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

Tesi Doctoral Internacional

RISK ASSESSMENT OF MYCOTOXINS IN CEREALS

EVALUACIÓN DEL RIESGO DE MICOTOXINAS EN CEREALES

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CERTIFIQUEN QUE:

Dª Ana Belén Serrano Serrano, llicenciada en Química, ha realitzat baix la seua direcció el treball **"Evaluación del riesgo de micotoxinas en cereales"**, i autoritzen la seua presentació per a optar al títol de Doctora per la Universitat de València.

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Aquest treball ha sigut plasmat en 11 articles, publicats o que es publicaran en les següents revistes:

1. Comparative assessment of three extraction procedures for determination of emerging *Fusarium* mycotoxins in pasta by LC-MS/MS. *Food Control, 32 (2013) 105-114. Impact factor: 2.819*
2. Dispersive liquid-liquid microextraction for the determination of emerging *Fusarium* mycotoxins in water. *Food Analytical Methods (under review). Impact factor: 1.802*
3. Development of a rapid liquid chromatography/tandem mass spectrometry method for the simultaneous determination of emerging *Fusarium* mycotoxins enniatins and beauvericin in human biological fluids. *Analytical and Bioanalytical Chemistry (under review). Impact factor: 3.578*
4. Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area. *Food Chemistry, 135 (2012) 423-429. Impact factor: 3.334*
5. Emerging *Fusarium* mycotoxins in organic and conventional pasta. *Food and Chemical Toxicology, 51 (2013) 259-266. Impact factor: 2.610*
6. Risk assessment associated to the intake of the emerging *Fusarium* mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin. *Food Control (2012) 28, 178-183. Impact factor: 2.738*
7. Risk assessment of beauvericin, enniatins and fusaproliferin present in follow-up infant formula by *in vitro* evaluation of the duodenal and colonic bioaccessibility. *Food Control, 42 (2014) 234-241. Impact factor: 2.819*
8. Degradation study of enniatins by liquid chromatography–triple quadrupole linear ion trap mass spectrometry. *Food Chemistry, 141 (2013) 4215-4225. Impact factor: 3.259*
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10. Development a mitigation strategy of enniatins in pasta under home-cooking conditions. *LWT-Food Science and Technology (under review). Impact factor: 2.468*
11. Evolution of enniatins levels and Maillard Reaction in pasta during one year of storage. *Journal of Agricultural and Food Chemistry (under review). Impact factor: 3.107*

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- 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).

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" No temas a las dificultades, lo mejor surge de ellas "

Rita Levi-Montalcini

A mi madre

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List of abbreviations

AcN	Acetonitrile
AcOEt	Ethil acetate
AECOSAN	Agencia Española de Consumo, Seguridad Alimentaria y Nutrición
AFs	Aflatoxins
ALARA	As Low As Reasonable Achievable
APPCC	Análisis de Peligros y de Puntos Críticos de Control
a_w	Water activity
BEA	Beauvericin
BPA	Buenas Prácticas de Agrícolas
BPF	Buenas Prácticas de Fabricación
bw	Body weight
C18	Octadecilsilice
CE	Comisión Europea
CONTAM	Panel on Contaminants in the Food Chain
DAS	Diacetoxyscirpenol
Deacetil-FUS	Deacetil-fusaproliferin
DL_{50}	Dosis Letal 50
DLLME	Dispersive Liquid-Liquid Micro-extraction
DON	Deoxynivalenol
EDI	Estimated Daily Intake
EFSA	European Food Safety Authority
ENs	Enniatins
ENA	Enniatin A

ENA_1	Enniatin A_1
ENB	Enniatin B
ENB_1	Enniatin B_1
EPI	Enhanced Product Ion
ER	Enhanced Resolution
ESI	Electrospray Ionization
FAO	Food and Agriculture Organization of the United Nations
FBs	Fumonisins
FUS	Fusaproliferin
GC	Gas Chromatography
HyLv	2-hidroxy-3-methylbutanoic acid
I	Incidence
IARC	International Agency of Research on Cancer
IDE	Ingesta Diaria Estimada
Ile	N-methyl-isoleucine
ILOD	Instrumental Limit of Detection
ILOQ	Instrumental Limit of Quantification
JECFA	Joint Expert Committee on Food Additives of the European Union
QUECHERS	Quick, Easy, Cheap, Effective, Rugged and Safe
L-L	Liquid-Liquid
LC-LIT-MS	Liquid Chromatography-Linear Ion Trap-Mass Spectrometry
LC-MS/MS	Liquid Chromatography-tandem Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantification

MAGRAMA	Ministerio de Agricultura, Alimentación y Medio Ambiente
MDL	Method Detection Limit
MeOH	Methanol
MQL	Method Quantification Limit
MON	Moniliformin
MR	Maillard Reaction
MRM	Multiple Reaction Monitoring
MS	Mass spectrometry
MSPD	Matrix Solid Phase Dispersion
nd	Non detected
NIV	Nivalenol
OTA	Ochratoxin A
PAT	Patulin
pc	Peso corporal
Ph	N-methyl-phenilalanine
PM	Peso Molecular
PMTDI	Provisional Maximum Tolerable Daily Intake
PRS	Pasta Resembling System
QqQ	Triple Quadrupole
RASFF	Rapid Alert System for Food and Feed
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SALLE	Salting-out liquid-liquid extraction
SCF	Scientific Committee for Food
SCIRI	Sistema Coordinado de Intercambio Rápido de Información
S-L	Solid-Liquid

SPE	Solid Phase Extraction
SPME	Solid Phase Micro-extraction
SRM	Selected Reaction Mode
TCs	Tricothecenes
TDI	Tolerable Daily Intake
t-TDI	Temporal Tolerable Daily Intake
t_R	Retention time
UE	Unión Europea
Val	N-methyl-valine
WHO	World Health Organization
ZEA	Zearalenone

RESUMEN

Se ha realizado la evaluación del riesgo de diferentes grupos de micotoxinas: aflatoxinas, fumonisinas, ocratoxina A, zearalenona, tricotecenos y micotoxinas emergentes de *Fusarium*.

Los procedimientos analíticos para determinar las micotoxinas en cereales, productos elaborados a base de cereales, agua y fluidos biológicos han sido validados de acuerdo a la normativa europea con resultados satisfactorios en cuanto a linealidad, exactitud, precisión y límites de detección y cuantificación. El nivalenol y las micotoxinas emergentes son las micotoxinas con una mayor prevalencia en cereales y productos derivados. Un elevado porcentaje de muestras han presentado simultáneamente varias micotoxinas, principalmente combinaciones de micotoxinas emergentes, de nivalenol y beauvericina, y de nivalenol y alguna de las aflatoxinas.

El estudio de bioaccesibilidad pone de manifiesto que el contenido de micotoxinas emergentes accesible en el tracto gastrointestinal es inferior al que se encuentra en papillas infantiles. Por tanto, la exposición de la población infantil tras el proceso de digestión podría ser menor a la estimada por consumo de alimentos.

La estimación de la ingesta diaria ha evidenciado la exposición de la población adulta a aflatoxinas, ocratoxina A y nivalenol por consumo de cereales y productos derivados, representando un riesgo potencial para la salud.

La población infantil presenta un riesgo de exposición a las micotoxinas emergentes de *Fusarium* superior al riesgo estimado en la población adulta, con una ingesta diaria estimada comparable a las ingestas diarias tolerables establecidas para las micotoxinas de *Fusarium*.

Los procesos de elaboración y cocción de la pasta reducen parte de los contenidos de micotoxinas emergentes de *Fusarium*. La estrategia de descontaminación basada en la modificación del pH en el agua de cocción de la pasta se ha propuesto para reducir en gran medida la concentración de las micotoxinas emergentes. El estudio de almacenamiento muestra que las concentraciones se reducen progresivamente con el tiempo siguiendo un modelo lineal. Se ha demostrado que los tratamientos aplicados dan lugar a la formación de nuevos productos de degradación de toxicidad desconocida.

Especial atención se debe prestar a la presencia de las micotoxinas en cereales y productos derivados, especialmente en alimentos infantiles, siendo necesarios estudios para evaluar la toxicidad y la influencia de los tratamientos tecnológicos para realizar una adecuada evaluación del riesgo.

SUMMARY

It has been performed the risk assessment of different groups of mycotoxins: aflatoxins, fumonisins, ochratoxin A, zearalenone, trichothecenes and emerging *Fusarium* mycotoxins.

The analysis methods to determinate mycotoxins in cereals, cereal-based products, water and biological fluids, have been validated according to the European regulation with satisfactory results in terms of linearity, precission, trueness and limits of detection and quantification. Nivalenol and emerging *Fusarium* mycotoxins were the mycotoxins with the highest prevalence in the samples of cereals and cereal-based products. Various mycotoxins co-occurred in a high percentage of samples. Among the most common combinations were of the emerging mycotoxins, nivalenol and beauvericin, and nivalenol with one of the aflatoxins.

The bioaccessibility study highlighted that the accesible concentrations of emerging mycotoxins were lower than those found in infant formula. Therefore, the exposure of infant population after the digestion process may be lower than those estimated by food consumption.

The estimated daily intake have shown the exposure of adult population to aflatoxins, ochratoxin A and nivalenol from consumption of cereals and cereal-based products, representing a potential health risk.

Infant population presents a risk of exposure to emerging *Fusarium* mycotoxins higher than the estimated risk on adult population, with a estimated daily intake comparable to the tolerable daily intake of other *Fusarium* mycotoxins.

Pasta processing and cooking process reduced part of the contents of emerging *Fusarium* mycotoxins. It has been prposed the mitigation strategy

based on pH modification in the cooking water, to greatly reducing the emerging mycotoxins concentration. The storage study showed that the concentrations are reduced gradually with the time according to a linear model. It has been demonstrated that the applied treatments resulted to the formation of novel degradation products with unknown toxicity.

Special attention should be paid to the occurrence of mycotoxins in cereals and products derived from them, specially to infant products. Studies to evaluate the toxicity and the influence of industrial treatments are required in order to perform a suitable risk assessment.

1. Introduction

1. INTRODUCCIÓN

La seguridad alimentaria es una de las grandes preocupaciones de la sociedad actual. El concepto seguridad alimentaria ha adquirido diferentes significados y ha ido evolucionando con el paso de los años. Según la FAO (Food and Agriculture Organization of the United Nations), existe seguridad alimentaria cuando "todas las personas tienen en todo momento acceso físico, social y económico a alimentos seguros, nutritivos y en cantidad suficiente para satisfacer sus requerimientos nutricionales y preferencias alimentarias, a fin de llevar una vida activa y saludable", término que fue ratificado en la última Cumbre Mundial sobre Seguridad Alimentaria de 2009 (FAO, 2009). Esta definición plantea cuatro componentes primordiales: la disponibilidad física, el acceso y la utilización de los alimentos (diversidad de la dieta, y correcta preparación, distribución y uso de los alimentos dentro de los hogares), y la estabilidad en el tiempo respecto a los tres componentes anteriores. Para que puedan alcanzarse los objetivos de seguridad alimentaria, deben cumplirse simultáneamente estos cuatro componentes. No obstante, el grado de importancia varía en función de la zona geográfica y de la población de referencia. El componente de mayor repercusión en los países desarrollados es la utilización de los alimentos, asociado habitualmente a la inocuidad, garantía de que un alimento no cause daño en la salud del consumidor cuando sea preparado y/o ingerido de acuerdo al uso que se destine (FAO, 2011). Es por ello que en las últimas décadas se han adoptado diferentes medidas para prevenir, controlar, y reducir los riesgos de origen alimentario para velar por la salud del consumidor. Los controles y el seguimiento del alimento se efectúan a lo largo de toda la cadena alimentaria y en todos los sectores de la alimentación: "de la granja a la mesa" (European Commission, 2015).

A nivel europeo, el sistema RASFF (Rapid Alert System for Food and Feed) cataloga los peligros en seguridad alimentaria sobre una base de datos semanal y anual. Este sistema representa la herramienta de intercambio de información más importante, proporcionando una elevada protección del consumidor ante los peligros para la salud que se ocasionan en el sector alimentario. De acuerdo con el informe más reciente, un total de 3205 notificaciones relativas a productos alimenticios fueron transmitidas a través del RASFF en el año 2013, de las cuales, 405 correspondieron a notificaciones relacionadas con la presencia de micotoxinas en alimentos (Annual Report RASFF, 2013). Como se puede observar en la Figura 1, las micotoxinas ocuparon el tercer lugar de los peligros alimentarios con un mayor número de notificaciones realizadas en el año 2013, ocupando el primer y segundo puesto las notificaciones relacionadas con la presencia de microorganismos patógenos y plaguicidas, respectivamente.

En España, el Sistema Coordinado de Intercambio Rápido de Información (SCIRI) coordinado por la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN), gestionó más de 3000 notificaciones en el año 2013. Los peligros originados por toxinas fúngicas dieron lugar a 375 notificaciones, de las cuales 8 correspondieron a alertas, 102 a informaciones y 265 a rechazos de productos alimenticios (SCIRI, 2013).

En la actualidad, la presencia de micotoxinas en los alimentos, constituye un problema de salud pública a nivel mundial. La FAO ha estimado que la contaminación por micotoxinas afecta una cuarta parte de las cosechas en el mundo (FAO/WHO, 2002). Las pérdidas por presencia de micotoxinas en productos alimenticios se han estimado en alrededor de mil millones de toneladas anuales. Todo ello repercute en la productividad agrícola y pecuaria,

dando lugar a considerables pérdidas económicas en el comercio de muchos países productores y en muchos casos, al encarecimiento del producto final. De hecho, algunos estudios han indicado que una reducción de las pérdidas causadas por la presencia de micotoxinas, podría provocar un impacto sustancial en los medios de subsistencia de países en vías de desarrollo y en la seguridad alimentaria (Ramos, 2011).

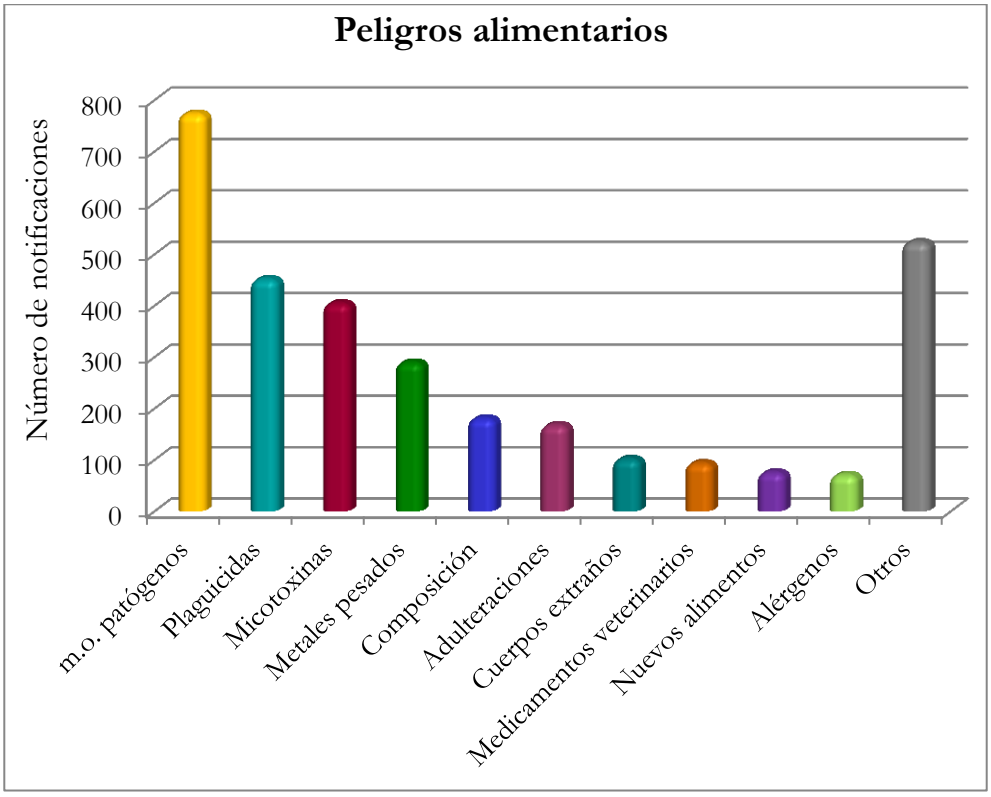


Figura 1. Notificaciones por peligros alimentarios (Annual Report RASFF, 2013).

1.1. Micotoxinas

1.1.1. Aspectos generales

Las micotoxinas son un grupo de sustancias biológicamente activas de bajo peso molecular (PM<700), producidas por diferentes especies de hongos filamentosos. La producción de micotoxinas tiene lugar durante la última fase de crecimiento del hongo o metabolismo secundario. Los hongos micotoxigénicos son capaces de crecer y producir micotoxinas en una amplia variedad de sustratos. Los principales hongos que producen micotoxinas en los alimentos, pertenecen a los géneros *Aspergillus, Fusarium, Penicillium* y *Alternaria*. Las especies que pertenecen a los géneros *Fusarium* y *Alternaria* precisan de un elevado contenido en humedad para crecer, y normalmente producen micotoxinas en el campo. Las especies de los géneros *Penicillium* y *Aspergillus* proliferan durante el trasporte y almacenamiento de la materia prima, ya que requieren bajos contenidos en humedad, aunque excepcionalmente se han dado casos de producción en campos de cultivo (Sanchis et al., 2007). Es importante destacar que el hongo puede crecer y producir micotoxinas en la planta o alimento, sin ser visible para el ser humano, lo que complica su detección precoz en el campo. El crecimiento del hongo y la producción de micotoxinas dependen de una serie de factores físicos, químicos y biológicos. Entre los factores físicos más destacados se encuentran la actividad del agua (a_w), la temperatura y la integridad física del grano o del alimento. Por lo que respecta a los factores químicos, el pH, la composición del sustrato y la disponibilidad de oxígeno, dióxido de carbono y nitrógeno son los factores más significativos. La presencia de invertebrados y microorganismos contribuye al deterioro biológico de los cereales, y también pueden actuar

como portadores de esporas de los hongos micotoxigénicos, aumentando así el riesgo de proliferación del moho (Brera et al., 2002).

Las micotoxinas son responsables de una serie de efectos adversos (agudos o crónicos) en los seres humanos y animales, llamados *micotoxicosis*. En el año 2001, la JECFA (Joint Expert Committee on Food Additives of the European Union), como órgano de asesoramiento científico para la FAO y la WHO (World Health Organization), evaluó los efectos tóxicos de diversas micotoxinas, considerando que el riesgo de intoxicación aguda es entre moderado y bajo en comparación con otras familias de compuestos de origen microbiológico. Sin embargo, el riesgo se incrementa cuando se consideran los efectos crónicos atribuidos a estas sustancias (FAO/WHO, 2001). En la actualidad, las micotoxinas representan un mayor riesgo de intoxicación crónica que el resto de contaminantes alimentarios, incluyendo plaguicidas, aditivos, medicamentos de uso veterinario, etc. (Kuiper-Goodman, 2004). La IARC (International Agency for Research on Cancer) ha clasificado al grupo de las aflatoxinas (AFs) como compuestos carcinógenos para el hombre (Grupo 1), a la ocratoxina A (OTA) y fumonisinas (FBs) como posibles carcinógenos (Grupo 2B), y al grupo de los tricotecenos (TCs) y la zearalenona (ZEA) como no carcinógenos para el ser humano (Grupo 3) (IARC, 1993).

