition of salt usually increases the solubility of analytes in the organic phase and the volume of the drop by reducing the solubility of the extractant (Fernández et al., 2014). The addition of salt to the aqueous sample can significantly improve the extraction of analytes in DLLME, possibly due to the salting-out effect. Hence, a series of experiments were performed in which NaCl was added at different concentrations (0.2g, 0,5g and 1g) to water samples extracted with MeCN-EtOAc. Results obtained are shown in Fig. 3. With a small amount of salt the separation phase was not clear, whereas with larger amounts (1 g) the separation between the phases was well defined by a solid protein material and peak shape was better defined (Fig. 4), as occurs in other studies (Campone et al., 2013).

The results showed that the highest recovery for all fifteen mycotoxins was obtained with 1g NaCl (Fig. 3). Thus, 1g NaCl was selected for subsequent experiments in watersamples.

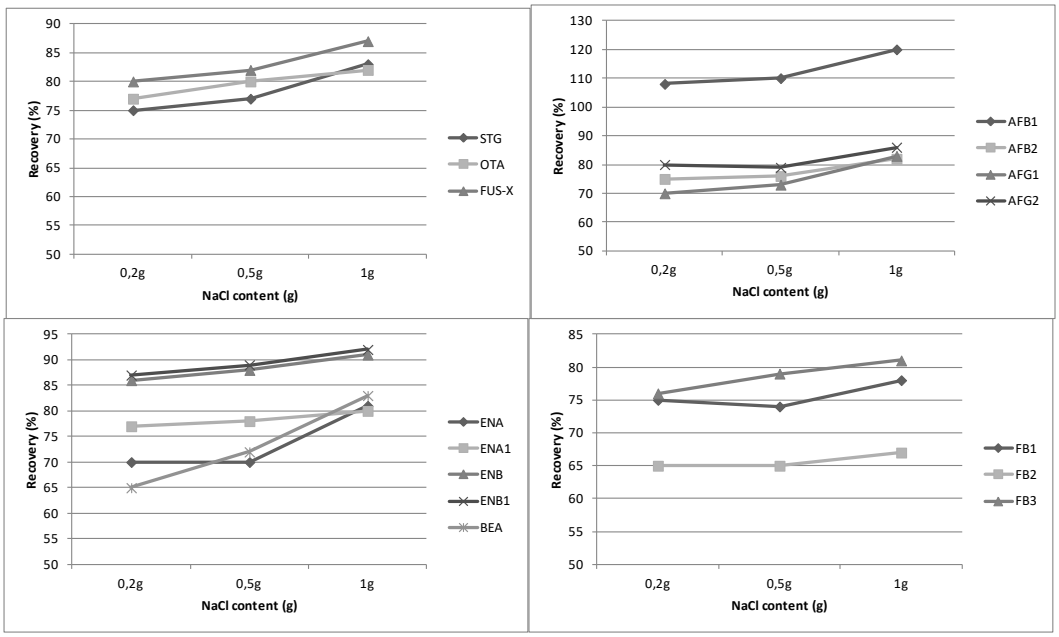

Fig 3. Recoveries obtained with different salt addition in water samples extracted with EtOAc.

In the case of plasma samples, only 0.2 g ofNaCl were added, due to the lower volume employed (250 μL). This NaCl content was selected based in other DLLME studies (Fernández et al., 2014).

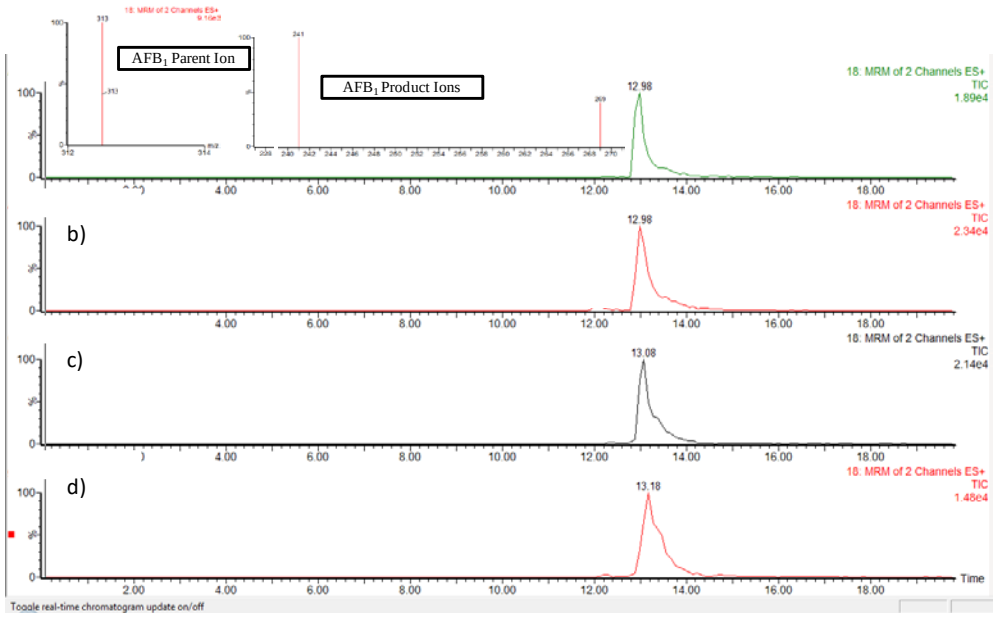

Fig. 4. Extracted chromatogram of AFB_1 with chloroform and 1g NaCl (a), EtOAc and 1g NaCl (b), EtOAc and 0.5g NaCl (c) and EtOAc and 0.2g NaCl (d).

4. Conclusions

A new dispersive liquid-liquid microextraction with lower-density extraction solvent has been developed in water and plasma samples. This method has shown to be appropriated for both water and plasma samples in multiclass mycotoxin determination, reducing extraction time and being environmentally friendly. Results showed that the method validated is adequate to be applied in real samples from toxicity studies, such as rearing water and fish plasma. However, for FBs and BEA extraction, future analyses are needed testing different solvent mixtures in acidic conditions in order to improve their recoveries.

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3.8. Mitigation of enniatins in edible fish tissues by thermal processes and identification of degradation products

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Mitigation of enniatins in edible fish tissues by thermal processes and identification of degradation products

Josefa Tolosa, Guillermina Font, Jordi Mañes, Emilia Ferrer

ABSTRACT

Emerging mycotoxins, such as enniatins and beauvericin, are common contaminants in vegetal matrices, but recently, the occurrence of mycotoxins in foodstuffs from animal origin has been also reported as they can be present in edible tissues of animals fed with contaminated feedstuffs. Sea bass, sea bream, Atlantic salmon and rainbow trout from aquaculture analyzed in the present survey showed contamination by emerging *Fusarium* mycotoxins enniatins (ENs). ENs were extracted from raw and cooked fish with acetonitrile and analyzed by Liquid Chromatography coupled to Mass Spectrometry. In this study, the stability of ENs was evaluated during food processing by the application of different cooking methods (broiling, boiling, microwaving and baking treatments). All treated samples showed a reduction in mycotoxin levels with different percentages depending on the type of EN and the fish species. Thus, the reduction obtained ranged from 30 to 100%. The thermal treatments have shown to be a good strategy to mitigate ENs content in edible fish tissues. On the other hand, some ENs degradation products originated during the application of thermal treatments were identified.

1. Introduction

Emerging mycotoxins, such as enniatins (ENs) and beauvericin (BEA), are depsipeptide molecules produced by the secondary metabolism of fungi from *Fusarium* genera. These mycotoxins occur as contaminants mainly in cereals, such as wheat, barley and maize, but also in oats, rye and rice. However, their presence has been also reported in other matrices from both vegetal and animal origin (Tolosa et al., 2013). The most prevalent ENs in food are ENA, ENA_1, ENB and ENB_1 (Serrano et al., 2012). The presence of these contaminants and their metabolites in products from animal origin, such as meat, milk, eggs and cheese could be consequence of a carry-over of these compounds into animal tissues after feeding of contaminated feed (Zhao et al., 2015).

In the last years, several studies have been focused in the development of strategies to reduce mycotoxin levels during food and feed production. As reported by other authors, food processing of cereals has effects on mycotoxins, leading to less-contaminated food compared to the raw materials (Hu et al., 2014). Different industrial processes have shown to be effective practices to reduce mycotoxin contents. In this sense, the content of some mycotoxins can be reduced due to thermal food processing applied, such as cooking, boiling, baking, frying, roasting and pasteurization (Kabak, 2009).

The content reduction achieved depends on several parameters. Some parameters are related with the nature and chemical structure of the mycotoxins and with the initial level of contamination in food. Other parameters are related with the treatment applied, such as temperature, time, pH, moisture content, etc (Bretz et al., 2006; Ryu et al., 2008; Serrano et al.,

2013). Regarding reduction achieved on other "traditional" *Fusarium* mycotoxins, Bretz et al. (2005) reported nivalenol (NIV) degradation at high temperatures and prolonged heating time. Beyer et al. (2009) have reported mycotoxin reduction depending on the interaction with other components such as sugar, starch and protein model. In the study conducted by these authors, the fate of T-2 toxin under typical food-processing conditions was assessed by applying different heating experiments. T-2 degradation was observed under all conditions, accelerating with rising temperatures, but the strongest degradation was observed in the protein model. However, studies performed with fumonisins (FMs) showed higher reduction percentages in sugar models (Bullerman and Bianchini, 2007). Most of the data indicate that FMs levels are decreased during heating, baking, frying, roasting, nixtamalizing and extrusion cooking of foods, concluding that reduction is directly related to time and temperature applied, as foods reaching temperatures greater than 150ºC during processing may have lower FMs levels (De Girolamo et al., 2016).

Limited data is available on the effects of food processing on ENs and BEA contents. As reported in the EFSA (European Food Safety Authority) Scientific Opinion on the risks to human and animal health related to the presence of BEA and ENs in food and feed, more data is necessary in order to assess the role of temperature applied in the mycotoxin content reduction and other factors related present in different foodstuffs (SCF, EFSA, 2014).

Thus, the content reduction of *Fusarium* mycotoxins ENs and beauvericin (BEA) by thermal treatments have been reported by some authors (Meca et al., 2012; Serrano et al., 2013; García-Moraleja et al., 2015). Furthermore, recent studies have evidenced that ENs contents are reduced through common

industrial processes, such as bread-making (Vaclavikova et al., 2013; Hu et al., 2014), beer-making, brewing or malting processes (Meca et al., 2013; Hu et al., 2014) and pasta production (Tittlemier et al., 2014; García-Moraleja et al., 2015; Serrano et al., 2016). Concerning fish, thermal treatments are applied to cook food; however, no data is available related with the employment of thermal processes to reduce mycotoxin contents in fish fillets. Furthermore, literature about natural occurrence of mycotoxins in edible fish fillets is still scarce (Tolosa et al., 2014).

Nevertheless, other studies have focused on the isolation and characterization of new mycotoxin products generated after thermal treatment application to food, as well as in the evaluation of the toxicity of these compounds. In most cases, degradation products are less toxic than their original molecules (Bretz et al., 2005; Beyer et al., 2009). In this sense, Shams et al. (2011) reported a new less-toxic derivative of diacetoxyscirpenol after thermal treatment applied to potatoes.

Considering the lack of data related to ENs and BEA degradation during food processing, the purpose of this study is to provide information on the fate of ENs and BEA during the fish cooking process and to determine the content reduction of emerging *Fusarium* mycotoxins in fish tissues achieved by four different thermal processes commonly used to cook fish (conventional oven, microwave, broiled and boiled treatments). Analyses were carried out by liquid chromatography coupled to tandem mass spectrometry with linear ion trap (LC-MS/MS-LIT).

2. Materials and methods

2.1. Materials

All solvents (acetonitrile (MeCN) and methanol (MeOH)) were purchased from Merck (Darmstadt, Germany). Deionized water (<18 MΩ/cm resistivity) was obtained from a Milli-Q water purification system (Millipore Corporation, Bedford, MA, USA). Ammonium formate (HCO_2NH_4, 97%) was supplied by Sigma-Aldrich (St. Louis, USA). All solvents were passed through a 0.22 μm cellulose filter from Membrane Solutions (Texas, USA).

ENs and BEA toxin solutions were provided by Biopure (Tulln, Austria). Individual stock solutions (1 mg) were diluted in 1 mL of MeCN (1000 mg/L). Then, intermediate solutions with decreasing concentration were prepared by dilution from the stock (from 1000 mg/L to 100 mg/L, from 100 mg/L to 10 mg/L, and from 10 mg/L to 1 mg/L). They were stored in glass-stoppered bottles and in darkness conditions at -20ºC. Working standard solutions (ranged from 0.01 to 100 μg/L) were prepared by the suitable dilution from 1 mg/L solution and were kept in darkness at 4 ºC.

2.2. Sampling

The species included in the survey were selected due to their important production in aquaculture. Atlantic salmon is economically the most important farmed fish in Europe, although other commercially reared species include rainbow trout, sea bass, sea bream, cod, halibut, tuna, eel and turbot. In this sense, fourty fish samples of sea bass, sea bream, Atlantic salmon and rainbow trout (*Dicentrarchus labrax* (n=10), *Sparus aurata* (n=10), *Salmo salar* (n=10) and *Oncorhynchus mykiss* (n=10), respectively) were purchased from different supermarkets located in Valencia (Spain), all of them from aquaculture

farming. The origin of the sea bass and sea bream samples were Spain and Greece, respectively, which is the main producer of sea bream in European aquaculture, while Atlantic salmon was from Norway and rainbow trout belonged from Spain. Samples were previously deboned and beheaded. All samples were stored in a dark and dry place at -20ºC until analysis. After their packages had been opened they were analyzed within the same day.

2.3. Thermal treatments (Cooking techniques)

Fish fillets were divided into 5 different parts, one of them was analyzed raw. Results for ENs contents in raw samples of sea bass and sea bream were previously reported by Tolosa, Font, Mañes, Ferrer, 2014. The other four parts were reserved for further processing. Four different thermal treatments were applied to those positive raw samples in all the species analyzed in the survey. The treatments applied were those most common employed to cook fish: boiled (BO), broiled (BR), baking in a conventional oven (CO) and baking in a microwave oven (MO). Parameters and thermal procedures were set based in literature reviewed about the effect of thermal treatments on nutritional parameters in different fish species (Nurhan, 2007; Ersoy & Özeren, 2009; Hosseiniet al., 2014). No salt, oil or other ingredients were added. Each fillet was cooked separately and raw fillets were used as the reference for calculating the percentage reduction achieved by the different thermal treatments applied. Thus, levels in raw fillets were considered to be the initial concentration.