Los mecanismos de acción de las diferentes micotoxinas son muy variables, principalmente debido a la diversidad de estructuras químicas que presentan. Es por ello, que los síntomas ocasionados en la salud humana y animal son muy diversos dependiendo del tipo de micotoxina responsable, pudiendo afectar a distintos órganos, aparatos o sistemas (hígado, riñón, sistema nervioso, endocrino e inmunitario). En la tabla 1 se muestran las principales micotoxinas, los efectos tóxicos que causan y las principales especies de

hongos responsables de su producción. No obstante, es importante considerar los posibles efectos de sinergismo, antagonismo y/o de adición que puede provocar la presencia simultánea de varias micotoxinas en el organismo (Speijers & Speijers, 2004).

Tabla 1. Tipos de micotoxinas, principales especies de hongos micotoxigénicos productores y efectos tóxicos causados por las micotoxinas (Martinez-Larrañaga & Anadón, 2006).

Micotoxina	**Principales hongos productores**	**Efectos tóxicos**
Aflatoxinas	*Aspergillus flavus* *Aspergillus parasiticus*	Carcinógenas, hepatotóxicas, nefrotóxicas, neurotóxicas
Ocratoxina A	*Aspergillus ochraceus* *Penicillium alutaceus*	Teratógena, hepatotóxica, nefrotóxica, neurotóxica
Tricotecenos	*Fusarium sporotrichioides* *Fusarium graminearum*	Teratógenos, citotóxicos, irritantes de mucosas
Zearalenona	*Fusarium graminearum* *Fusarium culmorum*	Hepatotóxica, estrógena, nefrotóxica, efectos endocrinos
Fumonisinas	*Fusarium moniliforme* *Fusarium proliferatum*	Teratógenas, hepatotóxicas, nefrotóxicas, neurotóxicas
Patulina	*Penicillium patulum* *Penicillium c-expansum*	Carcinógena, hemorragias en el pulmón

La principal vía de exposición del ser humano y los animales a las micotoxinas se produce por vía directa a través de la ingesta de alimentos contaminados. Excepcionalmente, la exposición puede ocurrir por vía indirecta, bien por inhalación a través del medio ambiente o por absorción cutánea a través del contacto con productos contaminados (Figura 2) (Rubinstein & Theumer, 2011). Los alimentos más habitualmente contaminados por micotoxinas son los productos del sector primario,

incluyendo cereales (maíz, trigo, cebada, avena, arroz, etc.), frutas y verduras (manzana, uva o tomates), café, cacao, especias y frutos secos. Algunas micotoxinas pueden resistir a los tratamientos físicos, químicos y biológicos aplicados durante el procesado de algunos alimentos, pudiendo persistir en el alimento procesado destinado al consumo humano o animal (piensos, pan, pasta, galletas, cerveza, vino, zumos, etc.). Por tanto, una vez la micotoxina ha contaminado la materia prima alimentaria, es muy difícil su eliminación de la misma (Delledonne, 2006). Además de los productos de origen vegetal, las micotoxinas pueden estar presentes en productos de origen animal, como leche, carne, huevos o pescado, procedentes de animales que han sido alimentados con piensos contaminados (Völkel et al., 2011).

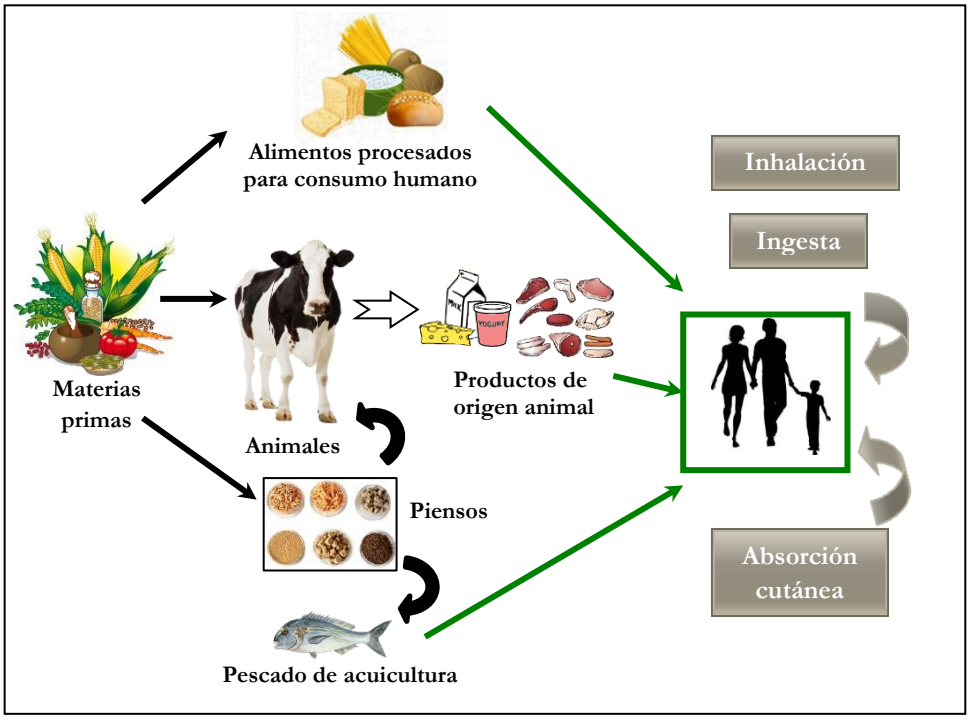


Figura 2. Vías de exposición de micotoxinas a animales y humanos.

1.1.2. Legislación

Respecto al marco legislativo, diferentes países presentan regulaciones para controlar los niveles de micotoxinas en alimentos y piensos. Según la última revisión completa realizada por la FAO, se estimó que alrededor de 100 países presentaban Reglamentos en los años 2002-2003 para estas toxinas de origen fúngico: 39 países de Europa (99% de la población del continente), 15 países de África (59% de la población), 26 países de Asia y Oceanía (88% de la población), 19 países de Latinoamérica (91% de la población) y Estados Unidos y Canadá (FAO, 2004). No obstante, se considera que el número de países con normativas para las micotoxinas ha aumentado considerablemente en la última década, lo que refleja la preocupación general de los gobiernos sobre los efectos potenciales de las micotoxinas.

Aunque en la actualidad se han identificado más de 400 micotoxinas, únicamente se han adoptado medidas legislativas para 13 de ellas. Según la FAO (2002), los factores que más influyen cuando se requiere fijar límites y reglamentar los contenidos de micotoxinas, son los siguientes:

- Disponibilidad de datos toxicológicos, de métodos analíticos, y de datos relativos a la presencia de las micotoxinas en diversos productos básicos.
- Conocimiento de la distribución de las concentraciones de las micotoxinas en un lote.
- Legislación de los países con los que existen contactos comerciales.
- Necesidad de un abastecimiento suficiente de alimentos.

En la Unión Europea existen diversos reglamentos de directa aplicación. En el año 2006, la Comisión Europea estableció el Reglamento (CE) Nº 1881/2006 de la Comisión, por el que se fija el contenido máximo de determinados contaminantes en los productos alimenticios. En este

Reglamento se establecieron contenidos máximos en piensos y alimentos para las llamadas "micotoxinas tradicionales", que comprenden el grupo de las AFs B_1, B_2, G_1, G_2 y M_1, OTA, ZEA, fumonisinas B_1 y B_2, deoxinivalenol (DON), y patulina (PAT). Esta normativa ha sido modificada posteriormente por los siguientes Reglamentos:

- Reglamento (CE) Nº 1126/2007 que establece límites para las micotoxinas de *Fusarium* en maíz y productos derivados.
- Reglamento (CE) Nº 165/2010 y (CE) Nº 1058/2012 en lo que respecta al contenido máximo de AFs.
- Reglamento (CE) Nº 105/2010 y (CE) Nº 594/2012, concerniendo a los contenidos máximos de OTA.
- Reglamento (CE) Nº 212/2014 en lo que concierne a los contenidos máximos de citrinina en complementos alimenticios.

Actualmente la Comisión Europea está estudiando la posibilidad de fijar contenidos máximos para las toxinas T-2 y HT-2, habiéndose establecido en el año 2013 la Recomendación 2013/165/UE, en la cual se fijaron diferentes niveles indicativos para las toxinas HT-2 y T-2 en cereales no elaborados y en productos a base de cereales para consumo humano y animal.

Además, la Unión Europea ha establecido recomendaciones para la prevención y reducción de micotoxinas de *Fusarium* en cereales y productos a base de cereales (Recomendación 2006/583/CE), para la prevención y reducción de la contaminación por PAT en zumo de manzana (Recomendación 2003/598) y sobre el control de la presencia de alcaloides de cornezuelo en piensos y alimentos (Recomendación 2012/154/UE).

1.2. Micotoxinas emergentes 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 (SCF, 2002). Los TCs, FBs y ZEA son las micotoxinas más comunes producidas por diferentes especies de *Fusarium*. El género *Fusarium* es también responsable de producir otro grupo de toxinas menos comunes, llamadas micotoxinas emergentes de *Fusarium*, que incluyen el grupo de las eniatinas (ENs), beauvericina (BEA), fusaproliferina (FUS) y moniliformina (MON). Estas micotoxinas son compuestos de creciente interés en la actualidad debido a su posible impacto negativo en la salud humana y animal.

1.2.1. Hongos productores

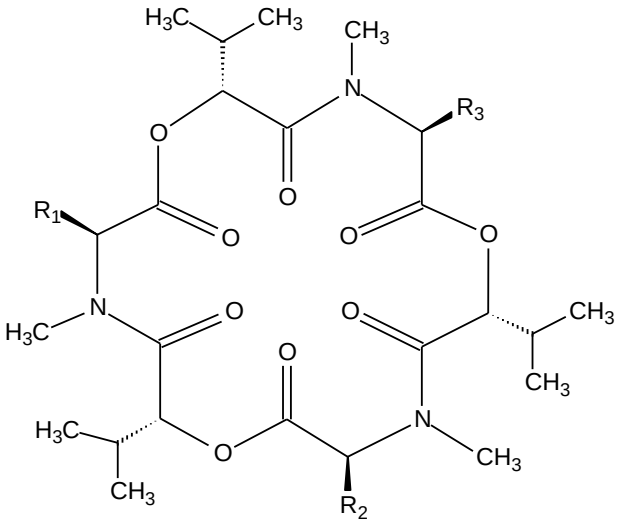
Entre las especies productoras de ENs y BEA, destacan *F. proliferatum, F. subglutinans, F. poae, F. avenaceum, F. oxysporum, F. sambucinum*, *F. tricinctum* y *F. vertillioides* (Herrmann et al., 1996; Logrieco et al., 1998; Shephard et al., 1999; Logrieco et al., 2002; Srobarova et al., 2002). La FUS fue aislada por primera vez de un cultivo de *F. proliferatum*, habiéndose comprobado posteriormente que también podía ser producida por *F. subglutinans, F. globosum* y *F. vertillioides*, entre otras especies productoras (Randazzo et al., 1993; Logrieco et al., 1996; Shephard et al., 1999; Srobarova et al., 2002). Estas especies del género *Fusarium* pueden proliferar en zonas de todo el mundo con diferentes características climáticas, tanto templadas como en zonas tropicales y subtropicales, habiendo sido aisladas en diferentes sustratos tales como los cereales, algodón, caña de azúcar, orquídeas, higos y suelos (Cabañes et al., 2007).

1.2.2. Estructura y propiedades químicas

El grupo de las ENs y la BEA se caracteriza por presentar una estructura de depsipéptido cíclico (Figura 3). Actualmente se conocen al menos 29 análogos de las ENs, los cuales se pueden agrupar en diferentes series en función de los grupos funcionales presentes en su estructura: eniatinas de tipo A, B, C, D, E, F, G, H, I, J, K, L, M, N, O y P (Uhlig et al., 2009; Sy-Cordero et al., 2012). De todas ellas, únicamente siete ENs se han encontrado en productos alimenticios de forma natural. La eniatina A, eniatina A_1, eniatina B y eniatina B_1 (ENA, ENA_1, ENB y ENB_1, respectivamente) son las de mayor interés por presentar una mayor incidencia en los alimentos, mientras que las ENs B_2, B_3 y B_4 solo se han detectado en algunos alimentos de manera excepcional (Tittlemier et al., 2013). En la Figura 3, se muestra la estructura química de la BEA, ENA, ENA_1, ENB y ENB_1. Como se puede observar en esta figura, presentan una estructura de hexadepsipéptido cíclico compuesto por tres aminoácidos alternos con ácido 2-hidroxi-3-metilbutanoico, unidos por enlaces peptídicos y enlaces éster intramoleculares. Los 5 compuestos únicamente se diferencian por el tipo de residuo de ácido N-metilamino: la BEA presenta tres residuos de N-metil-fenilalanina (Ph), la ENA tres residuos de N-metil-isoleucina (Ile), la ENA_1 presenta dos Ile y un residuo de N-metil-valina (Val), la ENB contiene tres Val, y la ENB_1 dos Val y una Ile.

En general, la BEA y las ENs se pueden considerar compuestos lipófilos, debido a los grupos apolares (grupos fenil, *sec*-butil, *iso*-propil y metil) que están orientados hacia la superficie externa con respecto al plano de la molécula, pero también presentan un cierto carácter hidrófilo debido a los grupos amida y carbonil éster orientados hacia el centro de la molécula (Khaled & Davies, 1982). Tanto los átomos de oxígeno de los grupos carbonilo, como los átomos

de nitrógeno de las amidas encadenadas, poseen pares de electrones libres que pueden actuar como nucleófilos, dando lugar a interacciones químicas débiles con cationes (ión-dipolo). Debido a ello, tanto la BEA como las ENs, son capaces de formar complejos estables con cationes metálicos o con moléculas cargadas o neutras (Hilgenfeld & Saenger, 1982; Krska et al., 1996). En la Tabla 2 se muestran algunas de las propiedades químicas de estas micotoxinas.



	R_1	R_2	R_3
BEA	fenilmetil	fenilmetil	fenilmetil
ENA	*sec*-butil	*sec*-butil	*sec*-butil
ENA$_1$	*sec*-butil	*sec*-butil	*iso*-propil
ENB	*iso*-propil	*iso*-propil	*iso*-propil
ENB$_1$	*iso*-propil	*iso*-propil	*sec*-butil

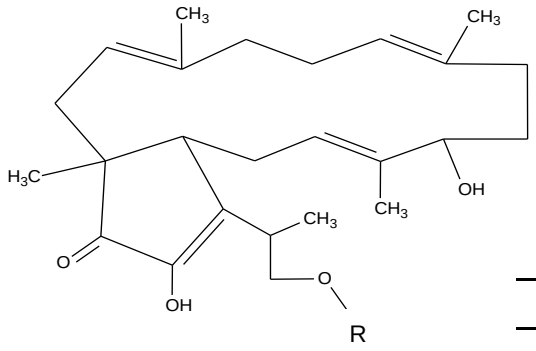
Figura 3. Estructura química de la BEA, ENA, ENA$_1$, ENB y ENB$_1$.

Tabla 2. Fórmula empírica, peso molecular y punto de fusión de las ENs, BEA y FUS (Jestoi, 2008).

Micotoxina	Fórmula empírica	Peso molecular (g/mol)	Punto de fusión (ºC)
ENA	$C_{36}H_{63}N_3O_9$	681.46	121-122
ENA_1	$C_{35}H_{61}N_3O_9$	667.44	66-67
ENB	$C_{33}H_{57}N_3O_9$	639.41	172-174
ENB_1	$C_{34}H_{59}N_3O_9$	654.42	- [(1)]
BEA	$C_{45}H_{57}N_3O_9$	783.00	93-97
FUS	$C_{27}H_{40}O_5$	444.00	142-147

[(1)] Dato no disponible

En la figura 4 se muestra la estructura de la FUS. La FUS es un sesterterpeno bicíclico compuesto por cinco unidades isoprénicas. La molécula presenta una naturaleza bipolar: los grupos hidroxilo, carbonilo y éster crean un entorno hidrófilo, mientras que los cuatro sustituyentes metilo posicionados en el plano opuesto del anillo, le confieren un entorno hidrofóbico. La molécula presenta una mayor estabilidad en condiciones básicas (pH=9) que en ácidas (pH=4). A temperatura ambiente (25ºC) se puede producir la degradación de la FUS para dar lugar mayoritariamente a la deacetil-fusaproliferina (deacetil-FUS) (figura 4), la cual presenta una toxicidad limitada en comparación con la FUS. La deacetilación de la FUS está favorecida en condiciones de humedad (Ritieni et al., 1999), mientras que el almacenamiento a bajas temperaturas es suficiente para que no se produzca (Wu et al., 2003).



	R
FUS	CH_3CO-
Deacetil-FUS	H-

Figura 4. Estructura química de la FUS y de la deacetil-FUS.

1.2.3. Toxicidad

Las ENs y la BEA presentan un amplio rango de actividades biológicas, habiéndose descrito una serie de propiedades antimicrobianas, insecticidas y herbicidas, así como actividad antibiótica sobre organismos patógenos (Klaric et al., 2010). Se ha comprobado que la BEA en combinación con miconazol y ketoconazol, presenta efectos sinérgicos antifúngicos (Fukuda et al., 2004). También se ha demostrado que esta micotoxina presenta una actividad fitotóxica en protoplastos de tomate (Paciolla et al., 2004) y raíces de maíz (Pavlovkin et al., 2006). Algunos estudios han afirmado que la FUS presenta propiedades fitotóxicas, pudiendo disminuir el contenido de clorofila en maíz (Nabudinska et al., 2003). Por otra parte, se ha demostrado que la FUS no presenta actividad antibacteriana frente a *Escherichia Coli* y *Saccharomyces Aureus* (Meca et al., 2009).

Diversos estudios de toxicidad *in vivo* e *in vitro* han indicado que las micotoxinas emergentes de *Fusarium* pueden causar una serie de efectos adversos.

Los estudios de toxicidad *in vivo* son muy limitados: únicamente existe un trabajo que evaluó la toxicidad aguda de la BEA en ratones, siendo la Dosis Letal 50 (DL_{50}) de 100 mg/Kg de peso corporal (Kg pc) por vía oral, y de 10 mg/Kg por vía intraperitoneal (Omura et al., 1991). En la mayoría de estudios donde se ha evaluado la toxicidad aguda de las ENs, se administró una mezcla de ENA, ENA_1, ENB y ENB_1. McKee et al. (1997) observaron una reducción de peso y la muerte de las ratas después de 2-5 días de administración por vía intraperitoneal de 10-40 mg de ENs/Kg pc. En un estudio más reciente, se evaluó la toxicidad de la ENA individualmente: un pienso contaminado por ENA fue suministrado a 5 ratas Wistar en una dosis diaria de 20.91 mg ENA/Kg pc durante 28 días, no observándose efectos adversos en las ratas tratadas (Manyes et al., 2014). Hasta el momento no existen estudios disponibles que evalúen los efectos de toxicidad crónica y subcrónica *in vivo*.

El número de estudios de toxicidad *in vitro* es considerablemente más amplio, habiéndose demostrado la acción citotóxica de las ENs y la BEA en diferentes líneas celulares, incluyendo células de ovario (CHO-K1) y células pulmonares (V79) de hámster chino (Behm et al., 2012; Lu et al., 2013), células epiteliales PK15 de riñón de cerdo (Klaric et al., 2006), células SF-9 de lepidópteros *Spodoptera frugiperda* (Fornelli et al., 2004), líneas celulares de macrófagos murinos RAW 264.7 (Gammelsrud et al., 2012) y células epiteliales Vero de riñón de mono (Ruiz et al., 2011). En cuanto a las líneas celulares de humanos, se ha indicado que las micotoxinas emergentes pueden causar citotoxicidad en células dendríticas, en células de colon Caco-2 y HT-29, y en la

línea celular de hígado humano Hep-G2 (Meca et al., 2011; Juan-García et al., 2013; Ficheux et al., 2013). En estos estudios *in vitro* se demostró que las ENs y la BEA pueden causar immunotoxicidad, mielotoxicidad y hematotoxicidad. En la mayoría se obtuvieron valores de IC_{50} (50% concentración inhibitoria) a bajos rangos de concentración (1.4-30 μM).

La BEA podría presentar un potencial efecto genotóxico, de acuerdo con diversos trabajos en los que se trataron linfocitos y leucocitos humanos, y células epiteliales de cerdo PK15 con esta micotoxina (Klaric et al., 2010; Celik et al., 2010).

Según Ivanova et al. (2006), la citotoxicidad de las ENs frente a hepatocitos de carcinoma humano HepG2 podría ser comparable a la evidenciada para el DON en un ensayo basado en medir la síntesis del ADN.

Aunque los diferentes autores han indicado una serie de efectos adversos ocasionados por estas micotoxinas de forma individual, es importante considerar las posibles interacciones que varias micotoxinas pueden ocasionar cuando están presentes en un mismo alimento, pudiendo dar lugar a un efecto sinérgico, aditivo o antagónico. En este sentido, diferentes estudios de toxicidad *in vitro* se han llevado a cabo para evaluar el efecto tóxico resultante de la combinación de varias micotoxinas. Recientemente, Prosperini et al. (2014) evaluaron el efecto que diferentes interacciones entre las ENs A, A_1, B and B_1, podrían ocasionar en la viabilidad de las células Caco-2 tras 24h de exposición. Los resultados obtenidos mostraron que las interacciones producidas por diferentes combinaciones binarias, ternarias y cuaternarias de estas ENs, pueden dar lugar a un efecto aditivo en la viabilidad celular. Únicamente la mezcla de ENB y ENB_1 dio lugar a un efecto antagónico. Resultados similares fueron obtenidos por Lu et al. (2013) en un estudio

desarrollado con células CHO-K1. Los autores observaron un efecto sinérgico o aditivo en la mayoría de los casos, con la excepción de las mezclas de las ENA+ENA_1+ENB_1 y de ENA_1+ENB+ENB_1, en las cuales se observó un efecto antagónico. Klaric et al. (2008) demostraron que diferentes combinaciones de BEA, OTA y FB1 dan lugar a efectos aditivos o sinérgicos sobre la actividad de la lactato deshidrogenasa y de la caspasa-3, y sobre el índice de apoptosis en células epiteliales de riñón de cerdo PK15.

Debido a los efectos tóxicos descritos, en los últimos años diferentes estudios han sido enfocados en la reducción del potencial citotóxico de estas micotoxinas mediante la adición de sustancias de origen natural. Según el estudio realizado por Lombardi et al. (2012), diferentes polifenoles como quercetina, rutina o miricetina, presentan un efecto citoprotector en las células CHO-K1 cuando se exponen a la presencia de ENs. Asimismo, se ha estudiado que el resveratrol puede disminuir el potencial riesgo toxicológico de la BEA en células CHO-K1, cuando ambos compuestos se encuentran en combinación (Mallebrera et al., 2015).

Por lo que respecta a la toxicidad de la FUS, existen limitados estudios que han evaluado su toxicidad. En el año 1997 se demostró que puede causar teratogenicidad así como una serie de efectos patógenos en embriones de pollo (Ritieni et al., 1997). En el año 2004, Fornelli et al. obtuvieron valores de IC_{50} y de CC_{50} (50% concentración citotóxica) superiores a 100 μM en células de insecto SF-9. Asimismo, se ha observado que es tóxica para *Artemia salina L. larvae* y para linfocitos B humanos IARC/LCL 171 (Jestoi, 2008). Sin embargo, los estudios realizados empleando líneas celulares de humanos (Caco-2 y HT-29), han indicado que la FUS no presenta efectos citotóxicos a las dosis ensayadas (Prosperini et al., 2012).

1.2.4. Mecanismo de acción

Las ENs y la BEA presentan acciones toxicodinámicas muy parecidas debido a su similitud estructural. Debido a sus propiedades ionóforas, son capaces de formar complejos estables tipo sándwich con iones de metales alcalinos, pudiendo transferir cationes monovalentes (K^+, Na^+) y divalentes (Ca^{+2}) a las membranas celulares (Kamyar et al., 2004; Tonshin et al., 2010). La formación de estos complejos da lugar a un aumento de la permeabilidad de las membranas biológicas y a la formación de canales catión-selectivos en las membranas lipídicas, los cuales pueden afectar a la homeostasis celular y al desacoplamiento de la fosforilación oxidativa. Asimismo, se ha demostrado que estas toxinas pueden causar daños del ADN, e inducción de la apoptosis y necrosis dando lugar a la muerte celular (Ruiz et al., 2011; Manyes et al., 2014).

Estudios recientes han evidenciado que el mecanismo de acción de las ENs y la BEA, podría ser mucho más complejo, ya que pueden actuar como inhibidores enzimáticos. Las ENs y la BEA presentan efectos inhibitorios en la actividad del colesterol aciltransferasa, así como en la actividad catalítica de las topoisomerasas, y de la enzima 3´,5´-nucleótido cíclico fosfodiesterasa (Escrivá et al., 2015).

La citotoxicidad de las ENs y la BEA también es debida a la generación de ROS (Reactive Oxygen Species) de forma dosis-dependiente en células Caco-2, que podría ser consecuencia de alteraciones mitocondriales, como la disminución del potencial de membrana mitocondrial (Prosperini et al., 2013a, Prosperini et al. 2013b). Existen estudios que han relacionado la generación de ROS con un incremento de la peroxidación lipídica y una disminución del nivel de glutatión (Mallebrera et al., 2014).

Respecto al mecanismo de acción de la FUS, diferentes estudios han evidenciado que los efectos teratógenos de la FUS en embriones de pollo, en parte son debidos a las interacciones no covalentes entre la FUS y las cadenas dobles y simples de los oligonucleótidos de ADN, con una relación estequiométrica 1:1 (Ritieni et al., 1997; Pocsfalvi et al., 2000).

1.2.5. *Toxicocinética*

Los estudios *in vitro* desarrollados hasta el momento han indicado que las ENs y la BEA se absorben y metabolizan rápidamente dando lugar a una serie de metabolitos, la mayoría de ellos sin caracterizar. Únicamente se han identificado 12 metabolitos de la fase I de metabolismo de la ENB en dos estudios realizados a partir de microsomas hepáticos de humanos, de rata y de perro (Fæste et al., 2011; Ivanova et al., 2011). Recientemente, Ivanova et al. (2014) identificaron un nuevo metabolito de la ENB en un estudio realizado con microsomas hepáticos de pollos. Los autores obervaron que el nuevo metabolito no se formó en las incubaciones de microsomas humanos, concluyendo así que la ENB presenta diferentes rutas de biotransformación dependiendo de la especie tratada.

Diferentes estudios de bioaccesibilidad *in vitro* mediante simulación de la digestión gastrointestinal, han sido llevados a cabo para evaluar la fracción de compuesto ingerido que está disponible para la absorción intestinal en el organismo. En estos estudios se ha evaluado la adición de diferentes sustancias como fibras, compuestos prebióticos o bacterias probióticas en la bioaccesibilidad de las micotoxinas. Mallebrera et al. (2013) evaluaron la bioaccesibilidad de la BEA en un sistema modelo, obteniendo porcentajes de reducción en el tracto gastrointestinal con respecto a la cantidad inicial, del 1.9-27% en presencia de fibras, del 60-80% con prebióticos, y del 30-85% en

presencia de bacterias probióticas. La bioaccesibilidad de las ENs fue evaluada por Meca et al. (2012a), obteniendo una fracción disponible del 10-60% en pan tostado. Asimismo, se han realizado algunos estudios para evaluar el transporte de las ENs y de la BEA a través de las monocapa celular en células Caco-2. El transporte de la BEA a través de la capa monocelular se estimó en un 25% y 52% después de 2 y 4 horas, respectivamente. El transporte de las ENs alcanzó porcentajes del 20-42% (2h) y 55-70% (4h), dependiendo del tiempo de incubación (Prosperini et al., 2012).

En cuanto a los estudios toxicocinéticos realizados *in vivo*, existen varios grupos que han desarrollado estudios en animales de experimentación y de granja. En un estudio piloto llevado a cabo por Devreese et al. (2013), la absorción oral de las ENs y la BEA fue estudiada en un cerdo, mediante el análisis de muestras de plasma a diferentes tiempos tras la administración intragástrica (0.05 mg/Kg pc). Las concentraciones más elevadas en plasma fueron detectadas 20-30 min después de la administración, mientras que niveles de trazas se detectaron tras 40 min, mostrando una rápida eliminación de estas micotoxinas. Según los autores, la BEA presentó la menor biodisponibilidad oral, siendo necesaria una mayor dosis para cuantificar la micotoxina en plasma. Se observó una gran diferencia en la absorción oral entre los diferentes análogos de las ENs, a pesar de que presentan una estructura química similar. La ENB presentó la mayor absorción oral (concentración máxima 73.4 ng/mL), seguida de la ENB_1, ENA_1 y ENA (concentración máxima 35.2, 11.6, y 6.8 ng/mL, respectivamente). La rápida eliminación de las ENs fue comparable con la obtenida para el DON en estudios similares.

En un trabajo posterior del mismo grupo, se llevó a cabo un estudio toxicocinético y de biodisponibilidad oral absoluta de la ENB_1 (0.05 mg/Kg

pc) en 5 cerdos. Tanto con la administración oral como con la intravenosa, la absorción de la ENB_1 fue rápida. Asimismo, fue rápidamente distribuida y eliminada, con un porcentaje de biodisponibilidad oral absoluta del 90.9% (Devreese et al., 2014).

Ivanova et al. (2014) realizaron dos estudios suministrando pienso contaminado por ENB a dosis de 12.7 mg/Kg en pollos, y de 11.2 mg/Kg en gallinas por vía basal durante 14 días. La ENB fue detectada en concentraciones de 3.6-7.0 ng/mL, en el 89% de las muestras de suero, y en el 63% de las muestras hígado. Los huevos de las gallinas del período de tratamiento fueron analizados, obteniéndose una incidencia del 100% con una máxima concentración de 19.4 μg de ENB/Kg. Según los autores, este resultado era de esperar debido a la naturaleza lipofílica de la ENB y al elevado contenido en lipoproteínas de los huevos. Asimismo, se detectaron diferentes niveles de concentración de la ENB en las muestras de huevos, hígado y suero.

En un estudio desarrollado en el año 2014, cinco ratas de experimentación fueron alimentadas por pienso contaminado por ENA (465 mg/Kg) durante 28 días, con una ingesta diaria estimada en 20.9 mg de ENA/Kg pc. Tras el estudio, los resultados demostraron la presencia de ENA en los siguientes órganos: hígado (22.7 mg/Kg), colon y duodeno (2.2-2.9 mg/Kg), y líquidos del tracto gastrointestinal (1.3-9.6 mg/Kg). Los niveles de ENA en plasma fueron analizados cada 7 días, obteniéndose los niveles más elevados (4.76 μg/mL) tras 28 días de exposición (Manyes et al., 2014; Juan et al., 2014a).

En general, todos los autores han descrito una rápida excreción de las micotoxinas emergentes de *Fusarium*, así como la formación de metabolitos de fase I de la ENB, existiendo diferencias significativas en cuanto a las rutas de biotransformación dependiendo del tipo de especie de animal tratada.

1.2.6. Presencia en alimentos

En los últimos años diferentes autores han estudiado la incidencia y niveles de concentración de las ENs, BEA y FUS en productos alimenticios procedentes de diferentes países. Estos estudios han puesto de manifiesto que las micotoxinas emergentes de *Fusarium* pueden estar presentes en alimentos procedentes de zonas geográficas con diferentes características climáticas como Sudáfrica, América (Argentina y EEUU), norte de Europa (Reino Unido, Finlandia, Dinamarca y Noruega), este de Europa (República Checa), Asia (China e Irán) y cuenca del Mediterráneo (España, Italia, Túnez y Marruecos) (Santini et al., 2012). Los alimentos con una mayor presencia de micotoxinas emergentes de *Fusarium* son los cereales sin procesar y productos a base de cereales para consumo humano y animal (Jestoi, 2008). En la tabla 3 se muestran los resultados de los estudios más recientes que han evaluado la presencia de FUS en diferentes alimentos. Las tablas 4-6 presentan los niveles de concentración y la incidencia de la FUS, ENs A, A_1, B y B_1, y de la BEA en diferentes cereales y productos derivados, descritos en los últimos 10 años.

Recientes estudios han mostrado que existe un amplio rango de matrices susceptibles de contaminación por ENs y BEA, como los frutos secos, el café y otros productos alimentarios menos comúnmente contaminados por toxinas de *Fusarium* (Tabla 7) (Tolosa et al., 2013; Hu y Rychlik, 2014; García-Moraleja et al., 2015). Asimismo, las ENs y la BEA han sido detectadas en productos de origen animal destinado a consumo humano (pescado, carne y huevos) procedentes de animales alimentados con piensos contaminados (Tabla 8) (Jestoi et al., 2007; Jestoi et al., 2009; Tolosa et al., 2014).

Los trabajos disponibles hasta el momento han evidenciado una elevada incidencia de las ENs y BEA en la mayoría de alimentos, habiéndose detectado

niveles de concentración muy elevados en cereales (procesados y sin procesar) procedentes de países caracterizados por un clima templado. Por lo general, las ENs tipo B presentan una mayor incidencia que de las ENs tipo A y la BEA. La FUS está presente ocasionalmente en alguno de los alimentos analizados, mostrando bajos porcentajes de incidencia en la mayoría de estudios (Tabla 3). Las máximas concentraciones de ENB_1, BEA y FUS se detectaron en muestras procedentes de Marruecos, con niveles de 795 mg/Kg de ENB_1 en una muestra de cereales para el desayuno (Tabla 5), 590 mg/Kg de BEA en una muestra de maíz en grano (Tabla 4), y 19.6 mg/Kg de FUS en una muestra de arroz (Tabla 3). La máxima concentración de ENA_1 fue de 814.42 mg/Kg en una muestra de arroz procedente de España (Tabla 4). Las máximas concentraciones de ENA y ENB se detectaron en una muestra de harina de trigo procedente de Túnez (121 mg/Kg y 295 mg/Kg, respectivamente) (Tabla 5).

En cuanto a la co-ocurrencia de varias micotoxinas en un mismo alimento, por lo general se ha observado la presencia conjunta de las micotoxinas emergentes de *Fusarium* en un mismo alimento. En la actualidad, las ENs y la BEA se han empezado a incluir en estudios multimicotoxina. En estos estudios se ha evidenciado la presencia simultánea de las ENs y la BEA con otras micotoxinas de *Fusarium*, como las FBs o el nivalenol (NIV), y con micotoxinas modificadas, como el deoxinivalenol-3-glucósido (Tansakul et al., 2013; Streit et al., 2013; García-Moraleja et al., 2015). Sin embargo, los estudios sobre la FUS han disminuido considerablemente en los últimos años, probablemente debido a los bajos porcentajes de incidencia descritos en los alimentos y a la dificultad de obtener estándares para realizar los estudios.

Tabla 3. Presencia y rangos de concentración de la FUS en alimentos.

Origen alimento	Alimento	Incidencia (n/N) [1]	Rango (µg/kg) [2]	Referencia
Eslovaquia	Maíz	8/22	<25 – 8200	Srobarova et al., 2002
Finlandia	Trigo, cebada, avena y centeno	0/38	nd	Jestoi et al., 2004a
	Productos a base de cereales	0/18	nd	Jestoi et al., 2004b
Italia	Productos a base de cereales	0/12	nd	Jestoi et al., 2004b
España	Trigo	3/21	3120 [3]	Meca et al., 2010
	Maíz	1/28	2470 [3]	Meca et al., 2010
	Cebada	0/4	nd	Meca et al., 2010
Túnez	Cereales en grano y productos a base de cereales	0/51	nd	Oueslati et al., 2011
Marruecos	Cereales para el desayuno	7/68	500 - 7400	Mahnine et al., 2011
	Trigo	4/25	2000 [3]	Zinedine et al., 2011
	Maíz	1/31	600 [3]	Zinedine et al., 2011
	Cebada	0/8	nd	Zinedine et al., 2011
	Arroz	3/70	200 - 19600	Sifou et al., 2011

[1] I: incidencia, n: nº muetras positivas, N: nº muestras totales

[2] nd: no se detecta

[3] Media (µg/kg)

Tabla 4. Presencia y rangos de concentración de ENs y BEA en cereales sin procesar.