Broiling (BR): Fish fillets were cooked in a pan for a total of 10 min (5 min each side) in a preheated pan. The temperature reached in the center of the fillet ranged between 64 and 70 ºC.

Microwave oven-cooked (MO): The fish fillet was placed in the microwave and cooked for 3 min at regular power (650W). The temperature reached in the center of the fillet ranged between 69 and 74 ºC.

Boiling (BO): Fish fillets were boiled in 500 ml of tap water for 5 min. The temperature reached in the center of the fillet ranged between 63 and 68 ºC.

Baking in a conventional oven (CO): Fillets were cooked in a conventional oven at 180ºC for 30 min. The temperature reached in the center of the fillet ranged between 64 and 69 ºC.

2.4. Mycotoxin extraction procedure

Sample preparation was performed according to a previous study (Tolosa et al., 2014). In brief, 10 g of homogenized fish sample was mixed with 50 ml of MeCN for 30 min and 30°C using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA). The extract was evaporated to dryness at 30ºC using a Büchi Rotavapor R-200 (Flawil, Switzerland). The solution is reconstituted in 10 mL of MeCN-MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate) and centrifuged at 3500g for 15 min and 5°C. The supernatant was purified using C_{18} cartridges (Waters, Milford, Massachusetts) by applying a slight vacuum. The extract was transferred to a 15 mL conical tube and evaporated to dryness at 30ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the solution was reconstituted with 1 mL of MeCN-MeOH 20 mM of ammonium formate 50:50 v/v and placed again in the ultrasonic bath (30 min, 30°C). Then, the solution is washed with hexane to eliminate fat particles and filtered through 13 mm/0.22 μm nylon filter (Membrane Solutions, Texas, USA) prior

to injection into the LC-MS/MS system. The samples were extracted in triplicate.

2.5. Analysis

2.5.1. LC-MS/MS analysis

LC–MS/MS-LIT analyses were conducted on a system consisting of an Agilent 1200 chromatographic system (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® mass spectrometer (AB Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionization (ESI) interface. The QTRAP® analyser combines a fully functional triple quadrupole and a linear ion trap mass spectrometer within the same instrument. Separation of analytes was performed using a Gemini-NX LC-column (Phenomenex, Aschaffenburg, Germany) (150 mm × 4.6 mm, 5 μm of particle size) preceded by a guard column utilizing the same packing material. The flow rate was set to 0.8 mL min^{-1}, and the oven temperature was 40°C, with mobile phase A slightly acidified with 0.1% formic acid and 5 mM ammonium formate and eluent B (mobile phase B) methanol with 5 mM ammonium formate and 0.1% formic acid. The elution gradient started with 0% of eluent B, increased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 70% in 2 min. During the subsequent 6 min, the column was cleaned, readjusted to the initial conditions and equilibrated for 7 min. The volume of the injections was 20 μL.

The analyses were performed using the Turbo V® ionspray in positive ionization mode (ESI+). Nitrogen served as the nebulizer and collision gas. The operating conditions for the analysis were the following: ion spray voltage, 5500 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 50 and 50 psi,

respectively; probe temperature (TEM), 450°C. MRM experiments were performed to obtain the maximum sensitivity for the detection of target molecules.

2.5.2. *Identification of degradation products by LC-MS-LIT*

Positive samples were analyzed by LC-MS-LIT in order to identified degradation products from ENs. For this purpose, an Applied Biosystems/MDS SCIEX Q TRAP TM Linear Ion Trap (LIT) mass spectrometer (Concord, Ontario, Canada), coupled with a Turbo Ion Spray source, was used. The analyses were carried out using the ER mode for newly formed compounds in the mass range from 200 to 900 Da, and the EPI mode to obtain a MS^2 scan of adducts fragments.

3. Results and discussion

3.1. Method performance

The method was previously house-validated and results were reported by Tolosa et al. (2014). Before the MS/MS conditions were optimized, full scan and daughter scan under positive and negative mode have been used. In addition, each compound was also characterized by the retention time (ENA 15.4 min; ENA_1 15.5 min; ENB 13.8 min; ENB_1 14.2 min; BEA 14.1 min). The criteria adopted for accepting the analysis was a retention time deviation <2.5% compared to the standard. The optimization of MS parameters as declustering potential (DP), collision energy (CE), and collision cell entrance potential (CEP) was performed by flow injection analysis for each standard mycotoxin, expressed in volts (V); entrance potential (EP) and collision cell exit potential (CXP) were also optimized for each analyte. Results for these parameters were as follows: DP (ENA 76V; ENA_1 66V; ENB 51V; ENB_1 66V

and BEA 116V).CE (ENA 25/59V; ENA_1 37/59V; ENB 39/59V; ENB_1 61/57V and BEA 27/39V). CXP (ENA 14/16V; ENA_1 8/10V; ENB 8/10V; ENB_1 10/12V and BEA 10/6V). The MS was operated in multiple reaction monitoring (MRM) mode and with the resolution set to unit resolution for Q1 and Q3. Method validation was carried out according to the guidelines established by the European Commission (Decision 2002/657/EC). The method validation included the determination of linearity, limits of detection (LODs), limits of quantitation (LOQs), recoveries, repeatabilities (intraday precision), reproducibilities (interday precision), and matrix effects. Good sensitivity was obtained for selected mycotoxins when the ESI+ mode was applied: the base peak observed was $[M + NH_4]^+$ for all of the mycotoxins studied. Optimum parameter values for each analyte and the two most relevant MRM transitions were selected. Precursor ions obtained $[M + NH4]^+$ and product ions were: ENA 699.4 ($210^Q/228^q$); ENA_1 685.4 ($210^Q/214^q$); ENB 657.3 ($196^Q/214^q$), ENB_1 671.2 ($214^Q/228^q$) BEA 801.2 ($784^Q/244^q$). The Q transition was used for quantification purposes, whereas the q transition was used to confirm the presence of target compounds in the sample. The recoveries, intraday precisions, and interday precisions were evaluated (Commission Decision, 2002). To determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in methanol and from the standards prepared in the extract of blank sample (fish from extractive fishing). All mycotoxins exhibited good linearity over the working range (10LOQ and 100LOQ), and the regression coefficient of calibration curves was >0.992. The intraday precision ranged was between 78-86% at 10LOQ and 82-91% at 100LOQ; interday precision ranged between 81-86% at 10LOQ and 82-93% at 100LOQ. For the evaluation of matrix

effects, a mixture of blank extracts of different fish was prepared as blank sample. A suppression of the signal for all mycotoxins was observed (ranging from 7 to 40%). Therefore, to minimize these matrix effects and for a selective and reliable mycotoxin quantification in different fish samples, the employment of liquid chromatography coupled to mass spectrometry with linear ion trap (LC-MS/MS-LIT) required matrix-matched calibration standards prepared with fish samples. The LODs and LOQs obtained were as follows: ENA and BEA 3μg/kg and 10μg/kg, respectively, and ENA_1, ENB and ENB_1, 0.3 μg/kg and 1.0μg/kg).

3.2. ENs contents in fish samples

The results obtained in sea bass and sea bream raw fillets reported by Tolosa et al., 2014 and the results corresponding to raw Atlantic salmon and rainbow trout analyzed in this study are showed in Table 1. The results obtained in the four species analyzed showed that ENA and BEA were not detected or were detected in traces level (<LOQ) in some samples, while ENB was the most predominant mycotoxin in all fish samples analyzed (40% of samples were contaminated). This fact is in accordance with other authors in animal derived products, such as Jestoi et al. (2007), who reported that emerging *Fusarium* mycotoxins were present in Finnish poultry tissues in traces levels for BEA and ENs except for ENB, which presented higher contents.

Contents of ENB_1 (33% of positive samples) and ENA_1 (25% of incidence) are also showed in Table 1. The highest incidence for all the mycotoxins analyzed corresponded to sea bass samples, while rainbow trout samples showed lower incidence and contents. These results confirmed the mycotoxin carry-over

from feed to edibles tissues and animal derived products belong from animals fed with contaminated feed.

Regarding ENs contents, ENB and ENB_1 showed higher levels than ENA_1 (Table 1). Besides, contents were higher in Atlantic salmon compared with sea bass and rainbow trout and also with sea bream. The results were in the following decreasing order of concentration ENB>ENB1>ENA1>ENA, which is in accordance with the results generally found by other authors, both in grains and in other foodstuffs, such as bread and beer (Vaclavikova et al., 2013; Habler et al., 2016) and also in feed (Uhlig et al., 2006; Sørensen et al., 2008).

Higher contents in Atlantic salmon can be explained due to the tendency of ENs to be bioaccumulated in lipophilic media (Faeste et al., 2011), thus, species with higher lipidic content (7.4 g/100g for Atlantic salmon) might show higher ENs contents than that with lower lipidic content (1.3 g/100 g of edible portion in sea bass) (Blanchet et al., 2005).

This pattern was also described by Jestoi et al. (2009). In this survey, ENs and BEA were detected in egg samples collected in the national residue monitoring programme in Finland at concentrations < LOQs. Overall, the prevalence and concentrations were higher in market egg yolk samples than in whole egg. Frenich et al. (2011), also analyzed ENs and BEA in eggs but only detected BEA in one sample. These results can be explained due to the solvent employed in the extraction step, because MeCN has shown better results in ENs extraction than MeOH.

Table 1. Incidence and contents (mean and concentration range) in raw samples.

Fish specie	Incidence (% positives/total)			Mean of positives (concentration range) (µg/kg)		
	ENA_1	**ENB**	**ENB_1**	**ENA_1**	**ENB**	**ENB_1**
Sea bass *(Dicentrarchus labrax)*	50.0	90.0	70.0	4.3 (1.7-6.9)	12.8 (1.3-12.8)	10.2 (1.4-31.5)
Sea bream *(Sparus aurata)*	30.0	40.0	30.0	4.0 (2.1-7.5)	14.9 (1.3-21.6)	12.7 (7.1-19.0)
Atlantic salmon *(Salmo salar)*	20.0	20.0	20.0	25.5 (22-29)	76.5 (50-103)	75 (56-94)
Rainbowtrout *(Onchorhynchus mykiss)*	-	10.0	10.0	-	3.6	2.9

3.3. Reduction by thermal processes

After thermal treatment application, ENs contents in edible tissues were surveyed. Comparing the contents between crude and processed samples, a reduction in mycotoxin contents was observed after all thermal treatments were applied. Reduction percentages were calculated taking into account that peak area in raw fillet corresponded to 100% of mycotoxin content, as contents after thermal treatment in some samples were under LOQ and it was not possible to quantify ENs contents.

For Atlantic salmon and rainbow trout the reduction was higher than for sea bass and sea bream. In the case of Atlantic salmon, the reduction percentage achieved for ENs and for all thermal treatments corresponded to 100%. These results can be explained by the higher fat content in the edible tissues of this specie, as fat suffers important modifications when applied cooking techniques.

On the other hand, for rainbow trout different reduction percentages were obtained. For ENB, reduction percentages ranged from 76% in microwave oven treatment, to 100% for broiled and boiled treatments. For ENB_1, the percentages of reduction ranged from 79% in microwave oven treatment, to 100% for broiled and boiled treatments. In this case, higher percentages can be explained due to the lower content reported in raw fillets.

The reduction of ENs is remarkable after thermal treatments were applied, as it can be observed in figure 1, where is showed the reduction percentage achieved in sea bass and sea bream after different thermal treatments applied. In figure 2 it can be observed a chromatogram in MRM mode showing ENs

contents in a raw sea bass sample and in the same sample after broiling. As it is showed, a reduction in peak area is remarkable.

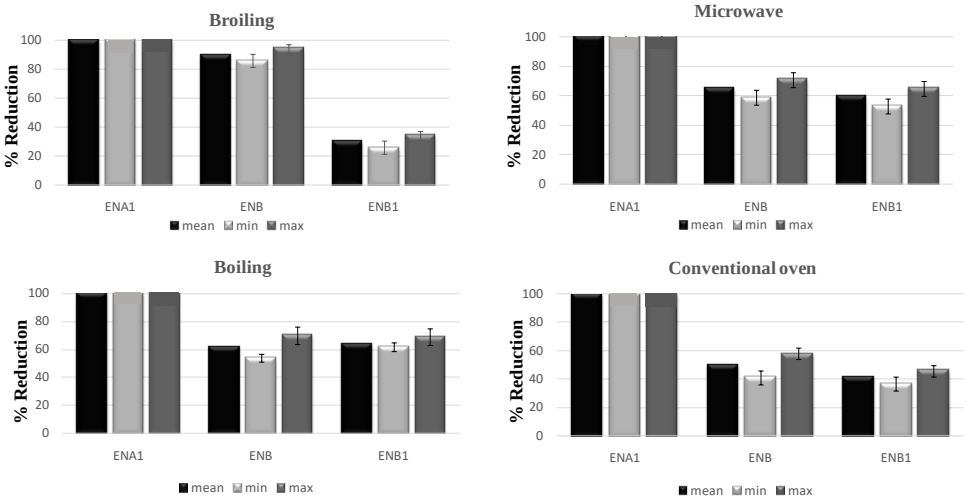
The reduction percentage achieved by different treatments depends on different factors, such as temperature, time of treatment and tissue composition. Results showed that after the broiling process was applied, reduction percentage ranged from 30 to 100%, after microwaving treatment, ranged from 60 to 100%, after boiling process was applied, the reduction ranged from 62 to 100% and after baking in conventional oven, ranged from 42 to 100%.

Results in broiled samples were variable, may be due to the different temperature reached in the fillet. Depending on the fillet size and thickness, in some cases, samples were maintained less time in the pan to avoid overcooking. The heating temperature together with the time employed affects the reduction process but also the degree of heat penetration (Kabak, 2009).