Cereal	Origen	ENA		ENA_1		ENB		ENB_1		BEA		Referencia
		I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	
Trigo	Noruega	23/80	<3-30	62/80	<4-190	80/80	<3-5800	78/80	<3-1600	10/80	<3-8	Uhlig etal.2006
	Finlandia	4/9	1-29	7/9	4-49	9/9	3.8-1920	9/9	10.8-399	8/9	nd-<10	Yli et al. 2006
	Suecia (4)	123/125	nd-4281	-	-	-	-	-	-	63/125	nd-12	Lindblad et al. 2013
	Italia	6/43	3.1-18.1	11/43	4.5-40.4	18/43	3.1-87.2	11/43	1.5-69.8	6/43	1.8-5.1	Alkadri et al. 2014
	Siria	4/40	1.5-2.2	4/40	0.6-2.1	1/40	nd-0.9	0/40	nd	5/40	1.5-1.7	
	Marruecos	11/80	49(3)	14/80	74(3)	49/80	93(3)	42/80	51(3)	8/80	9.4	Blesa et al.2014
Maíz	Burkina Faso y Mozambique	0/13	nd	2/13	0.1-0.1	0/13	nd	1/13	0.1	11/13	0.1-35.6	Warth et al. 2012
	Alemania	-	-	-	-	28/80	115 (3)	-	-	34/80	315 (3)	Goertz et al.2010
	Sudáfrica	0/42	nd	0/42	nd	0/42	nd	0/42	nd	3/42	<LOQ	Hickert et al. 2015
Cebada	Finlandia	12/29	1-7	25/29	<4-81	29/29	<3.8-614	29/29	10.8-338	27/29	nd-<10	Yli et al. 2006
	Noruega	23/75	<3-59	67/75	<4-500	75/75	<3-3200	75/75	<3-1900	14/75	<3-21	Uhlig et al.2006
	República Checa	28/28	5.96-12.36	28/28	3.6-174.3	28/28	14.5-1416	28/28	5.56-476.2	28/28	5.13-47.91	Bolechova et al., 2015
Avena	Noruega	12/73	<3-17	24/73	<4-75	73/73	<3-190	61/73	<3-270	49/73	<3-120	Uhlig et al.2006
	Suecia (4)	93/93	nd-862	-	-	-	-	-	-	91/93	nd-327	Fredlund et al. 2013
Maíz, trigo y cebada	Marruecos	1/64	nd-34000	25/64	14000-445000	14/64	5000-100000	4/64	8000-32000	17/64	1000-59000	Zinedine et al. 2011
Trigo, maíz, y arroz	España	0/64	nd	47/64	33380-814420	5/64	2230-21370	3/64	4340-45940	21/64	510-11780	Meca et al.2010
Sorgo, arroz, trigo y sésamo	Burkina Faso y Mozambique	2/7	0.2-2.0	2/7	0.2-4.1	1/7	nd-0.9	1/7	nd-4.1	7/7	3.5-486	Warth et al. 2012

(1) I: incidencia, n: nº muetras positivas, N: nº muestras totales

(2) nd: no se detecta, -: dato no disponible

(3) Media (µg/kg)

(4) Los datos de la ENA son referidos a la suma de las ENs A, A_1, B, B_1, B_2 y B_3

Tabla 5. Presencia y rangos de concentración de ENs y BEA en productos a base de cereales para consumo humano.

Producto	Origen	ENA		ENA_1		ENB		ENB_1		BEA		Referencia
		I (n/N) [1]	Rango (μg/kg) [2]	I (n/N) [1]	Rango (μg/kg) [2]	I (n/N) [1]	Rango (μg/kg) [2]	I (n/N) [1]	Rango (μg/kg) [2]	I (n/N) [1]	Rango (μg/kg) [2]	
Cereales desayuno	Marruecos	2/68	10100-29700	21/68	37500-688000	9/68	1000-89100	12/68	600-795000	4/68	nd-2000	Mahnine et al., 2011
	República Checa	7/7	36-278	2/7	18-410	7/7	18-941	6/7	10-785	-	-	Malachova et al., 2011
	Túnez	0/4	nd	4/4	156.1 [3]	4/4	57.4 [3]	4/4	25.1 [3]	0/4	nd	Oueslati et al., 2011
Snacks	República Checa	34/34	20-65	5/34	24-61	23-34	13-240	18/34	8-106	-	-	Malachova et al., 2011
Harina	República Checa	21/22	27-2532	16/22	7-100	21/22	26-256	21/22	7-71	-	-	Malachova et al., 2011
Pasta	Italia	1/25	<LOQ-8	1/25	< LOQ-9	11/25	<LOQ-106	1/25	<LOQ-4	0/25	nd	Juan et al., 2013
Harina, pasta y cous cous	Túnez	14/51	19600-121000	47/51	11100-480000	35/51	1500-295000	20/51	4800-120000	0/51	nd	Oueslati et al., 2011
Cereales desayuno, pan y harina	Portugal	10/61	2.6-71	32/61	3.4-789	30/61	1.6-491	27/61	2.8-369	1/61	nd-3.2	Blesa et al., 2012
Papillas	Italia	0/75	nd	3/75	6.58	10/75	101.30	1/75	7.80	1/75	1.18 [3]	Juan et al. 2014b
Arroz para consumo humano	Iran	0/65	0.06 [3]	5/65	nd	0/65	nd	0/65	nd	26/65	nd-0.47	Nazari et al., 2015
	Marruecos	16/70	8400-119500	4/70	56200-448700	21/70	4400-36200	17/70	3600-23700	53/70	3.8-26.3	Sifou et al. 2011
Galletas	Italia	2/20	<LOQ	2/20	<LOQ	2/20	<LOQ	2/20	<LOQ	-	-	Capriotti et al., 2014

[1] I: incidencia, n: nº muetras positivas, N: nº muestras totales
[2] nd: no se detecta, -: dato no disponible
[3] Media (μg/kg)

Tabla 6. Presencia y rangos de concentración de ENs y BEA en piensos.

Alimento (Origen)	ENA		ENA_1		ENB		ENB_1		BEA		Referencia
	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	
Maíz sin procesar para rumiantes (Dinamarca)	6/100	nd-106	20/100	nd-107	95/100	nd - 2598	64/100	nd-496	55/100	nd-988	Sørensen et al., 2008
Maíz sin procesar (Dinamarca)	-	-	-		7/20 28/99	nd-200 nd-365	-	-	-	-	Rasmussen et al., 2010 Storm et al., 2014
Piensos formulados con cereales y semillas (Austria, Hungría y Dinamarca, América, Australia)	72/83	nd-1745	79/83	nd-2216	76/83	nd-780	76/83	nd-2690	81/83	nd-2326	Streit et al., 2013
Piensos para pollos formulados con maíz y frutos secos (Mozambique y Burkina Faso)	4/10	0.6-7.9	4/10	3.4-43.9	4/10	2.2-114	7/10	0.1-94.4	10/10	3.3-418	Warth et al., 2012
Piensos para animales de granja formulados con cereales (República Checa y Reino Unido)	-/395	0.1-2816	-/395	0.1-400	-/395	0.3-4614	-/395	0.1-845	-/395	0.3-685	Zachariasova et al., 2014
Piensos de piscifactoría formulados con cereales y pescado (España)	20/20	0.6-3.4	20/20	0.3-6.5	20/20	0.1-3.2	20/20	0.15-10	19/20	0.15-10	Tolosa et al., 2014

(1) I: incidencia, n: nº muetras positivas, N: nº muestras totales

(2) nd: no se detecta, -: dato no disponible

Tabla 7. Presencia y rangos de concentración de ENs y BEA en otros alimentos de origen vegetal.

Alimento (Origen)	ENA		ENA_1		ENB		ENB_1		BEA		Referencia
	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	I (n/N) (1)	Rango (µg/kg) (2)	
Chufa (España)	1/47	nd-676.5	8/47	32.2-4440	1/47	nd-44.8	5/47	21.6-346	5/47	51.6-228.5	Sebastià et al., 2012
Cacahuetes (Mozambique y Burkina Faso)	0/23	nd	0/23	nd	0/23	nd	1/23	nd-0.3	16/23	0.1-24.0	Warth et al., 2012
Frutos secos (España)	27/30	39-23300	6/30	7-523	26/30	22-14610	10/30	22-784	12/30	1-42	Tolosa et al. 2013
Frutas deshidratadas (España)	10/28	180-369	13/28	nd-11	15/28	nd-58	1/28	nd-22	7/28	18.2-131.8	Tolosa et al. 2013
	0/14	nd	4/14	2.2-8.1	7/14	18.5-227.7	2/14	30-39.9	2/14	nd-7.0	Azaiez et al., 2014
Café molido e instantáneo (España)	0/6	nd	4/6	0.64-4.82	5/6	0.96-36.14	4/6	0.52-5.33	1/6	nd-0.37	García-Moraleja et al., 2015
Hierbas Medicinales (China)	4/60	<0.8-354.6	4/60	<1.1-252.5	7/60	<1.0-290.5	/60	<1.1-40.2	12/60	<1.2-124.8	Hu & Rychilk, 2014

(1) I: incidencia, n: nº muetras positivas, N: nº muestras totales

(2) nd: no se detecta, -: dato no disponible

Tabla 8. Presencia y niveles de concentración de ENs y BEA en productos de origen animal.

Alimento (Origen)	ENA		ENA_1		ENB		ENB_1		BEA		Referencia
	I (n/N) **(1)**	**Rango** (µg/kg) (2)	**I** (n/N) (1)	**Rango** (µg/kg) (2)	**I** (n/N) **(1)**	**Rango** (µg/kg) **(2)**	**I** (n/N) **(1)**	**Rango** (µg/kg) (2)	**I** (n/N) **(1)**	**Rango** (µg/kg) **(2)**	
Carne e hígado de pollo y pavo (Finlandia)	1/319	nd-<LOQ	2/319	nd-<LOQ	9/319	nd-2.0	12/319	nd-<LOQ	7/319	nd-<LOQ	Jestoi et al., 2007
Huevos (Finlandia)	4/479	nd-1.3	7/479	nd-7.5	66/479	nd-7.0	254/479	nd-3.8	66/479	nd-7.0	Jestoi et al., 2009
Huevos (Italia)	0/27	nd	0/27	nd	0/27	nd	0/27	nd	0/27	nd	Capriotti et al., 2012a
Pescado (España)	0/20	nd	8/20	1.7-7.5	13/20	1.3-44.6	10/20	1.4-31.5	0/20	nd	Tolosa et al., 2014

(1) I: incidencia, n: nº muetras positivas, N: nº muestras totales

(2) nd: no se detecta, -: dato no disponible

1.3. Prevención y reducción de la contaminación por micotoxinas

La aplicación de diferentes estrategias de prevención y de reducción de los niveles de micotoxinas, junto con el cumplimiento de las diferentes normativas que fijan contenidos máximos de estos tóxicos, es en la actualidad, la forma más factible de gestionar y controlar la contaminación por micotoxinas en productos alimenticios.

1.3.1. *Medidas de prevención*

La prevención se basa en la aplicación de diferentes sistemas preventivos. El Sistema de Análisis de Peligros y de Puntos Críticos de Control (APPCC) constituye un sistema de gestión de la inocuidad de los alimentos con el cual se pretende garantizar la seguridad de los alimentos. El sistema APPCC está encaminado a identificar, prevenir y controlar los peligros que pueden aparecer en cualquier etapa de la cadena alimentaria (Sanchis et al., 2011). Si el sistema APPCC se aplica de forma adecuada, debería permitir la reducción de los niveles de micotoxinas en materias primas, piensos y alimentos para consumo humano. El sistema APPCC para la prevención de micotoxinas, debe basarse en la aplicación de Buenas Prácticas Agrícolas (BPA), durante el período previo a la cosecha, y de Buenas Prácticas de Fabricación (BPF), durante el sistema de producción y elaboración de los alimentos (FAO/WHO, 2012).

- *Buenas Prácticas Agrícolas*

Las BPA constituyen la primera línea de defensa para la prevención de la contaminación por micotoxinas en el campo. Sin embargo, la mayoría de factores que influyen en la contaminación por micotoxinas en el campo están relacionados con las condiciones climáticas, no pudiendo ser controlados. El

Codex Alimentarius ha establecido un Código de Prácticas con una serie de pautas y recomendaciones para garantizar unas BPA en la prevención y/o reducción de la contaminación por micotoxinas (Codex Alimentarius, CAC/RCP 51-2003). Las prácticas a desarrollar previamente a la cosecha (prevención pre-cosecha) son las siguientes:

- Elaboración de un plan de rotación de cultivos.
- Selección del momento para la plantación de los cultivos.
- Selección de variedades vegetales resistentes y selección de semillas modificadas genéticamente para resistir al desarrollo de los hongos micotoxigénicos y a las plagas de insectos.
- El control del suministro de agua a través del sistema de riego
- El uso de fertilizantes y/o acondicionadores del suelo para garantizar un pH adecuado.
- El empleo de fungicidas con capacidad inhibitoria de los hongos productores de micotoxinas.
- La prevención de la infesta de insectos, malas hierbas y plagas mediante el uso de agentes químicos registrados (insecticidas, herbicidas, etc.), también puede ser una forma indirecta de controlar la presencia de hongos y por tanto, la aparición de micotoxinas.

Durante la cosecha se recomiendan las siguientes prácticas:

- La selección cuidadosa del momento de realizar la cosecha para recolectar los cereales, comprobando que el contenido de humedad es adecuado (inferior al 15%).
- La selección de la maquinaria adecuada de recolección para no causar daño físico al grano o reducir al mínimo los daños mecánicos, y evitar el contacto del cereal con el suelo.

- La selección de contenedores adecuados para el transporte de la materia prima, garantizando que estén limpios, secos, libres de insectos y de proliferación fúngica visible.

Las prácticas para la prevención de la proliferación de hongos y producción de micotoxinas tras la cosecha (prevención post-cosecha) comprenden:

- La determinación de los niveles de humedad de los cereales cosechados, y en el caso de presentar un contenido en humedad superior al 15%, la aplicación de una etapa de secado previa al almacenamiento para evitar la proliferación de los hongos.
- La aplicación de procesos de limpieza de los granos, mediante la eliminación de materias extrañas, así como de los granos dañados o deteriorados previamente al almacenamiento.
- El control en todo momento de la temperatura (< 20ºC), del aire de circulación y de los niveles de humedad (a_w<0.7) de las instalaciones. El empleo de atmósferas modificadas o gases alternativos como el dióxido de carbono, el nitrógeno, el monóxido de carbono o el dióxido de azufre, puede prevenir el desarrollo de mohos micotoxigénicos durante al almacenamiento.
- La adopción de buenos procedimientos de limpieza para reducir la presencia de hongos e insectos en las instalaciones del almacenamiento, mediante la utilización de sustancias adecuadas.

Con el fin de mejorar las BPA, es aconsejable documentar los procedimientos de plantación, recolección, trasporte y almacenamiento de cada temporada, para poder explicar las posibles causas de la proliferación de los hongos y la producción de micotoxinas en determinadas condiciones, evitando

así, los errores cometidos en futuras prácticas (Codex alimentarius, CAC/RCP 51-2003).

- *Buenas Prácticas de Fabricación*

La aplicación de las BPF engloba los procesos de producción y elaboración del alimento, el almacenamiento y la distribución de los alimentos destinados al consumo humano y animal. El principal objetivo de las BPF es evitar que estos procesos sean una fuente de contaminación por micotoxinas. Las micotoxinas son peligros químicos, pero de origen biológico, por lo que las medidas empleadas en las BPF también son aplicables para otros peligros de origen químico y biológico. Según Sanchis et al. (2011), las BPF comprenden el desarrollo de diferentes procedimientos para un adecuado mantenimiento, limpieza y desinfección de las instalaciones, para el control de plagas, así como el desarrollo de un plan de trazabilidad. Las BPF tambié comprenden el establecimiento de un plan de formación y evaluación de los trabajadores, incluyendo el cuadro de incompatibilidades para evitar contaminación cruzada, y el desarrollo de un plan de homologación de proveedores.

1.3.2. Estrategias de reducción

En ocasiones las medidas de prevención no logran detener el desarrollo fúngico y la producción de micotoxinas. En esta situación es recomendable aplicar procedimientos de descontaminación para reducir los niveles de las toxinas al mínimo. Por lo general, se considera que un proceso de descontaminación es efectivo cuando ha sido aceptado por las agencias regulatorias y cumple la función de reducir la exposición del consumidor a los alimentos contaminados por micotoxinas. Los requisitos generales que deben

de cumplir los métodos de descontaminación son los siguientes (Dalcero et al., 2011):

- Deben de destruir, eliminar o inactivar las micotoxinas, así como las esporas fúngicas y micelios de hongos, para asegurar que no se vuelvan a producir micotoxinas
- No deben producir residuos tóxicos, carcinógenos o mutágenos en el producto final.
- No deben alterar las propiedades tecnológicas del producto final, manteniendo el valor nutritivo, las características organolépticas y asegurando la aceptabilidad del producto.
- Deben de ser económicamente viables para permitir la aplicabilidad a gran escala.
- No deben producir alteraciones en el ambiente resultantes de los tratamientos aplicados.

Los procesos de descontaminación se pueden clasificar en función del tipo de tratamiento aplicado, existiendo métodos físicos, químicos, biológicos, y combinaciones de los mismos.

❖ *Métodos químicos*

Diversos métodos que emplean la adición de agentes químicos, han sido propuestos para lograr una efectiva reducción de los niveles de micotoxinas. El empleo de algunos ácidos y bases, de agentes clorados y de agentes reductores y oxidantes ha permitido minimizar la presencia de micotoxinas (Jard et al., 2011). Sin embargo, la mayoría de estos agentes químicos alteran las características del producto alimenticio, dando lugar a una importante pérdida del valor nutricional y a la formación de diferentes compuestos tóxicos. En

este sentido, de acuerdo con el Artículo 3 del Reglamento (CE) 1881/2006, es de destacar que en la Unión Europea, la detoxificación de micotoxinas mediante la aplicación de tratamientos químicos está prohibida.

En la actualidad existen diferentes alternativas al uso de sustancias sintéticas, habiéndose demostrado que las sustancias de origen natural, como los isotiocianatos o los compuestos fenólicos, pueden presentar un gran potencial en la reducción de diversas micotoxinas. No obstante, los mecanismos de actuación y transformación de estos compuestos son desconocidos, por lo que son necesarios más estudios para poder ser empleados en la industria alimentaria (Azaiez et al., 2013; Pani et al., 2014).

- *Métodos biológicos*

Los métodos biológicos se basan en la adición de un agente biológico, principalmente microorganismos y sus metabolitos, que actúan como agentes de adsorción o de transformación de la micotoxina. El empleo de microorganismos para la detoxificación de alimentos contaminados por micotoxinas, requiere en primer lugar tener conocimiento acerca de su patogenicidad y de su toxicidad. Asimismo, se debe considerar la formación de nuevos metabolitos derivados de la detoxificación microbiana, y de los efectos adversos que puedan producir.

En la literatura se han descrito una serie de estrategias para reducir los contenidos de micotoxinas emergentes de *Fusarium*, que engloban el empleo de enzimas y bacterias. La actividad de enzimas de *Saccharomyces cerevisiae* para degradar la BEA durante el proceso de fabricación del pan y de la cerveza se ha demostrado recientemente por Meca et al. (2013b). Asimismo, se ha

demostrado el potencial de ciertas bacterias acido lácticas para la reducción de las ENs (Roig et al., 2013).

Por lo general, los métodos biológicos presentan el inconveniente de requerir condiciones difícilmente aplicables en la industria alimentaria, comprometiendo en muchas ocasiones la aceptabilidad del producto final (Jard et al., 2011).

❖ *Métodos físicos*

Diversos métodos físicos han sido aplicados para reducir el contenido de micotoxinas. Las técnicas más difundidas son la separación de los granos dañados, la limpieza física de los granos, el empleo de técnicas de extrusión, la inactivación térmica, y la aplicación de diferentes radiaciones (Voss et al., 2008; Dalcero et al., 2011).