Regarding ENs reduction, it can be observed that ENB reduction was very similar between sea bass and sea bream samples when conventional oven, microwaving and boiling treatment were applied (Figure 1). However, ENB_1 performance after the application of those treatments was more variable between the species analyzed. In general, reduction achieved in sea bream samples was slightly higher than those obtained in sea bass samples, especially for ENB_1. This difference can be explained due to the different fat composition of tissues in all the species included in the study. In this sense, some authors have reported that BEA and ENs are ionophoric molecules that can form stable and lipophilic complexes with cations and transport them into

the lipophilic phase, so the changes produced in fat content after temperature application could also affect the ENs contents present in tissues (Beyer et al., 2009).

(a)



(b)

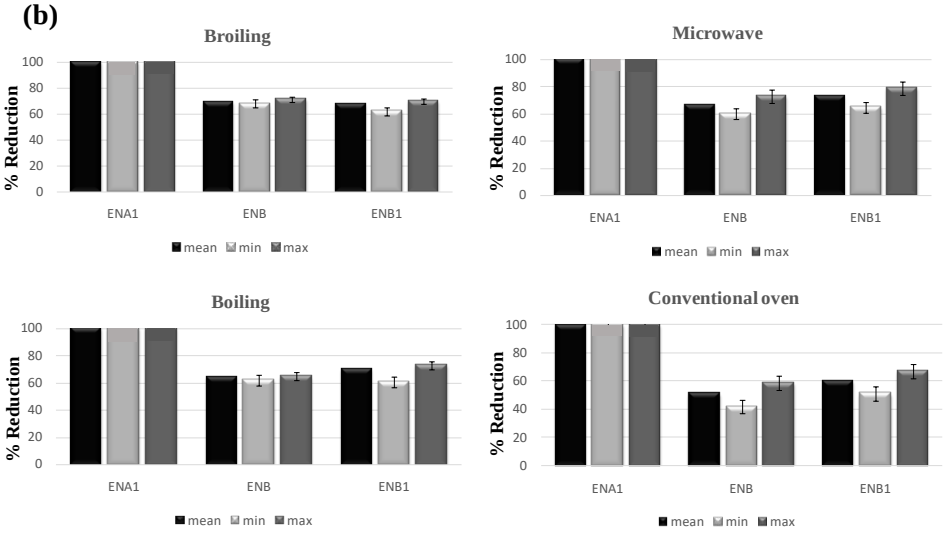


Figure 1. Enniatin reduction percentage after different thermal treatments applied to raw fillets in sea bass (a) and sea bream (b).

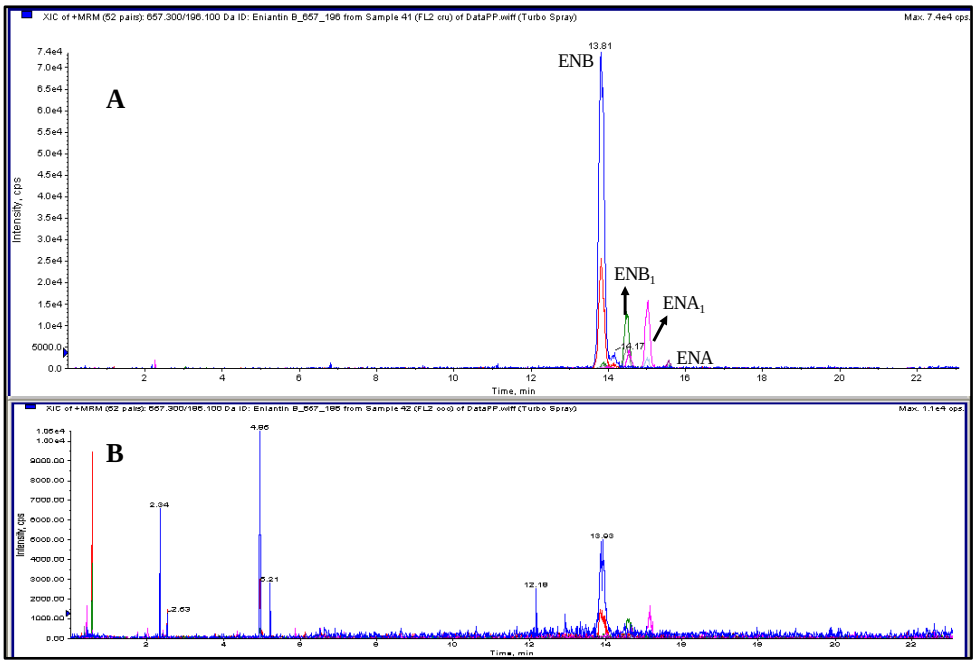


Fig. 2. Chromatogram in MRM mode showing enniatin reduction in a sea bass sample. A) Raw sea bass fillet; B) Sea bass fillet after broiling treatment.

The stability of emerging *Fusarium* mycotoxins during thermal food processing has been discussed by several researchers. Some studies have supported that the impact of high temperature is a decisive parameter in mycotoxin reduction (Serrano et al., 2013). According to these authors, after cooking processes in samples spiked with high amounts of ENs, a high percentage of reduction was observed, but ENA_1 and ENB showed higher thermal stability than other

ENs, which was not in accordance with results found in our survey. Compared with our results, ENs stability is similar but some differences have been observed. In this sense, ENA_1 showed lower stability in samples analyzed, as the percentage achieved corresponded to 100%, mainly due to low contents detected in raw samples (under LOQ), which also implies very low contents after thermal treatments application.

Comparing these results with other surveys on emerging *Fusarium* mycotoxins, Serrano et al. (2013) reported a reduction on ENs contents in pasta from 81% to 100% and Meca et al. (2012) reported a reduction on BEA contents that depended on temperature and time of thermal application, although they studied other foodstuffs with different composition. In both studies degradation products from ENs and BEA were identified. Pineda-Valdes and Bullerman (2000) reported a reduction on moniliformin (MON) content, an emerging *Fusarium* mycotoxin, dependent on temperature and pH, showing the highest percentage of reduction at 175ºC and pH 10 during 60 min of processing.

3.4 Characterization of ENs degradation products by LC-MS-LIT

Although ENs are structurally similar compounds, each type of EN may present a different response depending on their intrinsic stability. During the cooking processes, mycotoxins undergo different transformations, so it is necessary to identify the degradation products formed in order to study the possible risk associated with consumption of farmed fish.

The positive samples to ENs degradation were analyzed by LC-MS-LIT in the ER scan modality (m/z = 200-900) in order to determine ENs degradation products produced through the thermal treatments applied, as indicated in

section 2.5.2. In Fig. 3 is showed a chromatogram indicating the presence of ENs both in raw and cooked samples. Furthermore, in these chromatogram it can be observed the presence of other peaks, which did not correspond to any of the mycotoxins included in the method. For this reason, those samples were analyzed by LC-MS-LIT in scan mode.

Degradation product 1 corresponded to a potassium adduct characterized by the loss of the aminoacid isoleucine (Ile) (m/z 573.1) and degradation product 2, with the loss of two units of Ile (m/z 443.2) (Fig. 4). The formation of new ENA_1 degradation products could be another reason for the higher percentage reduction obtained for this mycotoxin in samples analyzed. In Fig. 4 is evidenced the MS-LIT spectra of the degradation products of ENA_1.

As the survey has been carried out in real samples with no mycotoxin addition, mycotoxins may interact with some components present in fish tissues, as reported by other authors in sugar, protein and starch models (Bullerman and Bianchini, 2007; Beyer et al., 2009), thus, results and identification of degradation products are difficult to compare with those obtained in water models. Besides, as co-occurrence is present in all positive samples and due to the related structure for all ENs, interactions between them may occur, forming new structures not identified.

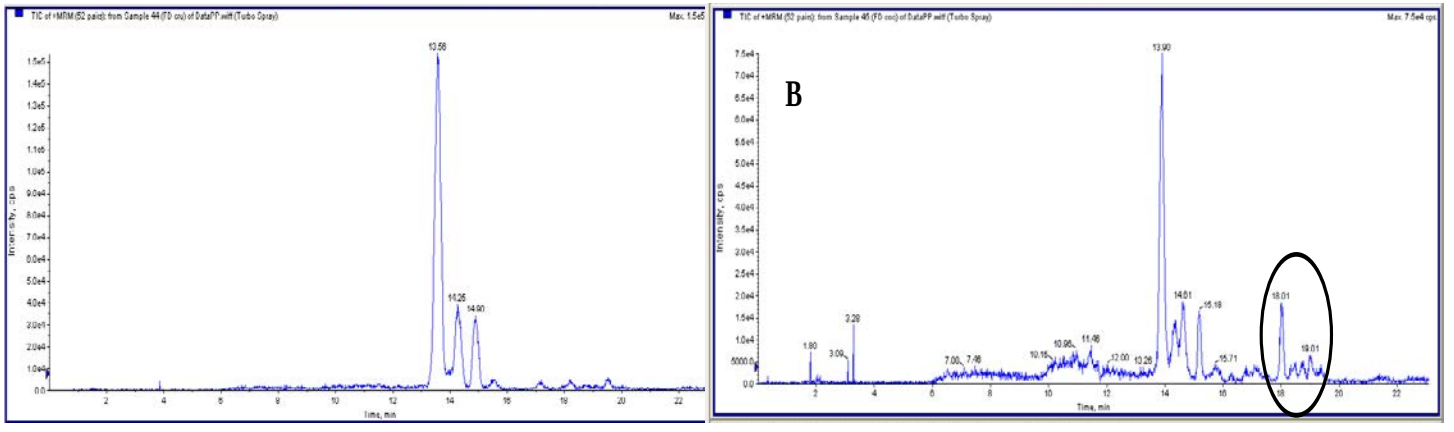


Fig. 3. TIC chromatogram in MRM mode showing enniatin reduction in a sea bream sample. A) Raw sea bream fillet; B) Sea bream fillet after broiling treatment showing degradation products remarked in a black circle.

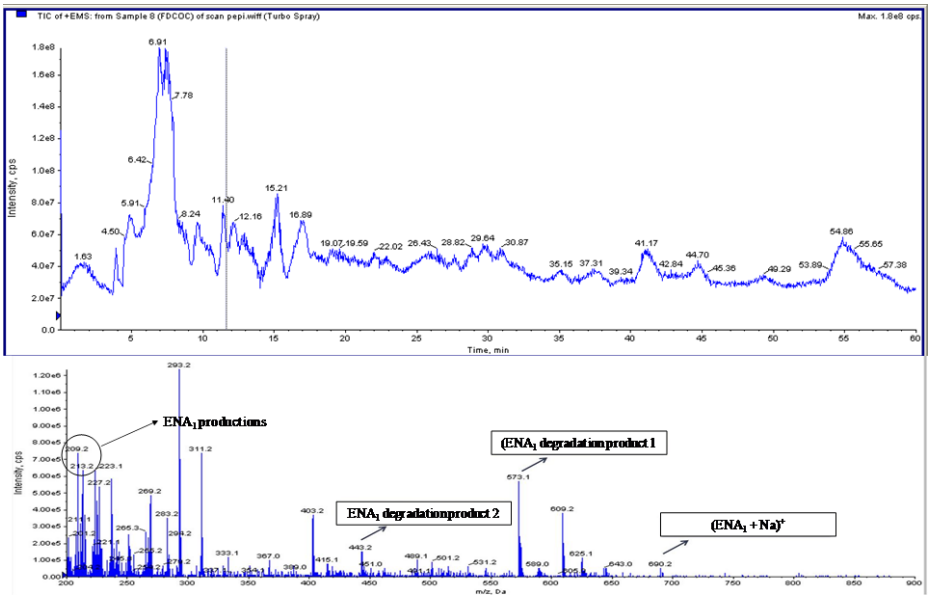


Fig. 4. ENA_1 degradation products identified by LC-MS-LIT in scan mode.

Other degradation products from ENA, ENB and ENB_1 have been described by other authors after thermal treatments applied to other foodstuffs, such as pasta (Serrano et al., 2013). Thus, further studies will be carried out in order to evaluate ENs performance in fish tissues after thermal treatments and the formation of new degradation products.

4. Conclusion

The influence of technological processing on ENs levels have been investigated, showing that cooking techniques commonly applied before fish consumption contributed to further decrease of ENs levels. Nevertheless, mycotoxin removal was not complete, as traces of ENB and ENB_1 were

detected in cooked samples at different concentrations. Furthermore, new metabolites from thermal treatments were identified.

Taking into account the large number of population consuming these products and that literature is scarce on mycotoxin contamination in fish, further research in the mechanisms of reaction and the new formation of degradation products is necessary to study the apparent reduction in mycotoxin levels observed after food processing.

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3.9. Presence of mycotoxins in Atlantic salmon (*Salmo salar*) from aquaculture

Food Chemistry (2017) Under review

Presence of mycotoxins in Atlantic salmon (*Salmo Salar*) from aquaculture

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ABSTRACT

A method consisting in liquid chromatography coupled with hybrid quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) has been employed for screening and identification of different mycotoxins in edible tissues of Atlantic salmon (*Salmo salar*). A non-targeted screening approach for the detection of 233 mycotoxins from a home-made spectral library was applied. The aim of the study was to evaluate the presence of these natural contaminants in fish tissues. Results showed that fourty mycotoxins were identified in salmon samples analyzed. The most prevalent were enniatins, rugulosin and ophiobolins, which are commonly contaminants presents in cereals and their by-products. Thus, aquaculture fish fed with contaminated feed can metabolize and retain mycotoxins and their metabolites in different organs and tissues.

1. Introduction

Mycotoxins are natural contaminants in agricultural feed and food, commonly found mainly in cereals and their by-products. Feed intended for different animal species include in its composition raw materials susceptible of fungal contamination, such as cereals and other protein sources from plant origin (Nizza & Piccolo, 2009). The increasing use of these sources in fish diets can introduce contaminants, mainly mycotoxins, which were not previously reported in fish tissues. Many studies have reported mycotoxin levels on a wide range of randomly sampled feed ingredients and finished feeds intended for terrestrial animals (Binder, 2007; Binder et al., 2007; Zinedine and Mañes, 2009; Rodrigues and Naehrer, 2012; Streit et al., 2012, 2013); however, studies in feedstuffs for aquaculture are still scarce, although, over recent years, earlier studies established feasible analytical approaches for mycotoxins in aquafeeds (Nácher-Mestre et al., 2015; Tolosa et al., 2014).