La aplicación de diferentes tipos de radiación como rayos X, rayos gamma o radiación UV puede reducir los niveles de ciertas micotoxinas según algunos autores, pero presenta el inconveniente de requerir un elevado coste, por lo que no es viable su aplicación en la industria (Dalcero et al., 2011).

Los tratamientos térmicos constituyen una herramienta efectiva y sencilla para la reducción de micotoxinas. Estos tratamientos son fácilmente adaptables a las diferentes etapas de procesado de los alimentos y piensos, tanto durante los tratamientos tecnológicos como durante las condiciones de cocinado en los hogares. Sin embargo, es importante considerar que algunas micotoxinas son compuestos termoestables, pudiendo persistir inalteradas a las elevadas temperaturas alcanzadas durante el procesado. En la actualidad, diversos autores han estudiado el efecto de los tratamientos tecnológicos en la reducción de los niveles de micotoxinas. El grado de reducción alcanzado

depende del tipo de micotoxina, de las concentraciones en el producto final y del tipo de tratamiento aplicado (Milani y Maleki, 2014).

Se han desarrollado diversos procesos para reducir las concentraciones de micotoxinas, aunque la mayoría de ellos no las pueden eliminar completamente (Bullerman & Bianchini, 2007). Así, los procesos de cocción-extrusión (80-200ºC) de los cereales pueden dar lugar a reducciones superiores al 80% en los contenidos de AFs, FBs y ZEA, y a reducciones del 30-55% en el caso del DON, OTA y MON (Castells et al., 2005).

Las AFs y la OTA son estables al tostado de diferentes alimentos. Las FBs son estables a diversos procesos como el pasteurizado (Kabak, 2008).

El DON es una de las micotoxinas más estudiadas durante el procesado de diversos cereales. La descontaminación por vapor sobrecalentado a temperaturas de hasta 185ºC, da lugar a reducciones de hasta el 50% en los contenidos de DON presente en trigo en grano (Cenkowski et al., 2007).

Diversos estudios se han centrado en la influencia de la fabricación y cocción de la pasta en la concentración de DON. El proceso de elaboración de la pasta no modifica los niveles de DON, mientras que el cocinado repercute más significativamente en los contenidos de DON (reducciones de hasta el 75%). Estos autores han evidenciado que las reducciones de DON alcanzadas en la pasta cocida, no son debidas a las elevadas temperaturas del tratamiento, si no a la transferencia del DON desde la pasta al agua de cocción, debido a su naturaleza hidrofílica (Visconti et al., 2004; Brera et al., 2013; Cano-Sancho et al., 2013).

El efecto de la elaboración del pan también ha sido estudiado por varios autores. La mayoría de los estudios han obtenido reducciones del 5-10% en los contenidos de DON a temperaturas de 170-210ºC (Scudamore et al., 2009;

Numanoglu et al., 2012; Vidal et al., 2014). Asimismo, la elaboración de galletas y de tortas a temperaturas de hasta 280ºC no da lugar a importantes reducciones de DON (Scudamore et al., 2009).

Hasta el momento, escasos estudios han demostrado la influencia del procesado en los niveles de las micotoxinas emergentes de *Fusarium*. Vaclavikova et al. (2013) realizaron un estudio de molienda de trigo convencional y de agricultura orgánica, contaminado de forma natural por ENs tipo B. En este estudio se estimó que alrededor del 75% del contenido inicial de ENs fue eliminado durante este proceso. Los autores explicaron este hecho, debido a la localización de las ENs en las capas externas de los granos de trigo, que son separadas para la obtención de la harina blanca. Resultados similares fueron obtenidos por Hu et al. (2014a), que indicó reducciones del 70-82% en los contenidos de las ENs B y B_1 después de la molienda de granos de trigo y de centeno. Estos autores también evaluaron la influencia del proceso de elaboración del pan a partir de las harinas resultantes de la molienda. Reducciones del 50-60% fueron obtenidas con el tratamiento térmico durante 14min a 240ºC, mientras que las reducciones fueron del 25-41% cuando se aplicó una temperatura de 200ºC durante 25min. Los contenidos de BEA fueron evaluados durante el fabricado del pan por Meca et al. (2013a), obteniendo reducciones del 92-95%.

El proceso de producción de la cerveza también ha sido evaluado en varios estudios, dando lugar a bajas concentraciones de ENs y BEA en el producto final (Meca et al., 2013a; Vaclavikova et al., 2013; Hu et al., 2014b).

❖ *Reducción de la absorción de micotoxinas en el tracto gastrointestinal*

Esta estrategia se basa en la adición de diferentes aditivos en el alimento, llamados secuestrantes o detoxificantes. Se trata de sustancias que pueden suprimir o reducir la absorción de las micotoxinas en el tracto gastrointestinal. Estos métodos presentan el inconveniente de que pueden absorber importantes micronutrientes. No obstante, el uso de ciertos aditivos en piensos está permitido en la Unión Europea. En el Reglamento (CE) Nº 386/2009 de la Comisión, se define a estos aditivos como "nuevos aditivos para piensos que suprimen o reducen la absorción, promueven la excreción de micotoxinas o modifican su modo de acción, mitigando así los posibles efectos nocivos de las micotoxinas en la salud animal". El Reglamento 1060/13 autoriza la adición de bentonita (un tipo de arcilla) a una dosis máxima de 20.000 ppm en los piensos de rumiantes, aves y cerdos, y el Reglamento 1016/13 autoriza la adición de ciertos microorganismos de la familia *Coriobacteriaceae* en los piensos de cerdos. Estos aditivos forman parte de un nuevo grupo funcional de aditivo dentro de la categoría de aditivos tecnológicos.

1.3.3. Productos de degradación y micotoxinas modificadas

Los diferentes tratamientos aplicados para la reducción de micotoxinas así como ciertos mecanismos de detoxificación naturales de algunas plantas, pueden dar lugar a diferentes compuestos de degradación así como a las llamdas micotoxinas modificadas o enmascaradas. Las micotoxinas modificadas se producen como consecuencia de reacciones entre las micotoxinas y diversos componentes de los alimentos, como azúcares, proteínas o lípidos. Por lo general, estos compuestos presentan un comportamiento muy diferente a las micotoxinas de origen, pudiendo

presentar una mayor o menor toxicidad. Un factor a tener en consideración es la posibilidad de que una micotoxina modificada sea ingerida, y posteriormente hidrolizada durante la digestión generando la micotoxina inicial. De esta forma, alimentos que inicialmente no presentaban elevados niveles de micotoxinas, pueden inducir efectos tóxicos acusados debido a la liberación de estos compuestos en el organismo (Broekaert et al., 2015).

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 tóxicos.

1.4. Análisis de micotoxinas

El análisis de micotoxinas en materias primas, piensos, alimentos y matrices biológicas, es un punto crítico de control en el ámbito de la seguridad alimentaria. Con el fin de alcanzar el nivel de protección deseado, se debe de disponer de datos fiables que permitan una adecuada evaluación de riesgos con la consiguiente toma de decisiones. Para ello, son necesarias metodologías analíticas apropiadas que permitan la correcta identificación y cuantificación de las micotoxinas en las diferentes matrices en las que pueden estar presentes. Muchas micotoxinas son tóxicas a concentraciones muy bajas y por tanto, los métodos analíticos deben de ser capaces de proporcionar una elevada sensibilidad y selectividad, así como una elevada fiabilidad en la cuantificación, incluso a niveles traza (Capriotti et al., 2012b).

Los criterios generales que deben reunir los métodos de muestreo y de análisis para el control oficial de micotoxinas en productos alimenticios, se han establecido en el Reglamento nº 401/2006, modificado por el Reglamento (UE) 519/2014 en lo relativo a los métodos de muestreo de los lotes de gran tamaño, las especias y los complementos alimenticios, y los métodos analíticos de cribado.

El análisis de micotoxinas requiere una primera etapa de muestreo, y una segunda etapa de tratamiento de la muestra que consiste en la extracción y/o purificación de acuerdo al tipo de matriz y las micotoxinas objeto de estudio. Por último, se aplica un método analítico para la separación, detección y cuantificación de las micotoxinas (Meneely et al., 2011).

Diversas técnicas de extracción han sido empleadas para la determinación de micotoxinas, destacando las técnicas tradicionales, como la extracción líquido-líquido (L-L) y sólido-líquido (S-L), seguidas de una etapa de purificación (clean-up) con el fin de reducir las interferencias presentes en la matriz. Las técnicas de extracción alternativas a las técnicas tradicionales comprenden la realización de la extracción y la purificación en un solo paso, como el QUECHERS (Quick, Easy, Cheap, Effective, Rugged and Safe) y la Dispersión de Matriz en Fase Sólida (MSPD, Matrix Solid Phase Dispersion), así como la aplicación de técnicas de micro-extracción, como la Micro-extracción Líquido-Líquido Dispersiva (DLLME, Dispersive Liquid-Liquid Micro-extraction) y la Micro-extracción en fase sólida (SPME, Solid Phase Micro-extraction). Estas técnicas presentan las ventajas de ser rápidas, económicas y de emplear un reducido volumen de disolvente.

Las técnicas de separación, detección y cuantificación de micotoxinas de mayor difusión son la cromatografía de gases (GC, Gas Chromatography) y la

cromatografía de líquidos (LC, Liquid Chromatography) acopladas a diferentes detectores. Los detectores de espectrometría de masas (MS, mass spectrometry), especialmente de MS en tándem (MS/MS), han supuesto un gran progreso en las últimas décadas, permitiendo la detección de diferentes sustancias en niveles de concentración extremadamente bajos con una óptima resolución y un elevado grado de fiabilidad.

Los métodos analíticos actuales suelen aplicar una sola etapa de tratamiento de muestra seguida del análisis por GC-MS/MS o por LC-MS/MS para la separación, detección y cuantificación de los compuestos de interés. No obstante, en los últimos años se ha observado una preferencia en el empleo de la LC-MS/MS para determinar micotoxinas, principalmente debido a que los métodos basados en la GC-MS/MS solo son adecuados para ciertas micotoxinas y suelen presentar un tiempo mayor de tratamiento de muestra, debido a las reacciones de derivatización, dando lugar a métodos más costosos y complejos (Li et al., 2013). La LC-MS/MS ha permitido el desarrollo de nuevos métodos multi-componente, ofreciendo un alto grado de sensibilidad, selectividad y fiabilidad, permitiendo la identificación inequívoca de la mayoría de las micotoxinas, incluyendo las micotoxinas emergentes de *Fusarium* (Santini et al., 2012).

1.5. 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 primaria para los reglamentos (FAO, 2002). El proceso de evaluación del riesgo presenta cuatro etapas: identificación del peligro,

caracterización del peligro, determinación de la exposición, y caracterización del riesgo. En la Unión Europea, la JECFA y la EFSA son los organismos que se encargan del proceso de evaluación del riesgo de contaminantes alimentarios. Los datos toxicológicos y los datos de presencia de micotoxinas en productos alimenticios proporcionan la información necesaria para la evaluación del riesgo y la consiguiente disposición de límites reglamentarios.

En la actualidad no existe normativa que regule los contenidos máximos permitidos de las micotoxinas emergentes de *Fusarium* en alimentos y piensos. Recientemente, la EFSA ha adoptado un dictamen científico a petición de la Comisión Europea, sobre los riesgos para la salud humana y animal asociados a la presencia de ENs A, A1, B, B1 y BEA en alimentos y piensos. Con este objetivo, el panel de expertos de contaminantes en la cadena alimentaria de la EFSA (CONTAM) evaluó los estudios toxicológicos existentes hasta el momento, y un total de 12.685 resultados analíticos con relación a la presencia de BEA y ENs en muestras de alimentos, piensos y materias primas (EFSA CONTAM Panel, 2014). En este dictamen científico se indicó que los productos alimentarios que más contribuyen a la exposición crónica a estas micotoxinas son los cereales y los productos a base de cereales, especialmente el pan, la pasta y los productos de bollería. El Panel de expertos CONTAM de la EFSA concluyó que:

- No existe un riesgo apreciable para la salud humana y animal con relación a la exposición aguda de la BEA y las ENs.
- Existen escasos datos para establecer la IDT y/o Dosis de Referencia Aguda.
- Existe cierta incertidumbre con respecto a la exposición crónica.

El panel CONTAM concluyó que son necesarios más datos de toxicidad *in vivo* para realizar una evaluación del riesgo con garantías. Asimismo, se

establecieron una serie de recomendaciones acerca de los estudios que se deberían realizar para poder llevar a cabo una futura evaluación del riesgo:

- Se recomienda la aplicación de métodos analíticos para determinar ENs y BEA, basados en el uso de la LC-MS/MS. Los métodos analíticos se deben evaluar mediante estudios de validación inter-laboratorio.
- Es necesario desarrollar material de referencia certificado para determinar ENs y BEA en alimentos y piensos.
- Se deben llevar a cabo más estudios sobre la co-ocurrencia de la BEA y las ENs con otras micotoxinas de *Fusarium* en alimentos y piensos, así como los posibles efectos combinados de estas toxinas.
- Son necesarios estudios de investigación sobre el comportamiento de las ENs y la BEA durante la preparación de productos a base de cereales, especialmente de pan, productos de bollería y pasta.
- Son necesarios datos de genotoxicidad *in vivo* e *in vitro*.
- Estudios de toxicidad *in vivo* son imprescindibles para llevar a cabo la evaluación del riesgo, siendo de gran interés el estudio de los efectos para la salud derivados de una exposición crónica, especialmente de los efectos en el sistema nervioso, endocrino, e inmune.
- Son necesarios estudios sobre los efectos adversos de las ENs y la BEA en animales de granja, especialmente en cerdos y caballos, para identificar el NOAEL.

2. Objectives

2. OBJETIVOS

El **objetivo general** de la presente Tesis Doctoral es la evaluación del riesgo de micotoxinas en cereales.

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

1. Realización de una revisión bibliográfica para obtener la información disponible acerca de la presencia de micotoxinas en alimentos.

2. Validación de métodos de análisis y determinación de micotoxinas en cereales, productos elaborados a base de cereales, agua y fluidos biológicos.

3. Evaluación de la bioaccesibilidad de las micotoxinas emergentes de *Fusarium* mediante un modelo de digestión *in vitro* estático.

4. Evaluación del riesgo de micotoxinas en diferentes grupos de población mediante la estimación de la ingesta diaria.

5. Estudio del comportamiento de las micotoxinas emergentes de *Fusarium* y de los productos de degradación formados durante los procesos de fabricación, cocción y almacenamiento de la pasta.

2. OBJECTIVES

The **overall objective** of this study was the risk assessment of mycotoxins in cereals.

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

1. To perform a bibliographic review in order to obtain information available on mycotoxins in foodstuffs.

2. To validate analysis methods and to determine mycotoxins in cereals, cereal-based products, water and biological fluids.

3. To determine the bioaccessibility of emerging *Fusarium* mycotoxins using a static *in vitro* digestion model.

4. To assess the risk of mycotoxins in different population groups by estimating the daily intake.

5. To study the fate of emerging *Fusarium* mycotoxins and their related degradation products, during pasta processing, cooking and storage.

3. Results

3.1. Comparative assessment of three extraction procedures for determination of emerging *Fusarium* mycotoxins in pasta by LC-MS/MS

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Comparative assessment of three extraction procedures for determination of emerging *Fusarium* mycotoxins in pasta by LC-MS/MS

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ABSTRACT

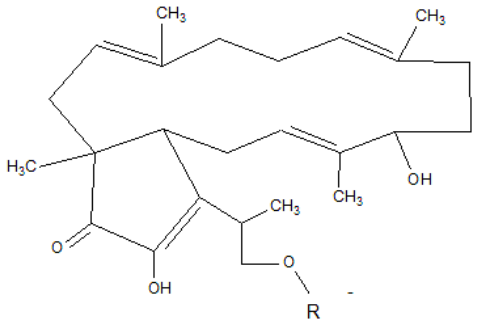
A new rapid, sensitive, reproducible and reliable method was developed for the quantitative determination of enniatins A, A_1, B and B_1, beauvericin and fusaproliferin in dry and fresh pasta by liquid chromatography-triple quadrupole-tandem mass spectrometry. A comparative study of different rapid and economical extraction procedures was performed for the extraction of these mycotoxins in pasta. For this purpose, three different approaches were studied during the extraction step (Ultra-Turrax, ultrasonic bath and microwave). Optimal extraction conditions were reached using Ultra-Turrax with acetonitrile for 3 min without purification step. The chromatographic separation of the six mycotoxins was accomplished in 15 min. The method was extensively validated with satisfactory results: recovery rates ranged from 86-112% and the relative standard deviations were lower than 15%. Limits of detection ranged from 0.02 to 0.15 μg kg^{-1}. The applicability of the method was assessed with the analysis of 30 samples of dry and fresh pasta.

Keywords: occurrence, pasta, emerging *Fusarium* mycotoxins, extraction procedures, *LC-MS/MS*

1. INTRODUCTION

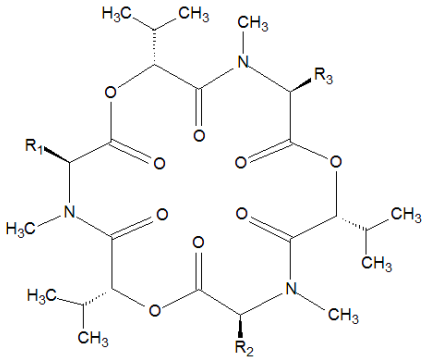
Mycotoxins are a group of natural substances produced as a result of the secondary metabolism of different species of filamentous fungi, which have been found occurring in foods and feeds. A wide range of undesirable effects on consumer health and economical losses in the world caused by the mycotoxins have been reported during years. The genus *Aspergillus, Fusarium, Penicillium, Claviceps* and *Alternaria* are the major species capable of producing mycotoxins in agricultural commodities during their growth period, and during harvest and post-harvest period under certain environmental conditions (Hussein & Brasel, 2001). According to the Scientific Committee on Food (SCF), the *Fusarium* fungi are probably the most prevalent toxin producing fungi in the temperate regions of America, Europe and Asia (SCF, 2002). The genus *Fusarium* includes more than 100 different phytopathogenic fungi species. Some of these species are known to produce mycotoxins such as deoxynivalenol, zearalenone or fumonisins, for which the European Union has set maximum levels (MLs) in certain foodstuffs (Commission Regulation, 2006). Also, several *Fusarium* species can produce other mycotoxins without established MLs, such as enniatins (ENs), beauvericin (BEA) and fusaproliferin (FUS), usually called emerging *Fusarium* mycotoxins. Over the last few years, emerging *Fusarium* mycotoxins have acquired importance because of their toxic effects in human and animal health such as cardiotoxicity, cytotoxicity or teratogenicity (Jestoi, 2008). Enniatins A, A_1, B and B_1 (ENA, ENA_1, ENB and ENB_1, respectively) and BEA are ionophoric compounds with similar toxicodynamic actions, which possess similar chemical structures with a cyclic hexadepsipeptide distinguished only by alternating amino acid residues (Fig. 2). FUS is a bicyclic sesterterpene derived from five isoprenic units (Fig. 1). These

compounds present similar polarity, both with a hydrophilic and hydrophobic environment (Jestoi, 2008).