Regarding fish studies, some authors have reported the carry-over of mycotoxins present in feed to edible portions of fish fed with contaminated feedstuffs (Nomura et al., 2011), showing that mycotoxins present in raw materials and feed for aquaculture fish can be fixed in edible portions and organs, and also metabolites from the original molecule.

However, those studies reporting mycotoxin presence in fish tissues come from dietary experiments in which fish are fed contaminated feed in order to observe mycotoxin metabolism and distribution. The major part of these studies are performed with aflatoxin B1 (AFB1) and deoxynivalenol (DON) (Guan et al., 2010; Nomura et al., 2011).

In addition, mycotoxins have the potential to enter human and animal food chains through milk, meat and eggs from livestock and poultry animals fed

with contaminated feed, although the exposure risk to humans by consumption of by-products and edible tissues of animals exposed is minor compared to the direct consumption of grain products (He et al., 2010).

Mycotoxins produce an impact on aquaculture farming; however, information on the consequences for reared fish species still remains lower than that for other terrestrial species (Caruso et al., 2013). Given the presence of mycotoxins in aquaculture feedstuffs and the expansion of this sector, is necessary to evaluate the presence of mycotoxins in fish produced by this sector, since more data is needed to perform a risk assessment evaluation for human consumption.

Atlantic salmon (*Salmo salar*) is economically the most important farmed fish in Europe, although other species commonly produced include rainbow trout, sea bass and sea bream, among others (EFSA, 2014). These species have been decisive for increase the demand and consumption, since this fact has converted their capture into their aquaculture farming in order to satisfy the increasing demand (FAO, 2016).

Regarding the carry-over of mycotoxins into animal derived products, only aflatoxin B1 (AFB1), and its metabolite aflatoxin M1 (AFM1), are legally monitored in these products by some countries (Comission Decision 1881/2006). However, no legal regulations are established for other mycotoxins in products of animal origin, because there is little information concerning their occurrence in these foodstuffs (Zhao et al., 2015). Therefore, the development of multi-residue methods for acquiring more occurrence data for risk assessment is essential. Within this context, multi-mycotoxin methods for mycotoxin analysis in feedstuffs have been developed by some researchers in order to get a complete survey of the contamination range (Streit et al.,

2012; Åberg et al., 2013). However, method development and validation for simultaneous mycotoxin determination in fish, which is also essential due to the carry-over from feed to edible tissues, has been scarcely reported.

Within each field of residues and contaminants, a clear trend toward to the use of multi-analyte methods can be observed. Such methods involving mass spectrometric (MS) detection are an efficient way of assessment for occurrence of undesirable substances and can provide both qualitative and quantitative information at the same time (Mol et al., 2008).

LC-MS/MS requires compound-dependent parameter optimization which is time-consuming and tedious if there are many analytes to be analyzed. To address this problem, LC coupled to full-scan instruments such as time-of-flight (TOF) can be used for screening for chemical contaminants and in structural elucidation, identification and characterization of chemical compounds (Quesada et al., 2013; Liao et al., 2015). LC-Q-TOF-MS has shown strong potential for screening and confirmation of organic contaminants and their metabolites (Nácher-Mestre et al., 2013).

Within this context, the aim of this study was to determine the presence of mycotoxins in fish tissues by applying a multianalyte method consisting in liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry (LC/Q-TOF MS).

2. Material and methods

2.1. Sampling

Ten samples of Atlantic salmon (*Salmo salar*) from aquaculture farming were acquired from valencian supermarkets and analyzed for mycotoxin contents. The origin of the samples was Norway. Samples were acquired as prepared

fillets in individual packages at different markets within one month in 2016. All samples were stored in a dark and dry place at -20ºC until analysis. After their packages had been opened they were analyzed within the same day. These samples were analyzed for emerging *Fusarium* mycotoxin detection by LC-MS/MS LIT and results were reported by Tolosa et al. (2017). The results showed some unidentified peaks, thus, positive samples showing ENs contents have been analyzed by LC-Q-TOF-MS in order to identify those compounds by exact mass.

2.2. Extraction procedure

The mycotoxin extraction used was that reported by Tolosa et al. (2014) for emerging *Fusarium* mycotoxins. Briefly, 10 g of homogenate fish tissue was mixed with 50 mL of MeCN for 30 min at 30°C using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., Danbury, CT, USA). The extract was centrifuged at 3540g for 15 min at 5°C. The supernatant was purified using C_{18} cartridges (Waters) by applying a slight vacuum. Cartridges were previously conditioned with 5 mL of MeOH and 5 mL of deonized water, washed with 5 mL of water, and then vacuum-dried for 5 min. Finally, mycotoxins were eluted with 5mL of AcN/MeOH 50:50 v/v (20 mM ammonium formate). The extract was evaporated to dryness at 30°C using a Büchi Rotavapor R-200. The solution was reconstituted in 10 mL of AcN/MeOH 50:50 v/v (MeOH with 20 mM of ammonium formate) and transferred to a 15 mL conical tube to be evaporated to dryness at 30°C using a multisample Turbovap LV Evaporator (Zymark). After solvent evaporation, the solution was reconstituted with 1 mL of acidified AcN/MeOH 50:50 v/v and sonicated again (30 min, 30°C). Then,

the solution was filtered through a 13 mm/0.22 μm nylon filter (Membrane Solutions) prior to injection.

2.3. LC-Q-TOF-MS analytical conditions

An Agilent 1290 HPLC system (Agilent, USA) was employed for chromatographic separation using an Acquity UHPLC BEH C18 1.7 μm particle size analytical column 50 × 2.1 mm (Waters) at a flow rate of 350 μL/ min. The mobile phase consisted in water (0.15 mM ammonium formate) and methanol 0.1% formic acid. The percentage of organic modifier (B) was changed linearly as follows: 0 min, 5%; 2 min, 25%; 13 min, 100%; 15 min, 100%; 15.1 min, 5%; 25 min, 5%. The column temperature was set to 60 °C and the injection volume was10 μl.

An hybrid quadrupole-orthogonal acceleration- TOF mass spectrometer (AB SCIEX TripleTOF™ 5600 LC/MS/MS System), with an orthogonal Z-spray-ESI interface operating in positive ion mode, was used. The date acquisition was used in positive mode, over a mass range from 100 to 1000 m/z. For automated accurate mass measurement an external calibrant delivery system (CDS) which infuses calibration solution was used prior to sample introduction. The MS was carried out using an IDA acquisition method with: the survey scan type (TOF-MS) and the dependent scan type (product ion) using 50V of collision energy (CE). Data was evaluated using the qualitatively evaluated using the PeakView™ software.

Q-TOF-MS resolution was approximately 10000 at full width at half-maximum (fwhm), at m/z 556.2771. The microchannel plate (MCP) detector potential was set to 2050 V. A capillary voltage of 3.5 kV and a cone voltage of 25 V

were used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The interface temperature was set to 450°C and the source temperature to 120°C. For MSE experiments, two acquisition functions with different collision energies were created: the low-energy function (LE), selecting a collision energy of 4 eV, and a second one, the high-energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV to promote in-source fragmentation. The LE and HE function settings were for a scan time of 0.2 s and an interscan delay of 0.05 s. Calibrations were conducted from m/z 50 to 1000 with a 1:1 mixture of 0.05 M NaOH/5% HCOOH diluted (1:25) with MeCN/water (80:20), at a flow rate of 10 mL/min. A triple-quadrupole analyzer (Waters Corp.) operating in MS/MS was used for the analysis of positive samples from the screening. Drying gas as well as nebulizing gas was nitrogen generated from pressurized air in a N_2 LC-MS (Claind, Teknokroma, Barcelona, Spain), and the collision gas was argon (99.995%; Praxair, Madrid, Spain) with a pressure of approximately 4×10^{-3} mbar in the collision cell. A capillary voltage of 3.5 kV in positive ionization mode was applied. The desolvation gas temperature was set to 500°C and column temperature was set to 40°C. The *Ion source gas 1 (GC1) and 2 (GC2) were 40 psi*. Ion Spray Voltage (ISVF) was 5500 and declustering potential 120.

Dwell times of 0.030 s/scan were chosen. TargetLynx application manager (MassLynx v 4.1) software was used to process the data obtained from standards and samples.

3. Results and discussion

The use of high-resolution Q-TOF-MS is an efficient tool for determining and verifying the molecular composition of many compounds. Thanks to the

accurate-mass full-spectrum acquisition capabilities of the TOF analyzer, it was feasible to investigate the presence of 233 mycotoxins from a wide list of validated compounds from a home-made spectral library. Mycotoxins have been detected as protonated ions $[M+H]^+$. Results showed that fourty mycotoxins were detected in salmon samples analyzed, wchich are shown in Table 1. To the best of our knowledge, this is the first study reporting the presence of these mycotoxins in fish samples directly purchased from supermarkets, and not from dietary studies or growing experiments with contaminated diets.

Among the most abundant mycotoxins detected in samples, some of them belong to commonly contaminants found in cereal based foodstuffs, such as enniatins (ENN A, ENN A1, ENN B and ENN B1, ENN B2), and fusaproliferin (FUS). Neverthless, other mycotoxins with less reported occurrence in foodstuffs were detected, mainly anisomycin, cytochalasin J (CJ), mycophenolic acid (MPA), ophiobolin A (OA) and B (OB), rugulosin and penicillic acid (PA).

Although the presence of these mycotoxins has been scarcely reported in feeds and animal products, their occurrence is described in grain cereal samples, such as wheat and corn (Aresta et al., 2003). Ophiobolins are phytotoxins produced by the pathogenic fungi *Bipolaris* spp, which usually infect rice, maize and sorghum, producing detrimental effects in plants; however, in animals, little is known about their toxicity, although ophiobolins have been described as toxic to animals in toxicity *in vivo* assays in mice (Au et al., 2000).

Other researchers also reported the presence of some mycotoxins detected in this study, such as chanoclavine, sulochrin,festuclavine, MPA, fumonisin B2 (FB2) and ENNs, among others, in different foodstuffs, mainly bread (Sulyok

et al., 2010). Regarding the survey reported by Rundberget & Wilkins (2002), the multi-mycotoxin method developed by these researchers allowed the simultaneous determination of MPA and other less reported mycotoxins in both food and feed, and the method developed by Sulyok et al. (2006) was able to detect 15 mycotoxins in common with this study in wheat and maize kernels. In feed and feed raw materials, as reported by Streit et al. (2013), some of the mycotoxins detected in this study have been also identified, such as MPA, cyclopiazonic acid, PA, radicicol, rugulosin, CJ, among others. In consequence, these mycotoxins could be present in edible tissues of animals who consume those contaminated feedstuffs (Nácher-Mestre et al., 2013).

Studies on natural mycotoxin occurrence in fish are still scarce, although recent reports have showed that different fish species in European aquaculture are commonly exposed to feed-borne *Fusarium* mycotoxins (Nácher-Mestre et al., 2015). Thus, emerging *Fusarium* mycotoxins have been previously detected in the study reported by Tolosa et al. (2014). According to Zhao et al. (2015), mycotoxin contamination in feed directly influenced the presence of mycotoxins in animal derived products.

Table 1. Identified mycotoxins in salmon samples.

Mycotoxin	Elemental composition	Exact mass (*m/z*)	RT* (min)
2-amino-14,16-dimethyloctadecan-3-ol	C20H43NO	314.3417	13.42
Anisomycin	C14H19NO4	266.1387	1.42
Chanoclavine	C16H20N20	257.1648	2.66
Curvularin	C16H20O5	292.1310	8.29
Cyclopenin	C17H14N2O3	295.1077	11.53
Cyclopiazonic acid	C20H20N2O3	337.1547	6.19
Cytochalasin J	C28H37NO4	452.2795	12.36
Deoxybrevianamide E	C21H25N3O2	352.2020	4.86
DOM-1	C15H20O5	281.1376	9.73
Dihydrolysergol	C16H20N2O	257.1648	2.66
ENN A	C36H63N3O9	682.4637	13.28
ENN A1	C35H61N3O9	668.4481	13.04
ENN B	C33H57N3O9	640.4168	12.47
ENN B1	C34H59N3O9	654.4324	12.78
ENN B2	C32H55N3O9	626.4011	12.50
Festuclavine	C16H20N2	241.1699	3.80
FK 506	C44H69NO12	804.4893	6.33
Fumigaclavine A	C18H22N2O2	299.1754	11.49
Fumitremorgin C	C22H25N3O3	380.1969	4.33
Fumonisin B2	C34H59NO14	706.4008	13.20

Fusaproliferin	C27H40O5	445.2949	13.19
Fusidic acid	C31H48O6	517.3524	14.40
Methysergide	C21H27N3O2	354.2176	12.20
Mycophenolic acid	C17H20O6	321.1333	2.97
Myriocin	C21H39NO6	402.2850	7.68
Ophiobolin A	C25H36O4	401.2686	13.28
Ophiobolin B	C25H38O4	403.2843	13.33
Oxidized luol	C28H39NO2	422.3054	12.97
Paspaline	C32H39NO4	502.2952	12.57
Penicillic acid	C16H18N2O5S	351.1009	11.26
Penicillin G	C27H33NO6	468.2381	11.06
Penicillin V	C37H44O6NCl	634.2930	9.79
Phomopsin A	C22H25NO8	432.1653	10.55
Pseurotin A	C16H24O6	313.1646	8.54
Radicicol	C22H23N5O2	390.1925	8.59
Rugulosin	C29H38O8	515.2639	13.66
Sulochrin	C24H34O9	467.2276	4.35
T-2 Toxin	C22H30N4O4	415.2340	7.74
Tetracycline	C17H24O4	293.1747	13.35
Vancomycin	C15H22O4	267.1591	12.92

*RT retention time

The metabolism of different mycotoxins in fish has not been completely elucidated; however, some studies have reported the identification of mycotoxins and/or their metabolites in different organs and tissues (El-Sayed and Khalil, 2009; Guan et al., 2009; Deng et al., 2010; Nomura et al., 2011). In the study reported by Nácher-Mestre et al. (2013), a screening method for the detection and identification of undesirable compounds in aquaculture products has been developed by UHPLC/Q-TOF-MS. This method has been applied to feed and fish fillets, showing that FB2 and zearalenone were detected in feed samples; however, no mycotoxin contamination was detected in fish fillets from growing experiments. In a posterior study, these authors have evaluated the carry-over of some mycotoxins, mainly aflatoxins (AFs), trichothecenes (TRC) and fumonisins (FBs), from feed to fish fillets in Atlantic salmon (*Salmo Salar*) and gilthead sea bream (Sparus aurata) (Nácher-Mestre et al., 2015); however, no mycotoxin carry-over was found from feeds to edible fillets of salmonids and gilthead sea bream fed contaminated diets. DON has been evaluated by Guan et al. (2009), who reported the transformation of TRC in different fish species mainly by deacetylation and/or deepoxydation reactions.
Due to QTOF characteristics, which allow compound identification by exact mass, a metabolite from DON metabolism, deepoxy-deoxynivalenol (DOM-1), has been identified in the present study in salmon edible tissues analyzed (Figure 1); however, no DON contents were detected. This could be explained because retention and accumulation of DON in animal tissues is generally low due to its rapid metabolization (Pietsch et al., 2014).

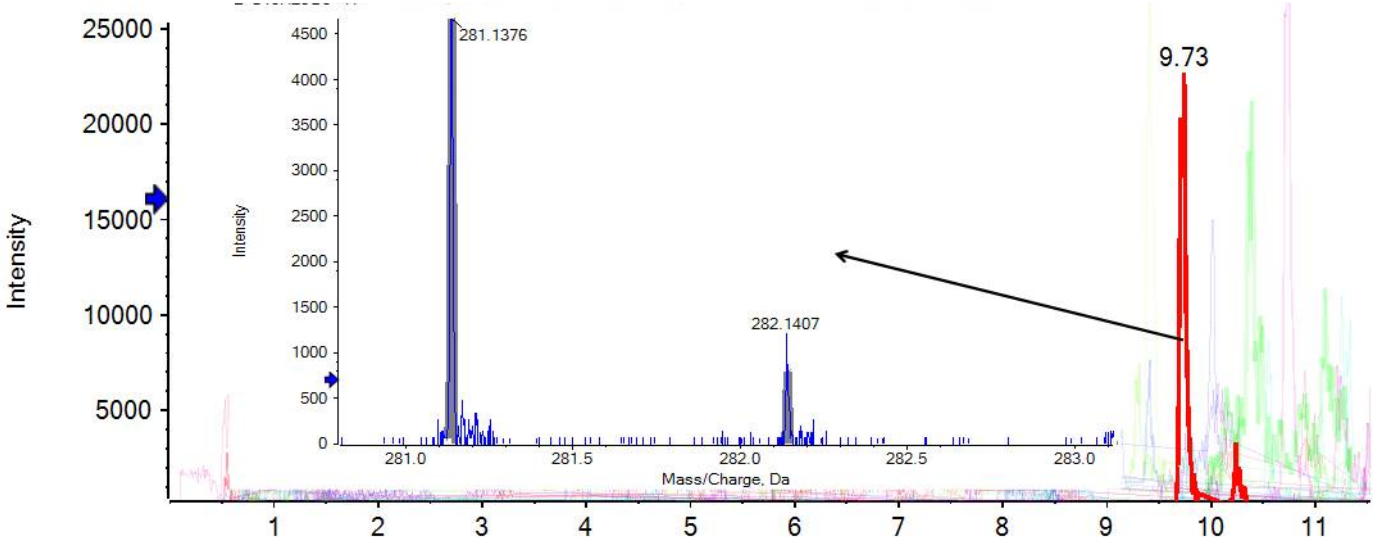

Figure 1. Chromatogram showing retention time and exact mass set to four decimals of DOM-1.

Regarding mycotoxin metabolism in fish, some studies reported the formation of trichothecene metabolites in different fish species. According to Tola et al. (2015), DON is transformed to de-epoxy deoxynivalenol (DOM-1) via deepoxidation and deacetylation by microorganisms from the digestive tract. Furthermore, hepatic microsomes in the liver of common carp (*Cyprinus carpio*), have been shown to transform DON to DON-3-glucuronide, while microbes from the digestive tract of brown bullhead (*Ameiurus nebulosus*) were more capable of transforming DON to DOM-1 than brown trout (*Salmo trutta*), pink salmon (*Oncorhynchus gorbuscha*) and other fishes.

Conclusion

A multiclass screening methodology has been applied for mycotoxin identification of 233 compounds from a validated list. Selectivity of the screening was supported by accurate-mass measurements provided by Q-TOF-MS. Fourty mycotoxins have been identified in aquaculture salmon. These mycotoxins have not been previously reported to occur in aquaculture fish; however, their occurrence has been reported in different cereal samples. This can be explained by the inclusion of these cereals as raw materials employed in feedstuffs elaboration, resulting in the carry-over from feed to edible parts of fish. Furthermore, a metabolite from DON was detected in salmon tissues, formed via DON deepoxidation and resulting in the formation of de-epoxy deoxynivalenol (DOM-1). Thus, it is necessary to ensure that farmed fish for human consumption is free from contaminants or contains concentrations lower than maximum limits established for legislated mycotoxins. In the light of these findings, the potential health risk associated with the consumption of

mycotoxin-contaminated aquacultured fish should draw public attention as these products are an important part of daily diets.

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4. GENERAL DISCUSSION

4. DISCUSIÓN GENERAL

De acuerdo con los objetivos planteados, el trabajo de investigación llevado a cabo a lo largo de esta Tesis Doctoral ha abordado la optimización y validación de diferentes métodos analíticos basados en LC y UHPLC acoplados a diferentes espectrómetros de masas para la determinación de micotoxinas en distintas matrices alimentarias. A continuación, los diferentes métodos validados han sido aplicados a muestras reales, optimizando previamente los parámetros analíticos mediante el empleo de diferentes técnicas de extracción dependiendo de las micotoxinas a determinar y de la matriz alimentaria, y así, llevar a cabo una monitorización de la presencia de diferentes micotoxinas (clásicas y emergentes) en diferentes alimentos, realizando así la evaluación del riesgo asociado a la ingesta de micotoxinas en productos de origen animal. Por último, se ha evaluado la presencia de productos de degradación formados tras la aplicación de diferentes procesos culinarios a muestras de pescado de acuicultura.

4.1. Validación de la metodología analítica para la determinación multi-micotoxina en alimentos de origen vegetal

Se ha optimizado un método LC-MS/MS para el análisis de micotoxinas emergentes de *Fusarium* en frutos secos. A continuación, se ha validado un método multi-micotoxina mediante el empleo de UHPLC-Orbitrap para la determinación de 17 micotoxinas, tradicionales y emergentes, en muestras de pasta de trigo y de maíz. Por último, se ha validado un método para la determinación de micotoxinas emergentes de *Fusarium* en materias primas y piensos mediante LC-MS/MS.

4.1.1. Optimización del método LC-MS/MS para la determinación de micotoxinas emergentes de Fusarium en frutos secos

Se ha optimizado un procedimiento analítico por LC-MS/MS para la determinación de algunas de las micotoxinas emergentes de *Fusarium*, concretamente, las mayormente descritas en alimentos: ENN A, ENN A1, ENN B, ENN B1 y BEA. En primer lugar se realiza la optimización de las condiciones del espectrómetro de masas mediante infusión del estándar de cada una de las micotoxinas objeto de estudio en modo "full SCAN", tanto en modo de ionización positivo (ESI+) como negativo (ESI-), con el fin de seleccionar el ión precursor con la relación m/z más abundante. Tanto las ENN s como la BEA han presentado una buena eficiencia en el modo ESI^+, sin embargo, además de las moléculas protonadas $[M + H]^+$, se detectan aductos de sodio $[M + Na]^+$ y potasio $[M + K]^+$, por lo que se considera necesaria la adición de modificadores como el formiato de amonio y el ácido fórmico, así como la selección de un voltaje de cono adecuado con el fin de suprimir la formación de dichos aductos, y así obtener una mayor sensibilidad del método y una mayor fiabilidad en la cuantificación.

El método se ha optimizado por inyección directa de cada analito, de acuerdo a las directrices establecidas por la Comisión Europea (EC, 2002c), donde se establece que una sustancia puede ser identificada mediante LC-MS/MS en modo de monitorización de reacciones múltiples (MRM) por, al menos, dos transiciones, una de cuantificación y otra de confirmación. Es decir, para la identificación de una micotoxina es necesaria la presencia del ión producto mayoritario (empleado en la cuantificación) y otro ión producto empleado para la confirmación de la identidad del analito. La fragmentación de

los iones precursores ha sido optimizada mediante la aplicación de diferentes energías de colisión, seleccionando las dos transiciones con mayor proporción entre la señal cromatográfica y el ruido de fondo (S/N) y con la mínima interferencia de los componentes de la matriz. La cuantificación de cada micotoxina se ha realizado con la transición de cuantificación, ratificada por la transición de confirmación.

Tras la optimización del método por espectrometría de masas, se procede a la optimización del método cromatográfico. La separación de las micotoxinas se lleva a cabo con una columna Gemini C18 (150 x 4.6 mm, 5 μm de tamaño de partícula), con diferentes combinaciones de disolventes como fases móviles. Se selecciona el modo de elución en gradiente con diferente composición de MeCN y MeOH en presencia de un tampón de ácido fórmico-formiato de amonio para lograr una eficiente separación de las micotoxinas en un menor tiempo de análisis. El método optimizado permite la separación de las ENNs y la BEA en un tiempo total de análisis de 20 min. Los métodos descritos en bibliografía por otros autores para las micotoxinas emergentes requieren tiempos de análisis superiores (Blesa et al., 2012; Juan et al., 2013; Hu & Rychlik, 2014).

- *Optimización del método de extracción en frutos secos*

El método de extracción para la determinación de micotoxinas emergentes de *Fusarium* (ENNs y BEA) en frutos secos y frutas desecadas se lleva a cabo mediante extracción sólido-líquido con ultrasonidos y MeCN como disolvente, ya que se obtienen resultados óptimos con el empleo de esta combinación. Los parámetros analíticos muestran la bondad del método. Las curvas de calibración muestran una linealidad adecuada en todo el rango de

trabajo (r^2>0.992). Los LODs y LOQs se calculan utilizando el criterio de S/N<3 y S/N<10, respectivamente, obteniendo valores que oscilan entre 0,02 y 0,15 μg/kg para el LOD y 0.1 - 0.5 μg/kg para el LOQ. El método empleado proporciona recuperaciones del 85-112%, una precisión intra-día entre 4 y 11% y una precisión inter-día entre 5 y 15%. Para todas las micotoxinas se observa un efecto matriz (ME) que oscila del 40 al 70%.

4.1.2. Optimización del método UHPLC-espectrometría de masas Orbitrap en muestras de pasta

El análisis mediante espectrometría de masas por Orbitrap (Q Exactive Orbitrap, Thermo Fisher Scientific, Waltham, MA, USA), se ha empleado en modo positivo y negativo simultáneamente. La separación de las micotoxinas se realiza mediante UHPLC (Thermo Fisher Scientific, Waltham, MA, USA) empleando una columna Accuore aQ C18 (100 x 2.1 mm, 2.6 μm de tamaño de partícula). Las fases móviles empleadas han sido agua y MeOH, ambas con ácido fórmico 0,1% y formiato de amonio 5mM. El flujo empleado es de 0,4 mL/min con el siguiente gradiente: 0 min - 60 % de fase B, 9 min - 100% de fase B, 12 min - 100% de fase B, 12.1 min - 60% de fase B, 15 min - 60% de fase B.

Los criterios de identificación empleados han sido el tiempo de retención (t_R) así como la masa exacta de cada una de las micotoxinas (o sus correspondientes aductos) con hasta cinco cifras decimales y su patrón de fragmentación típico para cada una de las micotoxinas incluidas en el estudio. La precisión en la masa detectada ha sido calculada de acuerdo a la relación entre la masa observada y la masa teórica y expresada en partes por millón

(ppm). Como se muestra en los resultados, la precisión es menor de 5 ppm para todas las micotoxinas objeto de estudio.

La sensibilidad del instrumento se evalúa a través de los cromatogramas en el método de exploración completa tanto en modo de ionización positiva como negativa. Al igual que en otros métodos previamente validados, se observa la formación de aductos con ácido fórmico o amonio ($[M+FAc]^-$ $[M+NH_4]^+$, respectivamente), siendo en algunos casos los iones más predominantes en el espectro de masas y, por tanto, seleccionados como iones precursores para algunas micotoxinas.

- *Optimización del método de extracción en pasta de trigo*

La determinación de las micotoxinas tradicionales AFs, TCs, FBs, OTA y ZON, y de las micotoxinas emergentes ENNs y BEA en muestras de pasta (elaborada a partir de trigo) y pasta sin gluten (a base de maíz y arroz) se ha llevado a cabo mediante el método de extracción QuEChERS. Todas las micotoxinas muestran buena linealidad en todo el rango de trabajo, y el coeficiente de regresión de las curvas de calibración (r^2) es mayor de 0.992, excepto en el caso de la FB2, que presenta un valor de r^2 de 0.9864 en muestras de pasta a base de trigo. Los LOD y LOQ oscilan entre 0.03-8.21 μg/kg y 0.09-24.6 μg/kg, respectivamente, en muestras de pasta de trigo, correspondiendo los valores más elevados a la FB2. Por otro lado, las recuperaciones oscilan entre 68-210%, correspondiendo el valor más elevado a la BEA, la cual muestra un marcado efecto matriz, al igual que NEO, NIV y DON. La precisión intra-día para los niveles de adición ensayados es de 5-8% y la precisión inter-día es de 8-13%.

- *Optimización del método de extracción en pasta sin gluten*

El método multi-micotoxina previamente optimizado para muestras de pasta a base de trigo ha sido aplicado a muestras de pasta sin gluten a base de maíz y arroz. Pese a tratarse de muestras de pasta, su diferente composición ha dado lugar a diferencias en algunos parámetros, por ello, los parámetros analíticos han sido optimizados nuevamente. Todas las micotoxinas muestran buena linealidad en todo el rango de trabajo, r^2>0.992, excepto para ZON (0.9870) y ENN B (0.9904). Los porcentajes de recuperación oscilan entre 74 y 173%; LOD y LOQ oscilan entre 0,001-7,84 μg/kg y 0,002-23,52 μg/kg, respectivamente. La precisión intra-día para los niveles de adición ensayados es inferior al 7% y la precisión inter-día es inferior al 10%.