	R
FUS	CH_3CO-
Deacetil-FUS	H-

Fig. 1. Chemical structures of fusaproliferin (FUS) and deacetilfusaproliferin (Deacetil-FUS)



	R_1	R_2	R_3
BEA	Phenilmethyl	Phenilmethyl	Phenilmethyl
ENA	*sec*-butyl	*sec*-butyl	*sec*-butyl
ENA_1	*sec*-butyl	*sec*-butyl	*iso*-propyl
ENB	*iso*-propyl	*iso*-propyl	*iso*-propyl
ENB_1	*iso*-propyl	*iso*-propyl	*sec*-butyl

Fig. 2. Chemical structures of beauvericin (BEA) and enniatins (ENA, ENA_1, ENB and ENB_1)

Limited studies exist about their incidence in food, which have indicated their presence mainly in grain cereals and in cereal products from different countries in the world (Argentina, South Africa, Morocco, Spain, Italy, Finland, etc.). The highest concentration levels have been found in samples from countries characterized by a Mediterranean climate such as Tunisia, Spain or Morocco (Meca, Zinedine, Blesa, Font, & Mañes, 2010; Oueslati, Meca, Mliki, Ghobel, & Mañes, 2011; Sifou et al., 2011). To carry out the analysis of mycotoxins, specific procedures are required for the extraction and removal of impurities using clean-up steps. Ultra-Turrax and rotatory shaker are the most widely used approaches for the extraction of emerging *Fusarium* mycotoxins. Ultra-Turrax provides extracts with few matrix interferences, so it does not need further steps of purification (Sifou et al., 2011), while rotatory shaker extraction usually requires clean-up of the sample (Jestoi, Rokka, Rizzo, & Peltronen, 2005; Zapata et al., 2010). Recently, new extraction methods have been developed for other mycotoxins (toxin T-2 and HT-2, ochratoxin A or aflatoxins) using different approaches such as the microwave and the ultrasonic bath (Liazid, Palma, Brigui, & Barroso, 2007; Romero-González, Garrido, Martínez, Prestes, & Grio, 2011). The methods employing ultrasonic bath and microwave are characterized by being economic, rapid and efficient. In microwave, the radiation causes motion of molecule and rotation of dipoles favoring the extraction of the studied compounds in the solvent (Wang, Meng, Lu, Liu, & Tao, 2007). Moreover, the use of the ultrasonic bath is probably the most simple and versatile tool for the disruption of cells. During the extraction with ultrasonic bath, the acoustic cavitation leads to local increase of pressure and temperature, and a reduction of the particle size producing an

enhancement on contact between solvents and compounds (Rostagno, Palma, & Barroso, 2003).

Usually ENs, BEA and FUS are analysed using liquid chromatography (LC) coupled to diodearray ultraviolet-visible detector (UV-Vis) (Serrano, Meca, Font, & Ferrer, 2012b), or LC coupled to tandem mass spectrometry (LC-MS/MS) (Jestoi et al., 2005). Studies using UV detection present several limitations. One of the main disadvantages is the analysis of mycotoxins in complex matrices, since LC-UV-Vis is a method vulnerable to interfering substances (substances that absorb at the same wavelength as the target mycotoxins). The analysis employing UV detection in complex matrices is not reliable and a method of confirmation is necessary, such as MS/MS detection (Sørensen, Nivelsen, Rasmussen, & Thrane, 2008). LC-MS/MS is one of the most suitable techniques for the analysis of mycotoxins: it provides a high selectivity, sensibility and reliability of the analysis. Matrix effects (ME) are the main problem of the MS/MS detection, since some compounds in the matrix frequently lead to reduce or enhance the analyte signal. The best way to address this problem is the use of isotopically labelled internal standards. Calibration curves prepared with matrix-matched standards is also a good alternative (Rubert, Soler, & Mañes, 2012; Soleimany, Jinap, & Abas, 2012). Therefore, the principal problem of the LC-MS/MS is easily solved resulting in a high confidence of the results.

Given that wheat products are frequently infected by *Fusarium* mycotoxins, a new LC-MS/MS-based method has been developed and validated for the simultaneous analysis of ENA, ENA_1, ENB, ENB_1, BEA and FUS. Different extraction procedures employing different approaches (Ultra-Turrax, ultrasonic bath and microwave) were tested for conducting to the study of the best

procedure to extract emerging *Fusarium* mycotoxins in pasta. Finally, a survey of several samples of dry and fresh pasta was carried out demonstrating the applicability of the method.

2. MATERIAL AND METHODS

2.1. Reagents and Materials

Acetonitrile (AcN), methanol (MeOH) and ethyl acetate (EtOAc) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) and formic acid (> 98%) were supplied by Panreac Quimica S.A.U. (Barcelona, Spain) (Madrid, Spain). Solid-phase used for purification was octadecysilica cartridges (C_{18}) (SEP-Pack Ligh C_{18} cartridge 55-105 μm) from Waters (Ireland). Deionized water was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

The standards of ENA, ENA_1, ENB, ENB_1 and BEA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard of FUS was kindly given by Professor A. Ritieni (Department of Food Science, University "Federico II" of Naples, Italy). Individual stock solutions of ENA_1, ENB, ENB_1, FUS and BEA with concentration of 1000 μg mL^{-1}, and the solution of ENA with concentration of 500 μg mL^{-1}, were prepared in methanol. They were stored in glass-stoppered bottles and darkness in security conditions at -20 ºC. These stock solutions were then diluted with pure methanol in order to obtain the appropriate working solutions and were stored in darkness at -20 ºC until the LC-MS/MS analysis.

2.2. Sample collection

The applicability of the method was assessed in thirty samples of pasta (20 of dry pasta and 10 of fresh pasta) that were collected from different supermarkets in Valencia (Spain). The composition of dry pasta samples was mainly durum wheat, and the composition of fresh pasta samples was durum wheat, water and eggs (20%). Before the analysis, all samples were ground and were divided in subsamples of 200 g. The subsamples were stored in a dark and dry place at 4 ºC until analysis.

2.3. Instrumental and Chromatographic Conditions

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 2 mm I.D., 3 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using an elution in gradient that started at 90% of A (AcN) and 10% of B (20 mM ammonium formate in MeOH), increased linearly to 50% B for 10 min. After, it was decreased linearly to 10% of B for 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 mL min^{-1}. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0,5 V; source temperature, 100 ºC; desolvation temperature, 300 ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L h^{-1}; cone gas 50 L h^{-1} (nitrogen 99.99% purity). Cone

voltages and collision energies were different at each mycotoxin and are reported in Table 1. 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 99.995% purity) pressure, 3.83 x 10^{-3} mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass spectrometer was operated in "full SCAN", Selected Ion Recording (SIR), and Multiple Reaction Monitoring (MRM) modes. All time measurements were carried out in triplicate. The MRM parameters and product-ions and precursor ion selected, are presented in Table 1.

Table 1. Product-ions observed in product ion scan mode for selected mycotoxins and MRM optimized parameters.

Mycotoxin	Precursor ion (m/z)	Product ion (m/z) [a]	Cone (V)	Collision energy (eV)	Retention time (min.)
ENA	681.9 $[M+H]^+$	228.2^{Q} 210.0^{q}	40	35 35	3.81
ENA1	667.9 $[M+H]^+$	228.2^{Q} 210.0^{q}	40	35 35	3.60
ENB	639.8 $[M+H]^+$	214.2^{Q} 196.2^{q}	40	35 35	3.41
ENB1	654.9 $[M+H]^+$	214.2^{Q} 196.2^{q}	40	35 35	3.53
FUS	445.0 $[M+H]^+$	409.0^{Q} 427.0^{q}	50	10 10	6.50
BEA	784.4 $[M+H]^+$	244.0^{Q} 262.0^{q}	40	40 40	3.22

[a] Q, Quantification transition q, Confirmation transition

2.4. Extraction Procedures

2.4.1. Extraction with Ultra-Turrax

Samples (5 g) were extracted with 50 mL of AcN using a Ika T18 basic Ultra-Turrax (Staufen, Germany) for 3 min. After, centrifugation for 15 min at 3554 g and 5 ºC, the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The extract was dissolved with 5 mL of AcN and then, it was evaporated to dryness by nitrogen gas at 35 ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and was filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) prior to injection in the LC-MS/MS system.

2.4.2. Extraction with microwave

Samples (5 g) were extracted with 50 mL of AcN using a microwave Saivod WP700P17-2 (Manisa, Turkey) for 2 min at 250 W. After, centrifugation for 15 min at 3554 g and 5 ºC, the supernatant was purified with cartridges of C_{18}. The clean-up procedure was based on three steps: conditioning with 2 mL of AcN, application of the supernatant into the column and elution of the analytes from the column with 10 mL of AcN/MeOH (50/50 v/v) 20 mM ammonium formate. The vacuum was adjusted to provide a flow rate of about 3 ml/min. The total eluate was evaporated to dryness by nitrogen gas at 35 ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and was

filtered through a 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) prior to injection in the LC-MS/MS system.

2.4.3. Extraction with ultrasonic bath

Samples (5 g) were extracted with 50 mL of AcN using an ultrasonic bath of 200 W (Branson, Germany) for 30 min at 40 ºC. After, centrifugation for 15 min at 3554 g and 5 ºC, the supernatant was purified with cartridges of C_{18}. The clean-up procedure was based on three steps: conditioning with 2 mL of AcN, application of the supernatant into the column and elution of the analytes from the column with 10 mL of AcN/MeOH (50/50 v/v) 20 mM ammonium formate. The vacuum was adjusted to provide a flow rate of 3 ml/min. The total eluate was evaporated to dryness by nitrogen gas at 35 ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and was filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) prior to injection in the LC-MS/MS system.

3. RESULTS AND DISCUSSION

3.1. Development of the LC-MS/MS analysis

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 were formed

because studied mycotoxins are ionophoric compounds and they are capable of forming complexes with monovalent and divalent cations through interactions with carbonyl groups oriented towards the centre of the molecule (Fig. 2). The monovalent and divalent cations can be presented during the analytical process in the solvents or in the instruments coatings, among others. If the formation of complexes is not under control, the resulting adducts may be present in the analysis by LC-MS/MS, leading to quantification problems. Several authors have reported that sodium adduct ions $[M+Na]^+$ and potassium adduct ions $[M+K]^+$ can be eliminated by addition of modifiers in the mobile phase, as ammonium acetate or ammonium formate (Jestoi et al., 2005; Kameník et al., 2010). On this account, different concentrations of ammonium formate added to the mobile phase were evaluated. The adducts of $[M+Na]^+$ and $[M+K]^+$ were removed with the addition of ammonium formate at a concentration of 20 mM to the mobile phase. However, ammonium adduct ions $[M+NH_4]^+$ were found with this addition. According to other authors, it was decided to change the cone voltage, since the application of the appropriate cone voltage of the MS-ion source has an effect on the abundance of the different adducts (Uhlig et al., 2004). The change of a cone voltage of 25 V to 40 V resulted in a decrease in the formation of ammonium adduct $[M+NH_4]^+$ compared to the abundance of the protonated molecule $[M+H]^+$. With this, higher sensitivity was achieved in the method, as well as more reliable quantification of the mycotoxins in the samples. The method was optimized according to the guidelines established in the 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.

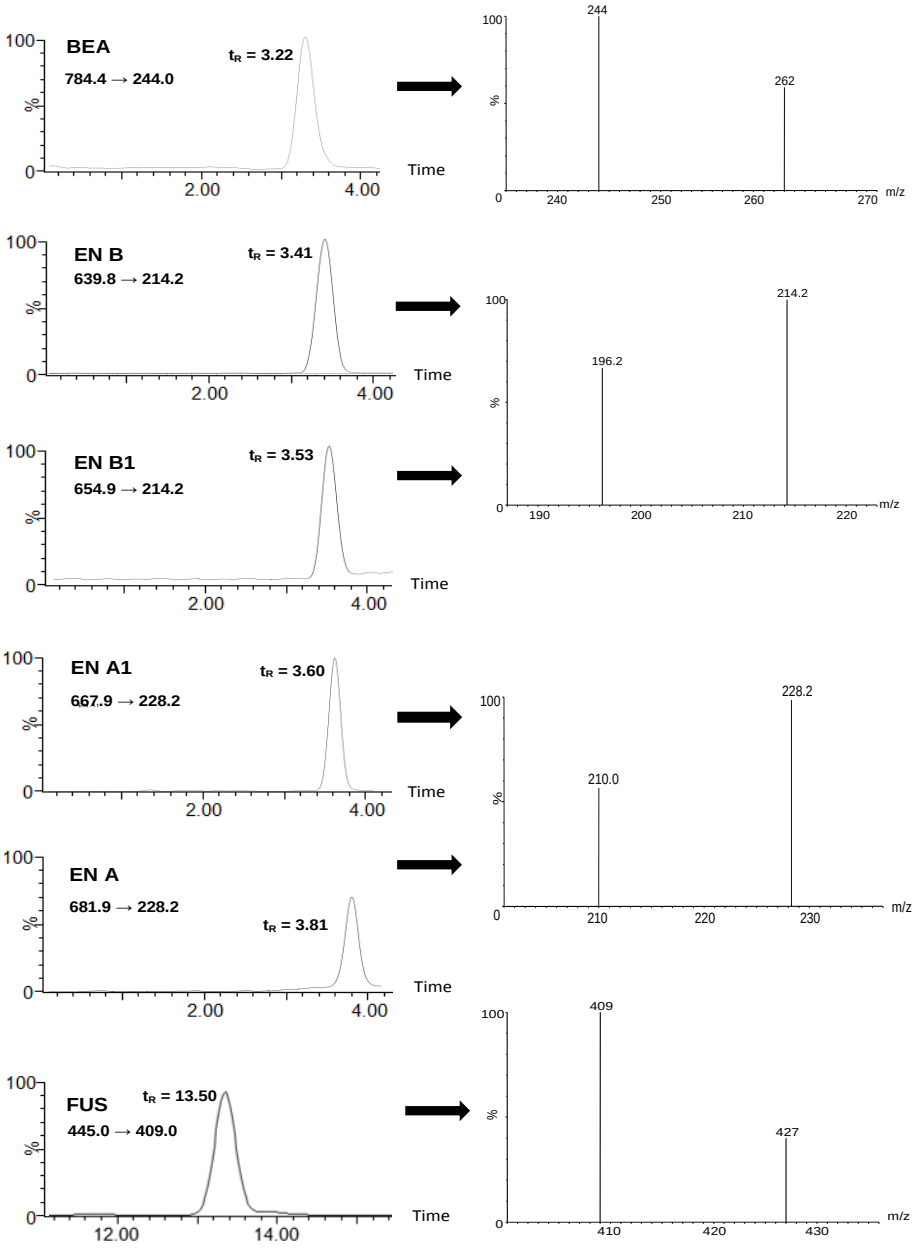


Fig. 3. LC-MS/MS QqQ MRM chromatograms and daughter ion spectrums obtained from an extract of the blank sample after spiking it with 25 μg kg^{-1} of ENA, and 50 μg kg^{-1} of BEA, FUS, ENA$_1$, ENB and ENB$_1$.

Therefore, the quantification of each mycotoxin was carried out with the primary transition (transition of quantification) and the confirmation with the second transition (transition of confirmation). Table 1 shows the MS/MS transitions as well as the cone voltages, collision energies and retention times (t_R) optimized for each compound. Fig. 3 shows the LC-MS/MS QqQ MRM chromatograms and daughter ion spectrums (with the structures of fragments) obtained from an extract of the blank sample after spiking it with 25 μg kg^{-1} of ENA, and 50 μg kg^{-1} of BEA, FUS, ENA_1, ENB and ENB_1. Fig. 4 shows the chromatograms of an extract of dry pasta naturally contaminated (1.26 μg kg^{-1} of BEA, 3.10 μg kg^{-1} of ENB_1, and 2.05 μg kg^{-1} of ENA).

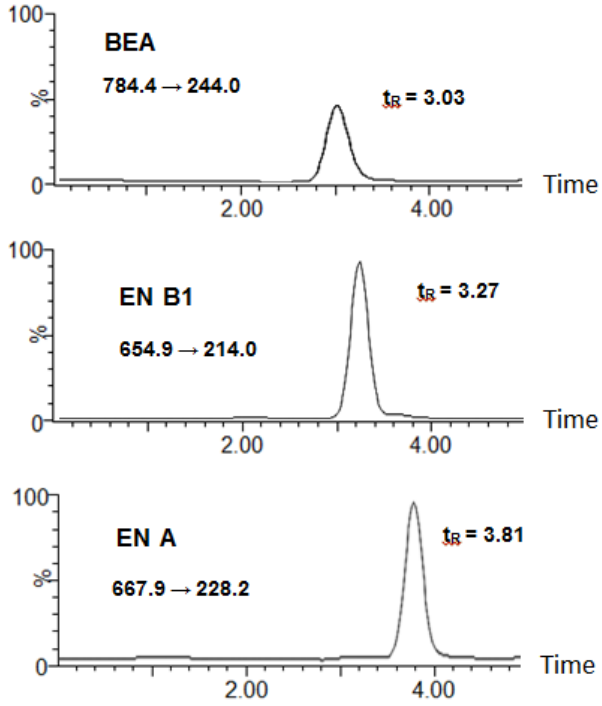


Fig. 4. Chromatograms of an extract of dry pasta naturally contaminated (1.26 μg/kg of BEA, 3.10 μg/kg of ENB1, and 2.05 μg/kg of ENA).

3.2. Extraction procedures

Three procedures (Ultra-Turrax, microwave and ultrasonic bath) were compared for the extraction of emerging *Fusarium* mycotoxins. For this purpose, different parameters such as the volume and nature of the extraction solvent or the extraction time, were evaluated for each technique separately. All experiments were carried out in triplicate using blank sample of pasta spiked at 2.5 μg kg^{-1} of ENA and 5 μg kg^{-1} of ENA_1, ENB, ENB_1, BEA and FUS. The extraction efficiency was evaluated in terms of recovery and the time achieved during the analysis. In Fig. 5 and in Tables 2 and 3, are shown the results for the critical parameters in the tested extractions.

3.2.1. Ultra-Turrax optimization

The critical parameters of this extraction were the volume and nature of the solvent and the time during the extraction. In order to select the most adequate solvent, the extraction was performed for five minutes, with 5 g of sample and 50 mL of solvent. These initial parameters were set based on other studies (Meca et al., 2010; Oueslati et al., 2011; Sifou et al., 2011). Solvents with different polarities were tested: AcN, MeOH, EtOAc and a mixture of AcN/MeOH 50/50 v/v. Because the melting point of the ENA_1 is 66-67 ºC (Jestoi, 2008), the evaporation of the solvents during the extraction process was carried out at temperatures lower than 65 ºC. Mixtures of organic solvents with water were discarded since the water evaporation at 65 ºC would result to long waiting times. Results are presented in Fig. 5a. Using MeOH, acceptable mean recoveries were obtained for ENB, ENB_1 and BEA (> 70%). When the extraction was performed with EtOAc and with the mixture of AcN/MeOH 50/50 v/v, mean recoveries were not acceptable for all mycotoxins. The best

results were obtained using AcN: the extracts and chromatograms were clearest and the recoveries of all studied mycotoxins were the highest (mean recoveries between 88.0 and 113.3%).