Los métodos multirresiduales para el análisis de micotoxinas en productos sin gluten se centran generalmente en FBs, AFs y ZON (Dall'Asta et al., 2009; Cano-Sancho et al., 2012; Huong et al., 2016). Sin embargo, en este estudio se ha desarrollado un método multi-micotoxina para la determinación simultánea de 17 micotoxinas en pasta sin gluten.

Respecto a la validación del método se observa una sensibilidad diferente en términos de LOD y LOQ entre pasta de trigo duro y muestras de pasta con maíz y arroz. En este sentido, LOD y LOQ son mayores en el análisis de pastas sin gluten, probablemente debido a que los parámetros calculados son consecuencia de un efecto matriz marcado, diferente entre las muestras de trigo y las de maíz. Otros autores han descrito este patrón tanto en muestras de trigo como de maíz (Liao et al., 2015), mostrando diferentes límites instrumentales, recuperaciones y ME (Zachariasova et al., 2010).

4.1.3. Optimización del método LC-MS-LIT en materias primas y pienso

Se realiza la optimización de las condiciones del espectrómetro de masas mediante infusión del estándar de cada micotoxina en modo "full SCAN", tanto en modo ESI+ como ESI-, con el objetivo de seleccionar el ión precursor con la relación m/z más abundante. Las micotoxinas emergentes de *Fusarium* (ENNs y BEA), han mostrado la formación de aductos de amonio $[M+NH_4]^+$ con buena sensibilidad, de manera que han sido los iones precursores seleccionados. De acuerdo con la normativa, se han tenido en cuenta las dos transiciones MRM más relevantes en la fragmentación de los iones precursores: la transición de cuantificación y la transición de confirmación, así como el t_R del patrón.

La optimización de los parámetros de MS como el voltaje del cono (DP), energía de colisión (CE) y potencial de entrada de células de colisión (CEP) se realiza mediante la inyección del estándar de cada una de las micotoxinas. El potencial de entrada (EP) y el potencial de salida de la celda de colisión (CXP) se fijan en 10V y 4V, respectivamente, para todos los analitos.

- *Optimización de la extracción en materias primas y pienso*

Para el análisis de micotoxinas emergentes de *Fusarium* en muestras de materias primas y piensos destinadas a diferentes especies animales, se emplea un método de extracción QuEChERS. El LOD para la ENA y la BEA es de 1 μg/kg y el LOQ, 5 μg/kg. Para la ENN A1, ENN B y ENN B1, el LOD es de 0,2 μg/kg y el LOQ, 1 μg/kg. El ME obtenido es el siguiente: 13%, 19%, 11%, 16% y 29% de supresión de señal para ENN A, ENN A1, ENN B, ENN B1 y BEA, respectivamente. Al igual que en los métodos descritos anteriormente, la

BEA es la micotoxina que muestra mayor ME. Los valores de la desviación estándar relativa (RSD) oscilan entre el 4 y el 11% para la precisión intra-día, y entre el 5 y el 15% para la precisión inter-día. Los valores de recuperación oscilan entre 89 y 136%.

- *Optimización de la extracción por Ultra-Turrax en pienso de peces*

El análisis de muestras de pienso destinado a la alimentación de peces de acuicultura se realiza mediante extracción sólido-líquido asistida por Ultra-Turrax. Los parámetros críticos evaluados son la naturaleza del disolvente, el volumen de éste (10, 20, 50 mL) y el peso de la muestra (2, 5 y 10 g). Los disolventes ensayados son: MeCN, una mezcla de MeCN/MeOH 50/50 v/v (20 mM formiato de amonio) y acetato de etilo (EtOAc). Los mejores resultados se obtienen con 50 ml de MeCN y 5 g de muestra, mostrando recuperaciones del 80 al 92%. La linealidad es adecuada para todas las micotoxinas ensayadas. Los LOD y LOQ oscilan entre 0,02–0,15 μg/kg y 0,10–0,5 μg/kg, respectivamente. El ME oscila entre 11 y 29%, mostrando una pequeña supresión de la señal. La precisión intra-día, para las dos concentraciones ensayadas, oscila entre 5 y 11%, mientras que la precisión inter-día oscila entre 7 y 13%.

4.2. Validación de la metodología analítica para la determinación de micotoxinas en productos de origen animal

4.2.1. Optimización del método LC-MS-LIT en pescado de acuicultura y en productos de la pesca

Las condiciones del método analítico han sido detalladas previamente en la sección 4.1.3.

- *Optimización de la extracción de micotoxinas emergentes de Fusarium en pescado de acuicultura*

La optimización del método se lleva a cabo por extracción sólido-líquido (S-L) mediante estudio comparativo con diferentes tipos y tiempos de agitación: Ultra-Turrax, microondas y ultrasonidos (Tolosa et al., 2014). Tras la extracción mediante MeCN y ultrasonidos, se observa una buena linealidad en el rango de trabajo ensayado, así como un buen coeficiente de regresión de las curvas de calibración (>0.992). La evaluación del ME muestra una supresión de la señal para todas las micotoxinas estudiadas (entre el 7 y el 40%). LOD y LOQ oscilan entre 0,3 y 3,0 μg/kg y entre 1,0 y 10,0 μg/kg, respectivamente. La precisión intra-día oscila entre 5 y 10%, mientras que la inter-día oscila entre 7 y 13%.

- *Optimización de la extracción por Ultra-Turrax*

Este método de extracción es eficiente y rápido, sin embargo, presenta el inconveniente de que el tejido del músculo de pescado, debido a su textura, no permite un correcto contacto entre las fases sólida y líquida.

- *Optimización de la extracción por microondas*

Los parámetros críticos ensayados han sido el tiempo de extracción, la potencia del microondas y la naturaleza del disolvente de extracción. Este estudio se ha realizado empleando tres mezclas de disolventes diferentes: MeCN, una mezcla de MeCN/MeOH 50/50 v/v (MeOH con formiato de amonio 20 mM) y MeOH. La temperatura aplicada es tan baja como ha sido posible debido a que la ENN A1 presenta un punto de ebullición menor que el resto de ENNs (66-67ºC) y, por otra parte, cuando la extracción ha sido

realizada usando alta potencia (500-1000 W), la temperatura es mayor que el punto de ebullición del disolvente. En consecuencia, se ha empleado una potencia continua de 250 W para la agitación. Las recuperaciones obtenidas con 10 g de muestra y 50 ml de MeCN son aceptables (70-89%), sin embargo, los extractos no son claros, mostrando cierta turbidez, probablemente debido al incremento de la solubilidad de varios compuestos por el aumento de temperatura en la muestra.

- *Optimización de la extracción mediante ultrasonidos*

Los parámetros críticos ensayados han sido la naturaleza del disolvente de extracción, la temperatura y el tiempo de sonicación. Las mejores recuperaciones se han obtenido cuando la temperatura aplicada es de 30ºC (para evitar alcanzar el punto de ebullición de las micotoxinas) y un tiempo de sonicación de 30 min. Los disolventes ensayados han sido MeCN, mezcla de MeCN/MeOH 50/50 v/v (MeOH con formiato de amonio 20 mM) y EtOAc. El disolvente ensayado que ha mostrado mejores resultados es el MeCN, obteniéndose extractos claros y transparentes y recuperaciones aceptables. Así, se observa que los métodos de extracción que incluyen una mayor proporción de MeCN como disolvente de extracción son apropiados para la extracción de las micotoxinas emergentes de *Fusarium*.

- *Optimización de las condiciones de preparación de hígado y vísceras y purificación de las muestras*

En las muestras de origen animal la presencia de lípidos en los extractos puede causar importantes interferencias que deben evitarse o reducirse en la medida de lo posible con el fin de mejorar los LOD y LOQ. La principal

diferencia entre las muestras de músculo y las de hígado es el contenido lipídico. Por lo tanto, para la preparación de estas muestras y las muestras de vísceras, se realiza un paso adicional mediante el empleo de n-hexano con el fin de eliminar los lípidos de la muestra. Las recuperaciones obtenidas tras este paso adicional son adecuadas (oscilan de 72 a 88%).

Con el fin de mejorar la eficiencia extractiva y reducir la presencia de compuestos que pueden interferir en la determinación, se ensayan diferentes tipos de agente dispersante para evaluar la eficacia de la purificación. Los agentes dispersantes ensayados para las muestras de pescado son: C8, sílice y C18. Los resultados muestran que el mejor agente dispersante para obtener buenas recuperaciones en muestras de pescado es el C18. La fase sólida se acondiciona previamente con 5 ml de MeOH y 5 ml de agua desionizada, se lava con 5 ml de agua y después se seca al vacío durante 5 min. Finalmente, las micotoxinas son eluídas con 5 ml de fase móvil. Las recuperaciones obtenidas con C18 oscilan entre 80 y 118%.

- *Optimización de la extracción de micotoxinas clásicas y emergentes en pescado ahumado y productos de la pesca*

El método QuEChERS ha demostrado ser un método fiable para ser aplicado con éxito a diferentes matrices alimentarias. Todas las micotoxinas muestran una buena linealidad en el rango de trabajo y el coeficiente de regresión de las curvas de calibración es superior a 0,992 para todas las micotoxinas analizadas. Los LOD y LOQ oscilan entre 1.0-20 μg/kg y 3.0-67 μg/kg, respectivamente, correspondiendo los mayores valores obtenidos a las FBs. Las recuperaciones oscilan entre 80 y 125%, y la precisión intra-día e inter-día obtenida son aceptables. Por lo que respecta al ME, éste oscila entre

56% y 99,8%, correspondientes a FB1 y FUS-X, respectivamente. Teniendo en cuenta la complejidad de estas matrices, que contienen lípidos y proteínas, ha sido necesario un paso adicional con C18 para eliminar las interferencias en los análisis cromatográficos y obtener recuperaciones adecuadas y ME mínimos. Así, en una segunda etapa se añaden 0,3 g de $MgSO_4$ y 0,1 g de C18. Además, de acuerdo con otros autores (Dzuman et al., 2014; Zachariasova et al., 2014), se ha empleado un mayor porcentaje de ácido fórmico en la solución acuosa para mejorar la eficiencia extractiva para algunas micotoxinas.

4.3. Optimización del método LC-MS/MS en agua de cría y plasma de peces

La optimización de un método para la determinación de micotoxinas en agua y en plasma de peces se ha realizado por micro-extracción líquido-líquido dispersiva (DLLME). Los parámetros instrumentales se describen en la sección 4.1.1. No obstante, para llevar a cabo este estudio se han incluido en el método otras micotoxinas además de las micotoxinas emergentes de *Fusarium*. Se han seleccionado los iones precursores y producto para cada una de las micotoxinas incluidas en el método, siendo los iones precursores AFB1 313.2; AFB2 315.2; AFG1 329.2; AFG2 331.2; OTA 404.1; FB1 722.0; FB2 706.4; FB3 706.6; FUS-X 355.3, STG 325.3 y los iones producto AFB1 269.0q/241.0Q; AFB2 243.0q/259Q; AFG1 215.0q/200.0Q; AFG2 217.0q/189.0Q; OTA 358.1q/341.2Q; FB1 334.0q/352.0Q; FB2 318.0q/336.0Q; FB3 336.4q/318.1Q; FUS-X 144.8q/267.1Q, STG 297.0q/281.0Q. Para optimizar el método se realizan pruebas con diferentes volúmenes de muestra y disolvente, y con diferentes tipos de disolventes.

- *Optimización de le extracción en agua y plasma de peces*

Mediante el empleo de MeCN como disolvente dispersante, se forma bien la interfase entre disolvente extractante y disolvente dispersante, coincidiendo con trabajos previos publicados por otros autores (Antep & Merdivan, 2012; Karami-Osboo et al., 2013).

Todas las micotoxinas muestran una buena linealidad en el rango de trabajo (r^2>0,992). Respecto a la evaluación del ME, se observa una supresión de la señal para todas las micotoxinas (entre el 8 y el 37%). Las recuperaciones presentan una RSD inferior al 20%, excepto para FBs y BEA en las muestras de plasma, que son inferiores al 70%, posiblemente debido al alto porcentaje que se necesita de disolvente orgánico para la extracción de la mayoría de las micotoxinas y el alto contenido de agua y/o las condiciones ácidas necesarias para la extracción de las FBs (Cao et al., 2013). Las precisiones intra-día para el agua oscilan entre 7 y 9% y para el plasma entre 5 y 9%. Las precisiones inter-día oscilan entre 7 y 9% para el agua y entre 6 y 9% para el plasma.

Se testan dos disolventes de extracción diferentes, cloroformo ($CHCl_3$) y EtOAc, en muestras de agua y plasma. Se elige EtOAc como una alternativa al cloroformo porque el EtOAc, al igual que otros éteres, son moléculas aceptoras de enlaces de hidrógeno y por lo tanto extraen los solutos donadores de electrones más fácilmente que el cloroformo. Respecto a las muestras de agua, las recuperaciones de AFB1 disminuyen de 120 a 90% cuando se emplea cloroformo como disolvente de extracción. Sin embargo, las recuperaciones de otras micotoxinas permanecen constantes, excepto en el caso de las micotoxinas emergentes de *Fusarium*, cuyas recuperaciones son menores

cuando se usa cloroformo. Así, el EtOAc ha sido seleccionado como el disolvente de extracción óptimo.

Algunos métodos publicados por otros autores obtienen buenas recuperaciones con cloroformo como disolvente extractante, posiblemente debido al empleo de una extracción previa al DLLME con otros disolventes, como MeOH u otras técnicas, tales como extracción de QuEChERS para muestras sólidas (Arroyo-Manzanares et al., 2013) o cartuchos Oasis en muestras acuosas (Campone et al., 2011b; Taherimaslak et al., 2015).

La adición de sal aumenta usualmente la trasferencia de los analitos desde la fase acuosa a la orgánica (Fernández et al., 2015). Así, la adición de sal a la muestra acuosa puede mejorar significativamente la extracción de analitos en DLLME, posiblemente debido al efecto "salting-out". Por lo tanto, se realizan una serie de experimentos con diferentes concentraciones de NaCl (0,2, 0,5 y 1 g) en muestras de agua extraídas con MeCN-EtOAc. Con una pequeña cantidad de sal la fase de separación no es clara, mientras que con mayores cantidades (1 g) la separación entre las fases queda bien diferenciada y se observa una forma del pico cromatográfico mejor definida, como ocurre en otros estudios (Campone et al., 2013). Sin embargo, en el caso de las muestras de plasma, únicamente se añaden 0,2 g de NaCl, debido al menor volumen de muestra empleado (250 µl).

4.4. Presencia de micotoxinas en alimentos

Los métodos previamente validados han sido aplicados a diferentes matrices, tanto de origen vegetal como de origen animal con la finalidad de determinar la presencia de diferentes familias de micotoxinas.

4.4.1. Micotoxinas en alimentos de origen vegetal

- *Micotoxinas en frutos secos*

La presencia de micotoxinas emergentes de *Fusarium* se ha evaluado en un total de 74 muestras de frutos secos y frutas deshidratadas adquiridas en diferentes mercados del área metropolitana de Valencia. Los resultados muestran elevada incidencia (63,5%), así como elevados contenidos en las muestras analizadas, principalmente ENN A y ENN B (23,3 y 14,6 mg/kg, respectivamente). Además, el 26% de las muestras analizadas presenta la coexistencia de 2 o más micotoxinas en una misma muestra. El mayor porcentaje de contaminación corresponde a los dátiles (92%), cacahuetes (82%), pipas de girasol y nueces (80%).

En las muestras con cáscara se analizan fruto y cáscara por separado, con la finalidad de conocer la distribución de las micotoxinas en los frutos. En los frutos con cáscara, el contenido de micotoxinas es mucho mayor que en el fruto, demostrando así el efecto protector que ejerce la cáscara sobre el fruto frente a la contaminación fúngica y la consecuente producción de micotoxinas. Este hecho ha sido descrito por Doster & Michailides (1994). El 80% de las muestras de cáscara analizadas presenta contenidos de alguna de las ENNs analizadas y tan solo el 7% presenta contaminación por BEA. Los contenidos más elevados de ENNs (ENN A, ENN A1 y ENN B) se detectan en muestras de cáscara de cacahuetes (23.300 mg/kg, 0,523 mg/kg y 14,610 mg/kg, respectivamente para cada una de las ENNs) y en pipas de girasol (2,62 mg/kg, 0,15 mg/kg y 2,75 mg/kg, respectivamente).

- *Micotoxinas en pasta*

Se han analizado un total de 58 muestras de pasta a base de trigo y 42 muestras de pasta a base de maíz y arroz (sin gluten) procedentes de la región italiana de Campania. Las micotoxinas incluidas en el estudio son las mayormente descritas en cereales y sus derivados: AFs, OTA, ZON, FBs, TCs, ENNs y BEA.

Se observa un patrón de contaminación por micotoxinas diferente entre el trigo duro y la pasta sin gluten, debido al empleo de diferentes ingredientes en su elaboración. En este sentido, en muestras de maíz las FBs son las micotoxinas mayormente detectadas en el presente estudio, principalmente FB1, mientras que no se detectan contenidos de FBs en las muestras de pasta de trigo analizadas (Tolosa et al., 2017a). Pese a que los TCs han presentado una elevada incidencia en la pasta de trigo duro, la pasta sin gluten presenta mayores contenidos medios de estas micotoxinas (241 μg/kg para NIV y 240 μg/kg para DON). Las ENNs muestran una elevada incidencia (90% para ENN B y 93% para ENN A1) y contenidos medios (326 μg/kg para ENN B y 64 μg/kg para BEA) en muestras de pasta de trigo duro, en cambio, en las muestras de pasta sin gluten no se detectan contenidos de ENN A ni ENNB y la incidencia de ENN A1 y BEA es menor.

- *Micotoxinas en materias primas y piensos*

Se analiza un total de 39 materias primas, 48 piensos destinados a diferentes especies de animales terrestres y 20 piensos destinados a peces de acuicultura, adquiridos en la zona metropolitana de Valencia. Las

materias primas analizadas son las más comúnmente empleadas en la elaboración de los piensos destinados principalmente a vacuno, ovino, caprino, porcino, aves, conejos, caballos y mascotas. La ENN B, ENN B1 y BEA muestran mayor frecuencia de contaminación , en cambio, las ENNs del tipo A (ENN A y ENN A1) no han sido detectadas en ninguna de las muestras analizadas en el estudio. Respecto a los contenidos medios, los niveles de ENN B oscilan entre 1,3 y 75,6 μg/kg, los de ENN B1 entre 36,3 y 113,2 μg/kg y los de BEA entre 3,0 y 64,8 μg/kg. Estos resultados están en concordancia con los datos publicados por otros autores (Sørensen et al., 2008), mostrando que las ENNs del tipo A presentan menor incidencia que las del tipo B, de acuerdo al siguiente orden decreciente: ENN B>ENN B1>ENN A1>ENN A.

Para los piensos, los resultados obtenidos muestran elevada prevalencia de las micotoxinas emergentes de *Fusarium* en las muestras analizadas (92%), siendo la ENN B y la ENN B1 las más abundantes. Los mayores contenidos medios corresponden a la BEA, ENN B, ENN B1 y, finalmente, ENN A1 (26,1, 24,2, 15,9 y 10,1 μg/kg, respectivamente). No se han detectado contenidos de ENN A en ninguna de las muestras analizadas. Los piensos con mayores contenidos de micotoxinas emergentes son aquellos destinados a conejos y ganado ovino, principalmente. La ENN A1 presenta contenidos similares en todos los piensos analizados, excepto en los piensos de animales domésticos en los que no se ha detectado. En estos últimos, la contaminación por ENN B1 también ha sido menor comparada con el resto de piensos, mientras que presenta contenidos más elevados en los destinados a conejos. Respecto a la ENN B, los mayores contenidos medios corresponden a pienso

destinado a conejos, ganado ovino y porcino. Para la BEA, los mayores contenidos se encuentran en los piensos destinados a ganado ovino, perros y ganado vacuno.

Se obtienen diferentes concentraciones en los piensos y en las materias primas empleadas en su elaboración, posiblemente debido a que, pese a que durante el procesado de las materias primas para la elaboración de los piensos (principalmente extrusión y peletización) se alcanzan altas temperaturas que pueden reducir el contenido de ENNs en el producto final (Dogi et al., 2011), una contaminación posterior durante el almacenado de los piensos puede resultar en la producción de micotoxinas.

En los piensos para peces de acuicultura se analizan 20 muestras de pienso adquiridas en diferentes establecimientos especializados del área metropolitana de Valencia. Se detecta la presencia de las cuatro ENNs y BEA en las muestras de pienso. Los contenidos medios obtenidos son 0,9, 1,1, 0,89, 1,77 y 1,4 μg/kg, para ENN A, ENN A1, ENN B, ENN B1 y BEA, respectivamente.

4.4.2. Micotoxinas en productos de origen animal

- *Micotoxinas en pescado de acuicultura*

En el estudio llevado a cabo se analizan un total de 40 muestras de pescado de las especies más consumidas (lubina, dorada, salmón y trucha arcoíris). Los resultados obtenidos en las muestras de músculo de pescado indican que las especies que presentan mayor incidencia son la lubina y la dorada (70 y 34%, respectivamente); sin embargo, las muestras de salmón presentan contenidos más elevados, siendo los contenidos máximos de 29, 103

y 94 μg/kg para ENN A1, ENN B y ENN B1, respectivamente. Esto puede ser debido al empleo de piensos con mayores contenidos de ENNs, ya que según algunos estudios llevados a cabo en cereales y materias primas de las regiones nórdicas muestran contenidos elevados de micotoxinas emergentes de *Fusarium* (Zachariasova et al., 2014). Además, las ENNs presentan cierto carácter lipófilo, por lo que pueden acumularse en mayor proporción en las especies con mayor contenido en grasas, como es el caso del salmón. La ENN A y la BEA no han sido detectadas en ninguna muestra, mientras que la ENN B presenta mayor incidencia, seguida de la ENN B1 y la ENN A1.

Por lo que respecta a las diferentes partes analizadas, en las muestras de lubina y dorada también se ha evaluado la presencia de ENNs y BEA en diferentes órganos y tejidos (hígado, vísceras y cabeza). La ENN A1 muestra mayor incidencia en las muestras de hígado y cabeza (20%). ENN B y ENN B1 presentan mayor incidencia en cabeza (25%), seguida por hígado y vísceras (15%). Estos resultados muestran la metabolización y distribución de las ENNs en los diferentes órganos y tejidos de los peces, por lo que se estima necesario el estudio de los posibles productos derivados del metabolismo de las ENNs en diferentes especies de peces procedentes de la acuicultura.

Las muestras de salmón se analizan por LC-Q-TOF, identificando 40 micotoxinas diferentes, además de las ENNs, en las muestras de músculo. Para ello, se realiza un análisis multirresiduo para la identificación de 233 micotoxinas mediante cromatografía líquida acoplada al detector de masas en tiempo de vuelo (LC-Q-TOF). Entre las micotoxinas detectadas más abundantes en las muestras, algunas de ellas son contaminantes comunes en los alimentos a base de cereales y también en los subproductos animales, como

ENNs (ENN A, ENN A1, ENN B, ENN B1 y ENN B2) y FUS. Pero también se detectan otras micotoxinas con menor presencia en los alimentos, como anisomicina, citochalasina J, ácido micofenólico, ofiobolina A y B, rugulosina y ácido penicílico, entre otras.

Hasta el momento, algunos autores han estudiado la presencia de micotoxinas en pescado, aunque la mayor parte de estos estudios se basan en la administración intencionada de piensos contaminados a diferentes especies de peces con la finalidad de estudiar su toxicidad y la presencia y/o la transferencia de éstas a las porciones comestibles (El-Sayed & Khalil, 2009; Deng et al., 2010; Nomura et al., 2011; Nácher-Mestre et al., 2015). Sin embargo, este es el primer estudio que detecta la presencia de estas micotoxinas en muestras de pescado de acuicultura ya listo para el consumo.

Teniendo en cuenta el aumento del consumo de pescado por la población y la escasez de datos sobre la contaminación por micotoxinas en esta matriz, se recomienda realizar más estudios para poder evaluar la exposición del consumidor y establecer el riesgo por exposición a micotoxinas asociado al consumo de pescado procedente de cría en cautividad.

- *Micotoxinas en productos de la pesca*

Se analizan un total de 72 muestras de salmón y trucha ahumados, diferentes tipos de sushi y sucedáneo de gulas adquiridos en supermercados del área metropolitana de Valencia, con el objetivo de determinar la presencia de AFs (AFB1, AFB2, AFG1, AFG2), FBs (FB1, FB2, FB3), FUS-X, OTA, ENNs (ENN A, ENN A1, ENN B, ENN B1), BEA y STG. Los resultados obtenidos en el presente estudio muestran que las micotoxinas analizadas no

están presentes ni en pescado ahumado ni en muestras de sushi. Por el contrario, en muestras de sucedáneo de gulas, se detectan pequeñas cantidades de FUS-X en una muestra (4 μg/kg) y FUS-X y ENN B en otra (4 y 7 μg/kg, respectivamente). Esta contaminación podría ser debida a los ingredientes utilizados.

La ausencia de micotoxinas en el pescado ahumado podría deberse a que durante el procesado, la grasa se elimina de los filetes, por lo que las micotoxinas lipófilas, como las ENNs, pueden ser eliminadas en este paso. Por otra parte, durante la etapa de salado, los filetes se colocan en solución acuosa con una concentración elevada de sal, de forma que las micotoxinas con carácter hidrófilo pueden transferirse de los filetes de pescado a la fase acuosa, produciéndose la transferencia del fluido intracelular al medio exterior (Cardinal et al., 2001). En este sentido, estos procesos pueden simular una etapa de extracción, por lo que sería posible encontrar micotoxinas hidrófilas, previamente presentes en filetes de peces de acuicultura alimentados con piensos contaminados, en agua y salmuera presentes en envases de pescado ahumado. Además, para el control químico de la actividad microbiana, se añaden algunos ácidos a los productos pesqueros (FAO, 2014). Estas sustancias también pueden interactuar con los contenidos de micotoxinas presentes en las partes comestibles del pescado, lo que sugiere que las sustancias conservantes y las hierbas comúnmente añadidas al salmón (eneldo) y también los tratamientos aplicados al pescado ahumado podrían afectar a la contaminación por micotoxinas en los filetes de pescado. Este efecto se ha observado previamente en muestras comerciales de altramuces comercializados con sal, acidulantes y sustancias conservantes (Tolosa et al., 2013).

4.5. Evaluación de la exposición

Se ha llevado a cabo la evaluación de la exposición a las micotoxinas emergentes de *Fusarium* basada en un enfoque determinista. Para ello, se calcula la EDI a partir de las concentraciones obtenidas en muestras de pescado de acuicultura, y de los datos disponibles de consumo de éste por la población adulta española (Ministerio de Agricultura y Pesca, Alimentación y Medio Ambiente, 2014). La ingesta diaria para los consumidores españoles es de 26,84 kg per cápita durante el año 2013. A partir de este dato de consumo y de las concentraciones medias obtenidas en músculo de pescado, se calcula la EDI. La ingesta diaria tolerable (TDI) no ha sido todavía establecida para las ENNs y BEA. Por esta razón, la aproximación a la evaluación del riesgo se lleva a cabo de conformidad con las directrices de seguridad para otras micotoxinas de *Fusarium*. De esta forma, los resultados son comparados con los valores de PMTDI establecidos para NIV (1 μg/kg pc/día), DON (0,7 μg/kg pc/día), la suma de HT-2/T-2 (0,1 μg/kg pc/día), y la suma de FB1 y FB2 (2 μg/kg pc/día). Se puede observar que las EDI calculadas fueron inferiores a las ingestas diarias tolerables (TDI) para NIV, DON, FB1/FB2 y HT-2/T-2. Los resultados obtenidos muestran que no es posible confirmar que las ENNs presentes en el pescado representen un riesgo para la población española, ya que son menores que las TDIs establecidas para otras micotoxinas de *Fusarium*.