Then, a study to determine the efficiency of extraction was carried out by testing different volumes of AcN (30, 50 and 70 mL). When 30 mL of AcN were used, mean recoveries (ranging from 54.3 to 103%) were lower than those obtained with 50 and 70 mL of AcN. Mean recoveries were acceptable both for volume of 50 mL (between 88.0 and 113.3%) and volume of 70 mL (between 91.9 and 110.0%). The differences between the mean recoveries obtained with both volumes were not statistically significantly (see Table 2). Considering the results, a volume of 50 mL was selected for the extraction. This was a good compromise between recovery and volume of extraction solvent.

Finally, the optimum extraction time was evaluated. In the previous assays for the optimization of volume and nature of the extraction solvents, acceptable recoveries were obtained with 5 min of extraction time. Therefore, it was decided to test the extraction efficiency for 1 and 3 min. The results are presented in the Table 2. When the extraction was carried out for 1 min, recoveries were not acceptable for ENA, ENB_1, BEA and FUS. The highest mean recoveries were obtained for a time of 5 min (ranging from 88.0 to 113.3%). Nevertheless, there were not remarkable differences between the recoveries obtained for 3 and 5 min. For this reason, 3 min was employed as the optimum time for a fast and efficient extraction. The mean recoveries with the selected parameters were 90.4, 86.0, 108.7, 97.1, 92.9 and 91.3% for ENA, ENA_1, EN B, ENB_1, BEA and FUS, respectively. The relative standard deviations (RSD) were < 10% in all cases. Further purification of the extract

was avoided in order to reduce analysis time, since the final extracts were transparent and suitable for LC-MS/MS analysis.

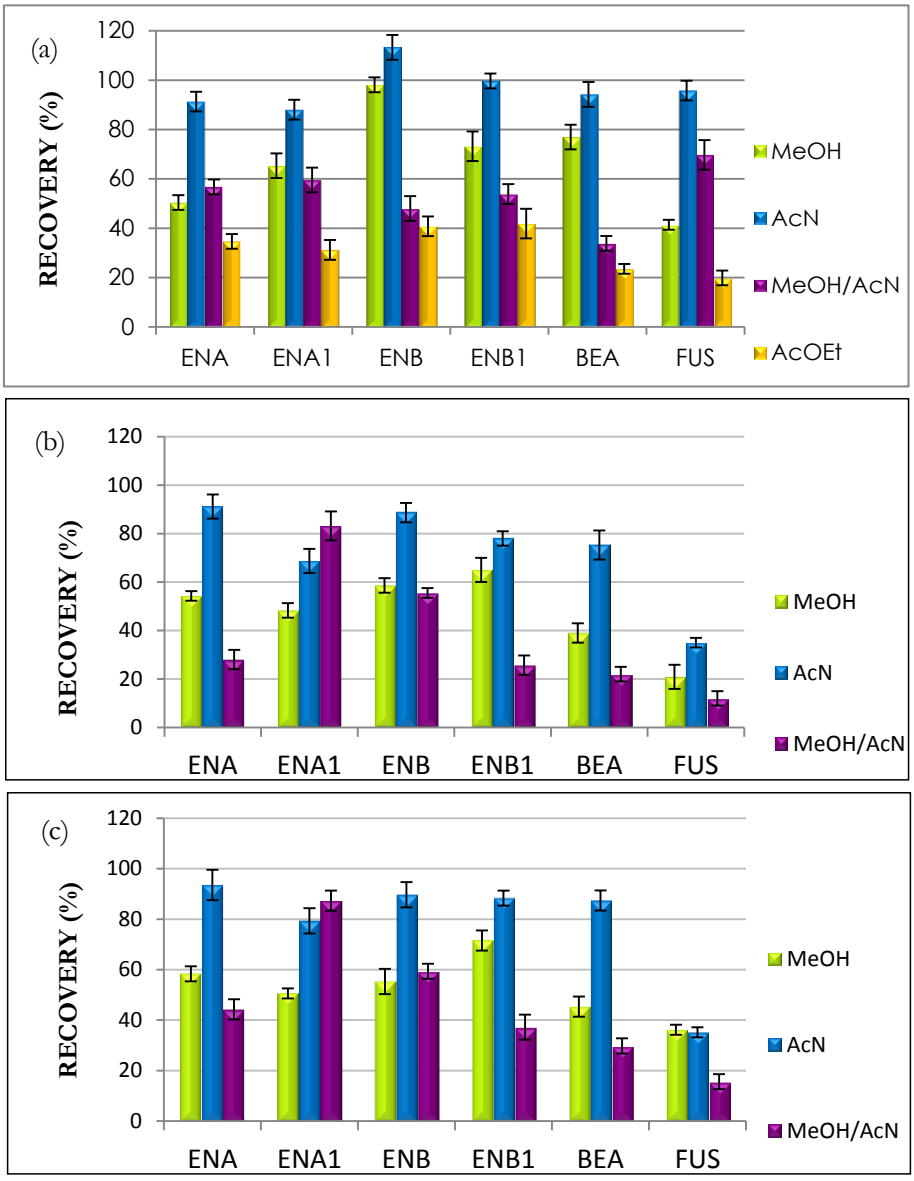


Fig. 5. Effect of the extraction solvent in analyte recoveries using: (a) Ultra-Turrax, (b) Microwave, (c) Ultrasonic bath.

Table 2. Recovery data and relative standard deviations (n=3) of the critical parameters in the extractions using Ultra-Turrax, microwave and ultrasonic bath.

Mycotoxin	Extraction method	Recovery ± RSD (%)									
		Volume			Temperature [a]				Time [b]		
		30 mL	50 mL	70 mL	25 ºC	30 ºC	40 ºC	50 ºC	1 min	2 min	3 min
ENA	Ultra-Turrax	64.4± 7.0	91.3 ± 4.3	93.4± 3.5	-	-	-	-	61.1± 3.4	90.4± 5.0	91.3 ± 4.3
	Microwave	60.7± 6.7	91.2± 5.4	90.2± 7.4	-	-	-	-	65.5± 4.8	76.4± 4.6	91.2± 5.4
	Ultrasonic bath	66.2± 5.9	93.2± 5.7	94.3± 5.5	82.7 ± 6.7	89.0 ± 3.0	92.1 ± 4.1	93.2 ± 5.7	83.4± 6.3	88.8± 4.6	93.2± 5.7
ENA1	Ultra-Turrax	54.3± 3.5	88.0± 3.9	91.9± 4.5	-	-	-	-	75.6± 4.1	86.0± 4.4	88.0± 3.9
	Microwave	58.7± 4.3	68.7± 5.0	70.8± 3.7	-	-	-	-	66.2± 3.9	71.5± 5.6	68.7± 5.0
	Ultrasonic bath	60.3± 5.0	79.3± 5.1	77.3± 6.8	66.2 ± 4.0	75.3 ± 5.8	82.6 ± 5.3	79.3 ± 5.1	74.2± 5.6	77.3± 6.3	79.3± 5.1
ENB	Ultra-Turrax	103.0± 4.9	113.3± 5.3	110.0± 6.7	-	-	-	-	86.2±3.0	108.7± 7.0	113.3± 5.3
	Microwave	76.2± 3.7	88.7± 4.0	92.1± 5.4	-	-	-	-	76.5± 4.2	85.3± 7.6	88.7± 4.0
	Ultrasonic bath	80.3± 8.6	89.7± 4.8	88.0± 6.4	74.6 ± 3.1	82.7 ± 6.4	86.4 ± 5.0	89.7 ± 4.8	82.4± 6.9	83.0± 4.6	89.7± 4.8

Table 2 cont.

Mycotoxin	Extraction method	Recovery ± RSD (%)									
		Volume			Temperature [a]				Time [b]		
		30 mL	50 mL	70 mL	25 ºC	30 ºC	40 ºC	50 ºC	1 min	2 min	3 min
ENB1	Ultra-Turrax	95.1± 5.6	99.7± 3.0	103.7± 5.0	-	-	-	-	63.4± 4.6	97.1± 6.4	99.7± 3.0
	Microwave	77.8± 6.0	78.3± 3.3	83.7± 3.8	-	-	-	-	69.5± 3.4	79.6± 4.9	78.3± 3.3
	Ultrasonic bath	80.4± 4.7	88.3± 2.7	85.6± 7.0	80.5 ± 4.6	86.5 ± 3.7	89.6 ± 6.4	88.3 ± 2.7	75.6± 5.3	83.6± 8.7	88.3± 2.7
BEA	Ultra-Turrax	75.9± 6.8	94.2± 4.9	93.1± 3.4	-	-	-	-	56.6± 4.5	92.9± 4.8	94.2± 4.9
	Microwave	70.6±3.8	75.3± 5.7	80.4± 4.3	-	-	-	-	60.6 ± 3.9	72.3± 7.6	75.3± 5.7
	Ultrasonic bath	73.5± 5.4	87.4± 3.6	82.6± 4.4	72.4 ± 2.4	78.7 ± .4	81.9 ± 2.6	87.4 ± 3.6	79.3± 7.6	78.9± 6.7	87.4± 3.6
FUS	Ultra-Turrax	89.7± 5.2	95.8± 4.0	96.0± 3.3	-	-	-	-	52.4± 4.3	91.3± 5.5	95.8± 4.0
	Microwave	34.7± 3.9	35.0± 2.3	33.6± 4.5	-	-	-	-	13.4±3.3	37.9± 6.8	35.0± 2.3
	Ultrasonic bath	33.0± 4.8	35.2± 2.0	38.7± 1.6	37.8 ± 6.2	42.6 ± 3.3	38.9 ± 5.0	35.2 ± 2.0	25.9± 4.8	29.6± 4.5	35.2± 2.0

[a] Data available for the extraction with Ultrasonic bath

[b] In the extraction with Ultrasonic bath, the times of thermal treatment are 10, 20 and 30 min instead of 1, 2 and 3 min, respectively

Table 3. Recovery data and relative standard deviations (n=3) of the critical parameters in the purification step.

Mycotoxin	Extraction method	Recovery ± RSD (%)							
		Eluting Solvent			Modifier		Concentration of modifier (mM)		
		AcN	MeOH	AcN/MeOH[a]	Ammonium formate	Formic acid	5	10	20
ENA	Microwave	75.4± 7.1	45.3± 6.3	83.7± 4.8	86.4± 3.2	76.6± 8.1	83.4± 4.4	86.4± 3.2	82.8± 4.6
	Ultrasonic bath	72.8± 6.1	42.7± 4.6	81.3± 6.4	86.6± 4.4	69.3± 4.9	80.0± 6.7	86.6± 4.4	89.7± 3.1
ENA1	Microwave	64.3± 2.9	36.4± 2.4	72.4± 4.3	73.0± 4.3	70.4± 7.4	70.3± 2.1	73.0± 4.3	75.9± 2.8
	Ultrasonic bath	65.7± 3.9	38.6± 4.6	75.9± 3.7	77.3± 6.4	72.1± 4.3	72.3± 3.8	77.3± 6.4	80.0± 3.6
ENB	Microwave	78.2± 4.6	50.9± 8.8	80.1± 3.0	84.3± 2.4	76.1± 3.8	77.4± 9.2	84.3± 2.4	82.4± 5.2
	Ultrasonic bath	76.5± 3.8	45.2± 1.9	85.3± 5.6	86.6± 7.3	65.3± 4.6	83.1± 8.5	86.6± 7.3	86.3± 6.0
ENB1	Microwave	77.7± 4.6	74.6± 3.8	83.4± 2.2	82.3± 3.9	78.3± 4.9	75.3± 6.4	82.3± 3.9	77.0± 2.3
	Ultrasonic bath	73.6± 5.5	70.2± 1.6	83.9± 4.7	81.3± 5.3	66.2± 6.6	82.3± 4.9	81.3± 5.3	85.6± 4.4
BEA	Microwave	69.3± 3.8	59.7± 2.9	66.4± 4.0	68.8± 6.9	63.4± 2.2	58.4± 6.6	68.8± 6.9	69.4± 2.1
	Ultrasonic bath	67.7± 4.4	56.3± 4.8	62.8± 6.6	66.5± 3.3	55.3± 1.6	64.3± 1.5	66.5± 3.3	75.1± 3.4
FUS	Microwave	34.9± 6.6	37.8± 7.1	40.5± 3.4	34.2± 8.4	30.1± 6.6	28.4±6.6	34.2± 8.4	37.0± 3.3
	Ultrasonic bath	36.4± 4.9	30.3± 6.6	36.8± 2.9	35.2± 4.6	24.8± 4.9	25.4± 4.6	35.2± 4.6	37.0± 3.3

[a] Mixture of AcN/MeOH 50/50 v

3.2.2. Microwave optimization

The critical parameters were the time, the potency, and the volume and nature of the extraction solvent. Initially, a study of the temperature during microwave extraction was carried out since ENA_1 presents a melting point relatively low (66-67 °C), while the other studied mycotoxins show highest melting points (between 93 and 174 ºC) (Jestoi, 2008). The variation of the T with the time and the potency (250, 500 and 1000 W) was studied during the extraction. This study was performed with two solvents: AcN and MeOH, with a boiling point of 82 and 65 ºC, respectively. The AcOEt was discarded due to its high volatility and the low recoveries obtained previously in the Ultra-Turrax extraction. When the extraction was performed using a potency of 1000 and 500 W, the T were higher than 65 ºC. T were of 70, 62, 50 and 40 ºC using a continuous potency of 250 W for 6, 5, 3 and 1 min of extraction, respectively. Finally, recovery experiments were performed using a continuous potency of 250 W testing times less than 3 min.

For the study of the extraction solvent, microwave extraction was performed for 3 minutes at 250 W, with 5 g of sample and 50 mL of solvent. As discussed above, the solvents tested were AcN, MeOH and the mixture of AcN/MeOH 50/50 v/v. Results are shown in Fig. 5b. Low recoveries (< 35%) were obtained for FUS in all cases, probably due to its deacetylation: the production of deacetyl-fusaproliferin (Fig. 1) is favored at high temperatures, although it has been reported to occur even at 25 ºC (Wu, Leslie, Thakur, & Smith, 2003). With regard to ENs and BEA, acceptable mean recoveries were obtained when AcN was used in the extraction (between 75.3 and 91.2%), while no acceptable results were obtained using MeOH (< 65%). The mixture of AcN/MeOH 50/50 v/v provided the highest mean recovery for ENA_1

(83.2%), but low mean recoveries for the other mycotoxins (< 55.5%). AcN was selected since it provided the best mean recoveries for most mycotoxins. The optimum volume of solvent was studied in the same way as that of the Ultra-Turrax extraction. The obtained results were similar to those obtained in the Ultra-Turrax extraction and the volume of solvent was set at 50 mL (see Table 2).

Then, three different times (1, 2 and 3 min) were explored. Mean recoveries obtained during 1 min of extraction (65.5-76.5%) were lower than those obtained during 2 and 3 min. Results were very similar for 2 min (mean recoveries between 71.5 and 85.3%) and 3 min of extraction (mean recoveries between 68.5 and 91.2%). The best recoveries for the majority of the studied mycotoxins (ENA_1, ENB, ENB_1 and FUS) were reached for 2 min of extraction (see Table 2). Therefore, a time of 2 min was selected to carry out the extraction.

The purification of the extracts was necessary because the sample extracts from microwave extraction had not been transparent, probably due to increased solubility of several compounds during the thermal treatment. Clean-up was performed in order to reduce the presence of compounds that may interfere in the determination. SPE employing cartridges of octadecyl-silica phase (C_{18}) was selected for the purification step, since in further experiments it gave satisfactory results for studied emerging *Fusarium* mycotoxins (Jestoi et al., 2004; Turner et al., 2009; Zapata et al., 2010). The optimum eluting solvent was evaluated testing the following solvents: MeOH, AcN and a mixture of MeOH/AcN 50/50 v/v. Recovery experiments were performed employing a constant volume of 10 mL. Results are shown in Table 3. The mixture of MeOH/AcN 50/50 v/v was the best eluting solvent, since it provided the

higher mean recoveries for ENs (ranging from 72.4 to 83.7%). With regard to BEA and FUS no acceptable mean recoveries were obtained with the three tested solvents (< 70%). Several authors have suggested that the addition of modifiers to the eluting solvent may result in better extraction efficiencies for some mycotoxins, although it must always be considered that the polarity of the eluting solvent is the most relevant parameter (Krska, Schubert-Ullrich, Molinelli, Sulyok, Macdonald, & Crews, 2008; Serrano, Font, Ruiz, & Ferrer, 2012a). In order to improve the recoveries, the addition of several modifiers (ammonium formate and formic acid) was studied in the purification step. The recoveries were not improved when formic acid was added in the eluting solvent. However, the addition of ammonium formate resulted in an increase of the recoveries for ENA, ENB and BEA, whereas the recoveries for the other mycotoxins remained constant. Finally, different concentrations of ammonium formate were evaluated giving the best results with the concentration of 20 mM. The mixture of MeOH/AcN (50/50 v/v) 20mM ammonium formate was used as eluting solvent, which provided final mean recoveries of 82.8% for ENA, 75.9% for ENA_1, 82.4% for ENB, 77.0% for ENB_1, 69.4% for BEA and 42.0% for FUS. The RSD were acceptable for all mycotoxins (< 10%).

3.2.3. Ultrasonic bath optimization

The critical parameters in this extraction were the sonication time, the T and the volume and nature of the extraction solvent. For the study of the solvent, the extraction was carried out in an ultrasonic bath for 30 min at 50 ºC, with 5 g of sample and 50 mL of solvent. These initial parameters were set based on other studies (Liazid et al., 2007; Romero-González et al., 2011). As

discussed above, the solvents tested were AcN, MeOH and the mixture of AcN/MeOH 50/50 v/v. Results are presented in Fig. 5c. No acceptable mean recoveries (< 37%) were obtained for FUS, probably due to its deacetylation. Mean recoveries were better for ENs and BEA using AcN (ranging from 79.3 to 93.5%) than using the other evaluated solvents (ranging from 29.8 to 87.3%). The optimum volume of solvent was studied in the same way as the extractions with Ultra-Turrax and microwave, obtaining similar results to those obtained in both extractions (see Table 2). The volume of AcN was set at 50 mL.

Then, different T´s were evaluated (25, 30, 40 and 50 ºC) to establish which provided the best recoveries. The results are presented in Table 2. Low mean recoveries were obtained for FUS, ranging from 35.2 to 42.6%. For ENs and BEA acceptable recoveries were obtained with all tested T (> 70%). The highest recoveries were provided employing 40 ºC as operating T for the extractions (between 81.9 and 92.1%).