Pese a que es necesaria más información sobre los datos toxicológicos de las micotoxinas emergentes de *Fusarium*, debe prestarse especial atención a estos resultados, ya que algunos autores indican que la citotoxicidad de DON no es significativamente diferente a la de ENNs y BEA.

Por otra parte, la detección e identificación de los compuestos de degradación generados tras el procesado de los alimentos, así como la evaluación de su toxicidad, también son aspectos importantes a tener en consideración para la evaluación del riesgo (Broekaert et al., 2015).

4.6. Mitigación de micotoxinas emergentes de *Fusarium* mediante tratamientos culinarios

Los estudios acerca del comportamiento de las micotoxinas emergentes de *Fusarium* durante los diferentes procesos a los que es sometido el alimento previamente a su consumo son escasos. De acuerdo a las recomendaciones del reciente dictamen científico de EFSA, para realizar la evaluación del riesgo de las micotoxinas emergentes (EFSA, 2014), se ha estudiado el comportamiento de estas micotoxinas y de los posibles productos de degradación y/o conjugación formados durante los procesos de cocinado del pescado.

Estudios recientes han demostrado que los contenidos de ENNs se reducen a través de procesos industriales comunes, como la fabricación de pan (Vaclavikova et al., 2013; Hu et al., 2014b), elaboración de cerveza (Meca et al., 2013; Hu et al., 2014a) y la producción de pasta (Tittlemier et al., 2014; García-Moraleja et al., 2015; Serrano et al., 2016). En este sentido, algunos autores han descrito la reducción del contenido de ENNs y BEA por tratamientos térmicos (Meca et al., 2012; García-Moraleja et al., 2015; Serrano et al., 2016). Por otra parte, en cuanto a la reducción lograda en otras micotoxinas tradicionales de *Fusarium*, algunos autores han descrito la degradación del NIV (Bretz et al., 2005), degradación de la toxina T-2

(Beyer et al., 2009) y de FBs (Bullerman & Bianchini, 2007; De Girolamo et al., 2016).

Sin embargo, no hay datos disponibles relacionados con la reducción de los contenidos de micotoxinas en pescado. En este sentido, en el presente trabajo se ha realizado el estudio de cuatro tratamientos térmicos diferentes comúnmente empleados para cocinar el pescado: hervido (BO), asado (BR), horno convencional (CO) y horno microondas (MO). Los parámetros y procedimientos térmicos estudiados se han basado en los descritos en la literatura respecto al efecto de los tratamientos térmicos sobre los parámetros nutricionales en diferentes especies de peces (Nurhan, 2007; Hosseini et et al., 2014).

Los porcentajes de reducción han sido calculados asumiendo que la concentración inicial en la muestra cruda es el porcentaje máximo (100%). Estos valores oscilan en función del método culinario aplicado, de la especie y de la micotoxina evaluada. Así, la ENN A1 presenta el mayor porcentaje de reducción (100%), debido a que la concentración inicial en las muestras era ya poco elevada. Para el salmón, la reducción obtenida (100%) es mayor que en lubina y dorada, posiblemente debido a que el salmón presenta un contenido lipídico más elevado y la grasa sufre importantes modificaciones durante el procesado térmico, acumulando más temperatura, lo cual puede afectar al contenido de micotoxinas. Por lo que respecta a la trucha arcoíris, diferentes porcentajes de reducción se obtienen dependiendo del método culinario aplicado, obteniendo un 100% de reducción en las muestras asadas y hervidas. En este caso puede deberse a la baja concentración inicial presente en las muestras de esta especie.

Estos resultados muestran que la estabilidad de las ENNs se ve afectada por diferentes tratamientos térmicos, tal y como han evidenciado también otros autores (Serrano et al., 2013).

4.7. Productos de degradación

Durante los tratamientos térmicos las micotoxinas pueden sufrir modificaciones en su estructura química, así como interactuar con diferentes sustancias presentes en la matriz alimentaria, dando lugar a nuevos compuestos, por lo que tras la aplicación de los procesos culinarios a las muestras de pescado, se ha procedido a la búsqueda de productos relacionados. La mayor parte de estudios llevados a cabo con el fin de monitorear los efectos de la cocción sobre el contenido de micotoxinas en los alimentos, solo muestra la disminución de la contaminación inicial, sin embargo, la identificación de los productos de degradación es escasa (Köppen et al., 2010). Algunos estudios se han centrado en el aislamiento y caracterización de nuevos productos de micotoxinas generados después del tratamiento térmico aplicado a los alimentos, así como en la evaluación de la toxicidad de estos compuestos. En la mayoría de los casos, los productos de degradación son menos tóxicos que sus moléculas originales (Bretz et al., 2005, Beyer et al., 2009; Shams et al., 2011).

El empleo de LC-MS-LIT en la modalidad de barrido completo (SCAN) ha permitido elucidar estructuralmente compuestos de degradación de las ENNs, formados tras la aplicación de los tratamientos térmicos. En los cromatogramas obtenidos se observa un aumento del tamaño del pico correspondiente a los productos de degradación al mismo

tiempo que se reducen los picos correspondientes a las micotoxinas madre (Tolosa et al., 2017b).

Como se ha descrito anteriormente, la ENN A1 es la micotoxina que ha sufrido mayor reducción tras el sometimiento de las muestras de pescado a diferentes procesos culinarios. Este elevado porcentaje de reducción alcanzado puede deberse a la transformación de la ENN A1 en productos de degradación, formados por las altas temperaturas a las que se han sometido las muestras. Así, se han identificado dos productos de degradación caracterizados por la pérdida de unidades de isoleucina (Ile).

Hasta el momento ningún estudio ha evaluado la toxicidad de los productos derivados de la degradación de las ENNs formados tras los tratamientos tecnológicos. No obstante, existen diversos estudios para otras micotoxinas de *Fusarium* que han identificado productos de degradación (Humpf & Voss, 2004; Bretz et al., 2006; Meca et al., 2012) y tras comparar la toxicidad de estos compuestos con la toxicidad de la micotoxina inicial, concluyen que la mayoría de los productos de degradación presentan una toxicidad inferior a la de la molécula inicial (Bretz et al., 2006).

Los resultados obtenidos en el presente estudio confirman que la estructura de las ENNs sufre modificaciones durante los tratamientos a los que son sometidos los alimentos, por lo que son necesarios nuevos estudios para la total identificación de estos productos de degradación en alimentos, así como para evaluar su toxicidad.

La identificación de los productos de conjugación y de degradación es esencial, ya que, mientras que para las micotoxinas libres existen métodos analíticos fiables, aquellas micotoxinas que han sufrido transformaciones pueden no ser detectadas por la metodología analítica de rutina. En la mayoría de estos casos, los compuestos resultantes no pueden detectarse debido a que las micotoxinas hidrolizadas y/o conjugadas pueden perderse durante la extracción y/o purificación, debido a su mayor polaridad y a que los detectores de MS no reconocen la transición específica monitorizada al carecerse de patrones.

4.8. Identificación de metabolitos

El análisis de las muestras de músculo de salmón mediante LC-Q-TOF ha permitido la identificación de deepoxi-deoxinivalenol (DOM-1), metabolito del DON. DOM-1 se forma por acción de las bacterias del tracto digestivo de diferentes especies mediante una reacción de de-epoxidación en el grupo epoxi de los TCs (Yang et al., 2015). Respecto a su toxicidad, de acuerdo con McCormick (2013), el DOM-1 es 55 veces menos tóxico que el DON.

Sørensen & Elbæk (2005) han descrito la presencia de DOM-1 en animales de abasto, principalmente en rumiantes, así como en leche procedente de vacas alimentadas con pienso contaminado con DON, aunque los contenidos detectados son bajos, ya que según estos autores, el ratio de excreción es mayor en orina y heces que en leche.

En peces, la conversión de TCs por parte de la flora bacteriana del tracto digestivo ha sido estudiada en diferentes ensayos in vivo. Guan et al.

(2009) ha descrito la formación de DOM-1 a partir de DON en el pez gato (*Ameiurus nebulosus*). En el estudio llevado a cabo por Pietsch et al. (2014), DOM-1 no se detecta en ninguna de las muestras de pienso administrado a carpas (*Cyprinus carpio L.*), ni de DOM-1 en diferentes tejidos de los peces tras la ingesta de pienso contaminado con DON. De acuerdo a estos resultados, en el estudio llevado a cabo por De Baere et al. (2011), en el que se administró DON a pollos y cerdos, no se detectó DOM-1 en ninguna de las muestras de plasma, aunque sí se detectó su presencia en bilis tras tres semanas de consumo de pienso contaminado.

5. CONCLUSIONS

5. CONCLUSIONES

1. La metodología analítica validada basada en la extracción y concentración mediante QuEChERS, extracción sólido-líquido y microextracción dispersiva líquido-líquido seguida de LC-MS/MS ha mostrado resultados satisfactorios para las micotoxinas clásicas y emergentes en alimentos destinados al consumo humano y a la alimentación animal.

2. El análisis multimicotoxina en pasta muestra una elevada incidencia de las micotoxinas clásicas y emergentes. El patrón de contaminación varía entre muestras de pasta a base de trigo y muestras de pasta sin gluten a base de maíz y arroz. En ambas se encuentran presentes deoxinivalenol, nivalenol, zearalenona, HT-2, eniatina A1 y beauvericina. Si bien, en los alimentos a base de trigo además están presentes T-2, 3-acetil deoxinivalenol y 15-acetil deoxinivalenol, eniatina A y eniatina B, mientras que en las de maíz y arroz están presentes las fumonisinas B1 y B2.

3. Las micotoxinas emergentes de *Fusarium* están presentes en todos los alimentos estudiados, tales como frutos secos, pasta, materias primas, piensos y productos de origen animal.

4. Los contenidos de eniatinas detectados en muestras de frutos secos son más elevados que en el resto de matrices alimenticias estudiadas, alcanzando valores del orden de mg/kg y presentando concentraciones más elevadas en las cubiertas que en la parte comestible.

5. Los contenidos de eniatinas y beauvericina en materias primas y productos compuestos destinados a la alimentación animal presentan variaciones apreciables. Entre las materias primas, la alfalfa deshidratada muestra los contenidos más elevados, mientras que para los piensos, los destinados a conejos y ganado ovino presentan mayor concentración.

6. El análisis de eniatinas en cuatro especies de pescado procedente de acuicultura ha mostrado la presencia de eniatina A1, B y B1 en músculo, siendo la lubina la que presenta mayor incidencia y el salmón la que presenta mayores contenidos.

7. Los tratamientos térmicos aplicados a las muestras de pescado, asado, hervido, microondas y horno, han mostrado ser eficaces en la mitigación de los contenidos de eniatinas, obteniéndose una disminución de entre el 30 y el 100% dependiendo del tratamiento aplicado, de la micotoxina en cuestión y de la especie animal.

8. Tras la aplicación de dichos tratamientos térmicos se han identificado dos productos de degradación de la eniatina A1, caracterizados ambos por la pérdida de unidades de isoleucina. Sin embargo, son necesarios más estudios para la identificación de otros productos de degradación presentes en los alimentos cocinados.

9. El empleo de LC-Q-TOF ha permitido la identificación de cuarenta micotoxinas en salmón de acuicultura, pertenecientes a muy diversas familias químicas, entre ellas el deepoxi-deoxinivalenol, producto de la metabolización del deoxinivalenol. Esto es debido a la ingesta de

piensos contaminados y/o al crecimiento de hongos en los tanques de producción.

10. La presencia de micotoxinas en muestras de origen vegetal y en productos de origen animal supone un problema de seguridad alimentaria. Por lo que, de acuerdo con la Autoridad Europea de Seguridad Alimentaria, deben continuar los trabajos que abordan la cuantificación de las micotoxinas en los alimentos destinados a la alimentación humana y animal, incluyendo los metabolitos y productos de degradación, además de estudios referentes a su mitigación.

5. CONCLUSIONS

1. The validated analytical methododology based in extraction and concentration by QuEChERS, solid-liquid extraction and dispersive liquid-liquid microextraction followed by LC-MS/MS analysis have shown satisfactory results for both classic and emerging mycotoxins, in food intended for human consumption and feed intended for animal feeding.

2. The multi-mycotoxin analysis in pasta samples showed high incidence of classic and emerging mycotoxins. The contamination pattern varies between wheat and gluten-free pasta elaborated with maize and rice. In both matrices deoxynivalenol, nivalenol, zearalenone, HT-2, enniatin A1 and beauvericin were detected. However, in durum wheat pasta T-2, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, enniatin A and enniatin B were also present, while in maize and rice samples, were present fumonisin B1 and B2.

3. *Fusarium* emerging mycotoxins were present in all studied foodstuffs, such as nuts, pasta, raw materials, feed and animal derived products.

4. Enniatin contents in nut samples were higher than those detected in the other food matrices surveyed, reaching values in the order of mg/kg and higher contents in the shell respect to the edible part.

5. Enniatin and beauvericin contents in raw materials and compound feedstuffs intended for animal feeding showed considerable variations. Among the raw materials, dehydrated alfalfa showed the highest

contents, while for feed, those intended to rabbits and sheep showed higher concentrations.

6. Enniatin analysis performed on four different fish species from aquaculture showed the presence of enniatins A1, B and B1 in muscle. Sea bass showed the highest incidence, while the highest contents were reported in salmon.

7. Thermal treatments applied to aquaculture fish samples, broiled, boiled, microwave and oven, have shown to be effective to mitigate enniatin contents, showing a reduction percentage between 30 and 100% in analyzed samples depending on the treatment applied, the mycotoxin and the fish species evaluated.

8. After thermal treatment application, 2 degradation products from enniatin A1 (ENN A1), both characterized by the loss of isoleucine units, were identified. However, further studies are needed to identify new degradation products in cooked foodstuffs.

9. The LC-Q-TOF analysis has allowed the identification of fourty mycotoxins with different chemical structures in farmed salmon, including the deepoxy-deoxynivalenol, a product from deoxynivalenol metabolism. These results are due to the ingestion of contaminated feedstuffs and/or fungal growing on the production tanks.

10. Mycotoxin occurrence in both plant foodstuffs and animal derived products suppose a food safety problem. Thus, according to the European Food Safety Authority, studies regarding the mycotoxin quantification in foodstuffs intended for human and animal

consumption, including metabolites and degradation products, as well as their mitigation are needed.

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6. REFERENCES

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