The time of extraction was the last parameter studied. When the extraction was carried out for 10 and 20 min, mean recoveries were inferior than those obtained for 30 min (Table 2). Therefore, a time of 30 min. was used as optimum time for the extraction.

The purification of the extracts was necessary to minimize the interferences, since no transparent extracts were obtained in the extraction. The acoustic cavitation caused a local increase of P and T, resulting in a reduction of the particle size and an increase of the solubility of several compounds. The optimization of the purification step was performed in the same way as the optimization for the extraction with microwave. The results are reported in Table 3. Results were very similar than those obtained by

microwave extraction, and the elution solvent selected was the mixture of MeOH/AcN (50/50 v/v) 20mM ammonium formate. The final mean recoveries with the selected parameters were 89.7, 80.0, 86.3, 85.6, 75.1 and 37.0% for ENA, ENA_1, ENB, ENB_1, BEA and FUS, respectively. The RSD were acceptable for all mycotoxins (< 10%).

3.2.4. Comparison and selection of the optimum procedure for the extraction

Fig. 6 shows the mean recoveries and the RSD achieved for pasta samples with the three tested procedures. All procedures presented good recoveries for ENs and BEA ranging from 69.5 to 109.0%. With regard to FUS, recoveries using microwave, ultrasonic bath and Ultra-Turrax were 41.6, 37.1 and 91.0%, respectively. Low efficiency was obtained for FUS when microwave and ultrasonic bath were employed in the extraction. As discussed above, the deacetylation of the FUS is favored at the T achieved in both extractions (Wu et al., 2003). On the other hand, the extraction with Ultra-Turrax was the most suitable procedure for the extraction of FUS. Furthermore, the use of Ultra-Turrax provided recoveries for ENs and BEA higher than those obtained using microwave and ultrasonic bath, probably because the Ultra-Turrax promotes the contact of the matrix with the solvent. Besides, the extraction with Ultra-Turrax was the fastest of the three procedures, since the procedures using microwave or ultrasonic bath showed a high number of manipulations. Therefore, the extraction using Ultra-Turrax may be advised as a convenient procedure for the extraction of ENs, BEA and FUS in pasta samples. For this reason, validation experiments were carried out with this procedure.

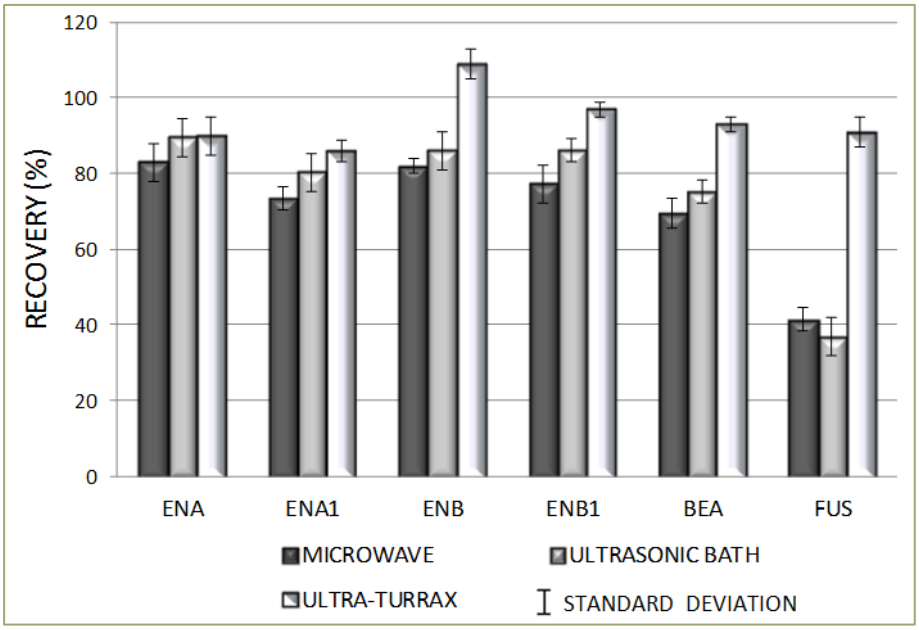


Fig. 6. Results from the comparative study by the three extraction procedures.

3.3. Evaluation of matrix effects

Some substances present in the matrix can change the instrumental response of the analyte, resulting in an enhancement or suppression of the analyte signal. For a reliable and selective LC-MS/MS quantification of the mycotoxins, in the literature has been described the use of isotopically labeled internal standards (Rubert et al., 2012). When isotopically labeled internal standards are not available for target compounds, it is best to use of matrix-assisted calibration curves, which is an accepted method for the correction of ME (Uhlig & Ivanova, 2004; Soleimany et al., 2012). As isotopically labeled internal standards were not available for ENs, BEA and FUS, the evaluation of the ME was performed by the use of matrix-assisted calibration curves: the slopes from the calibration curve prepared with known amounts of standards

and extracts of blank sample (with matrix), were compared with the slopes from the calibration curve prepared with the same amounts of standards and methanol (without matrix). According to other authors (Rubert, Soler, & Mañes, 2011), a mixture of extracts of dry and fresh pasta, where none of the studied mycotoxins were detected, was used as a blank sample in order to ensure results representatively. The standards were prepared at six concentration levels: 0.025-25 μg kg^{-1} for ENA and 0.05-50 μg kg^{-1} for ENA_1, ENB, ENB_1, BEA and FUS. The calculation of the signal suppression/enhancement (SSE) due to matrix effects was performed as follows:

$$SSE\ (\%) = 100 * slope_{with\ matrix}/slope_{without\ matrix} \qquad \text{Eq. (1)}$$

SSE values higher than 100% reflect the enhancement in the signal. SSE values lower than 100% reflect the suppression of the signal. SSE values of 100% indicate that there are not absolute ME. Results are presented in Table 4. There was a suppression of the signal for all mycotoxins (between 53.0 and 78.6 %). For more certain results, matrix effects were evaluated using blank extracts of fresh pasta and dry pasta separately, since they have different compositions. For this purpose, a blank sample of dry pasta and a blank sample of fresh pasta were selected and the same procedure described above was applied for the evaluation of ME. The results indicated that the SSE for dry pasta (SSE_d) and SSE for fresh pasta (SSE_f) were very similar to SSE for the mixture of both types of pasta (SSE_m) (Table 4). Therefore, there was no difference in the use of standards prepared in extracts of dry pasta, fresh pasta or in a mixture of both types. For the evaluation of the other parameters

(linearity, precision, …), matrix-assisted calibration curves were prepared with a mixture of fresh and dry pasta.

Table 4. Linearity (r^2) from calibration curves prepared with and without matrix. Evaluation of matrix effects: signal suppression/enhancement (SSE).

Mycotoxin	r^2		Slope		SSE_m (%) [a]	SSE_d (%) [b]	SSE_f (%) [c]
	Without matrix	With matrix	Without matrix	With matrix			
ENA	0.999	0.997	51.869	40.770	78.6	76.3	79.5
ENA1	0.999	0.999	34.431	23.449	68.1	70.5	69.6
ENB	0.996	0.994	69.706	36.934	53.0	54.1	51.7
ENB1	0.999	0.998	56.057	32.649	58.2	60.9	59.8
FUS	0.998	0.995	72.364	47.390	65.5	63.4	66.2
BEA	0.999	0.992	32.453	22.999	70.9	67.6	67.9

[a] SSE_m: SSE of mixture of blank samples from dry and fresh pasta.
[b] SSE_d: SSE of blank sample from dry pasta.
[c] SSE_f: SSE of blank sample from fresh pasta.

3.4. Method validation

Method validation was carried out according to the guidelines established by the European Union (Commission Decision, 2002; Commission Regulation, 2006). 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. The standards were prepared at six concentration levels (each level was prepared in triplicate):

0.025-25 μg kg^{-1} for ENA, 0.05-50 μg kg^{-1} for ENA_1, ENB, ENB_1, BEA and FUS. Calibration curves were constructed using the analyte peak area versus the concentration of the analyte. The calibration curves showed good linearity with correlation coefficients $r^2 > 0.992$ (Table 4).

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 were calculated using a signal-to-noise ratio of 3. The LOQs were calculated using a signal-to-noise ratio of 10. LODs for ENA, ENA_1, ENB, ENB_1, BEA and FUS were 0.15, 0.08, 0.15, 0.15, 0.02 and 0.03 μg kg^{-1}. LOQs for ENA, ENA_1, ENB, ENB_1, BEA and FUS were 0.5, 0.25, 0.5, 0.5, 0.1 and 0.05 μg kg^{-1}. Values obtained for LOQ and LOD from this study were significantly lower than the values obtained in other published methods for determination of ENs, BEA and FUS in cereal samples (Jestoi, 2008). As far as we know, the LODs of the developed method were only comparable with those obtained in the method developed by Jestoi et al. (2005), which analyzed ENs and BEA in eggs obtaining LODs ranged between 0.1 and 0.7 μg kg^{-1}.

Recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision) were evaluated through recovery studies using spiked blank samples at two concentration levels (LOQ and 100 x LOQ). For this purpose, 1 mL of working solution was added to each blank sample before and after extraction. This addition was performed five times at two concentration levels. Recovery experiments were carried out by comparison of absolute peak areas from each analyte in blanks spiked before the extraction, and the absolute peak areas in blanks spiked after the extraction. Intra-day precision was assessed by five

determinations at each addition level in the same day. Inter-day precision was assessed by one determination at each addition level for five days. The mean recoveries and the RSDs are presented in Table 5. RSD values ranged between 4 and 11% for the intra-day precision, and between 5 and 15% for the inter-day precision. Recovery ranges at the low spiked level (LOQ) and the high spiked level (100 x LOQ) were 85-110% and 86-112%, respectively. Therefore, the results were satisfactory and in accordance with the limits set in the Commission Decision 2002/657/EC: a mean recovery (n=5) between 70% and 120%, and a RSD lower than 20%.

3.5. Commercial samples

The developed method was applied to the analysis of 30 samples of pasta. The results are presented in Table 6. Fig. 4 shows the chromatograms of an extract of dry pasta naturally contaminated (1.26 μg kg^{-1} of BEA, 3.10 μg kg^{-1} of ENB_1 and 2.05 μg kg^{-1} of ENA). Emerging *Fusarium* mycotoxins were detected in thirteen of the total samples (43%). The prevalence of mycotoxins was slightly superior in samples of fresh pasta (50%) than in samples of dry pasta (40%). Two or more mycotoxins were present in 92% of the positive samples (12 samples), while one mycotoxin was detected in only one sample. According to other authors, BEA and FUS were detected in a lower number of samples in comparison with the samples contaminated by ENs (Mahnine et al., 2011). The incidence of ENs type B (100%) were higher than the incidence of ENs type A (77%). This is in accordance with the frequency of contamination obtained in similar works (Mahnine et al., 2011; Sifou et al., 2011).

Table 5. Recovery values (%) and relative standard deviations given in brackets (%) at two concentration levels (μg kg^{-1}).

Mycotoxin	Intra-day precision [a]				Inter-day precisión [b]			
	Low level (LOQs)		High level (100 x LOQs)		Low level (LOQs)		High level (100 x LOQs)	
	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery
ENA	0.5	92 (5)	50	91 (4)	0.5	93 (8)	50	90 (6)
ENA1	0.25	88 (7)	25	86 (9)	0.25	85 (11)	25	88 (8)
ENB	0.5	109 (8)	50	112 (6)	0.5	110 (8)	50	109 (9)
ENB1	0.5	99 (10)	50	97 (11)	0.5	97 (13)	50	95 (15)
BEA	0.05	93 (6)	5	94 (4)	0.05	96 (7)	5	94 (5)
FUS	0.1	94 (13)	10	91 (11)	0.1	93 (14)	10	91 (13)

[a] Number of replicates: 5

[b] Different days: 5

Table 6. Incidence and concentration range of enniatins, beauvericin and fusaproliferin in dry and fresh pasta.

Mycotoxin	Dry pasta		Fresh pasta	
	I (n/N) [a]	Concentration range (μg kg^{-1})	I (n/N) [a]	Concentration range (μg kg^{-1})
ENA	3/20	<0.5 -12.98	5/10	<0.5 -6.93
ENA1	4/20	<0.25 – 0.29	5/10	<0.25 – 1.23
ENB	7/20	<0.5 – 0.58	5/10	0.85 – 3.99
ENB1	7/20	<0.5 – 3.10	5/10	0.52 – 3.63
BEA	2/20	0.62 -1.26	3/10	<0.1 – 0.12
FUS	3/20	<0.05 – 0.13	2/10	<0.05 – 0.11

[a] I: incidence, n: number of positive samples, N: number of total samples

Concerning the contamination levels, the maximum value for the sum of ENs (12.59 μg kg^{-1}) was found in a sample of fresh pasta. The maximum concentrations of ENA (12.98 μg kg^{-1}), BEA (1.26 μg kg^{-1}) and FUS (0.13 μg kg^{-1}) were detected in three different samples of dry pasta, while the highest concentrations of ENA_1 (1.23 μg kg^{-1}), ENB (3.99 μg kg^{-1}) and ENB_1 (3.63 μg kg^{-1}) were found in one sample of fresh pasta. In general, ENs were present at concentration levels higher than BEA and FUS, which is usual in other studies. In 2004, Jestoi et al. (2004) analysed ENs, BEA and FUS in grain-based products from Finland and Italy, and detected ENs in a wide range of concentrations (<0.6-170.0 μg kg^{-1}), while FUS and BEA were not detected in any sample. Recently, several authors have reported high levels of emerging *Fusarium* mycotoxins in cereal-based products. In the year 2010, Meca et al. (2010) found ENs, BEA and FUS in cereals from Spain with concentration ranges of 2.2-814.4, 0.5-11.8 and 1.0-6.6 mg kg^{-1}, respectively, while in

Mahnine et al. (2011) showed the contamination of ENs (0.6-795.0 mg kg^{-1}), BEA (2.1-10.6 mg kg^{-1}) and FUS (0.5-7.4 mg kg^{-1}) in breakfast and infant cereals from Morocco. Analyzing the results from this study, a general conclusion is that emerging *Fusarium* mycotoxins levels were lower than those found in previous studies carried out in different geographic areas. At the moment, the European Union has not proposed MLs for emerging *Fusarium* mycotoxins, and for this reason it was not possible to establish a relationship between the results from this study and the MLs.

4. CONCLUDING REMARKS

The main advantages of the proposed extraction procedure are the rapidity, the application facility and the low cost, since Ultra-Turrax requires economic equipment readily available in any laboratory. Good recoveries and precision in a short period of time were obtained indicating the reliability of the developed method by LC-MS/MS QqQ. This resulted in increased confidence in the mycotoxin identification when the developed procedure was applied to real samples. This is a novelty method for the simultaneous analysis of emerging *Fusarium* mycotoxins applied in samples of dry and fresh pasta. The results showed the presence of ENs, BEA and FUS in 43% of the analysed samples. Further studies are necessary in order to determine the potential presence of emerging *Fusarium* mycotoxins in foodstuffs from different geographical areas.

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3.2. Dispersive liquid-liquid microextraction for the determination of emerging *Fusarium* mycotoxins in water

Food Analytical Methods (Under review)

Dispersive liquid-liquid microextraction for the determination of emerging *Fusarium* mycotoxins in water

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ABSTRACT

A new method was optimized for the determination of emerging *Fusarium* mycotoxins enniatins (ENs) and beauvericin (BEA) in different types of water. Mycotoxin analysis was performed by dispersive liquid-liquid microextraction (DLLME) combined with liquid chromatography-tandem mass spectrometry (LC-MS/MS). Mycotoxins were efficiently extracted from water into carbone tetrachloride by DLLME technique using acetonitrile as disperser solvent. Detection limits were in the range of 0.06-0.17 μg L^{-1}. Quantification was performed using matrix-matched calibration curves over a linear range from LOQ to 200 μg L^{-1}. Acceptable recoveries were obtained between 78.5% and 100.1% with relative standard deviations of <14%. The proposed method may be advised as an easy, sensitive and accurate method for determining emerging *Fusarium* mycotoxins in water. The method was successfully applied to the analysis of different kinds of water. No detectable levels were achieved in surface, ground, tap and bottled water. Concentration levels up to 50 μg L^{-1} were detected in cooking water related to the pasta cooking process.

Keywords: analysis, enniatins, beauvericin, water, DLLME, LC-MS/MS.

1. INTRODUCTION

Mycotoxins play an important role in the human and animal health worldwide. Enniatins (ENs) and beauvericin (BEA) are a group of *Fusarium* mycotoxins receiving importance in the last years. Although adverse effects have not been attributed to these mycotoxins in humans, different toxicological studies have evidenced the cytotoxic activity of ENs and BEA (alone or in combination) lower than micromolar range (Prosperini et al. 2014). The human exposure to ENs and BEA commonly occurs through different cereals and their products, such as bread, pasta or biscuits (Serrano et al. 2013; Capriotti et al. 2014; Hu et al. 2014). Recent studies have showed other less common matrices such as feed and fish, which could be contaminated by ENs and BEA (Tolosa et al. 2015). These studies revealed the diversity of matrices susceptible to mycotoxin contamination. On this account, it has been described that other mycotoxins (deoxynivalenol and zearalenone) can occur in different water sources since large volumes of water are used in agricultural practices and to manufacture foods and feeds (Bucheli et al. 2008; Gromadzka et al. 2009). Mata et al. (2015) reported the presence of aflatoxins and ochratoxin A in commercial bottled waters.

To the moment, no studies have been conducted on the presence of ENs in water, although one study revealed the presence of BEA in environmental waters (Schenzel et al. 2010). In order to improve the data on the human and animal exposure to ENs and BEA, it could be of interest to study the presence of these mycotoxins in different kinds of water, including surface and ground waters, drinking water or water involved in cooking food processes. To achieve this purpose, it is imperative to develop a new analytical methodology to determine ENs and BEA in aqueous samples.

The determination and quantification of ENs and BEA has been usually performed by high resolution liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Concerning the sample treatment, most of the methods available in the literature are based in traditional techniques such as liquid-liquid extraction or solid-liquid extraction, which implicate several disadvantages, mainly the high volume of solvent, high amounts of sample and the long times required for the analysis (Santini et al. 2012). In the last years, alternative methods have been proposed to improve the efficiency of the extraction techniques. One of the most important tendencies in the sample preparation is the simplification and miniturization of the methods, since they provide important advantages in comparison to the conventional techniques. These novel methods imply low sample and solvent volume, fast analysis and great efficiency (Cruz-Vera et al. 2011). Dispersive liquid-liquid microextraction (DLLME) has gained prominence in the recent years as easy, efficient, economical and environmentally responsible methodology to sample treatment. DLLME involves a simple microextraction technique based on the use of an extraction solvent mixed with a disperser solvent. To the date, limited number of studies have been developed to determine mycotoxins by DLLME (Arroyo-Manzanares et al. 2013; Karami-Osboo et al. 2013; Arroyo-Manzanares et al. 2015). To the authors knowledge, no studies have been conducted to the development of miniaturized techniques for the emerging *Fusarium* mycotoxins analysis. The purpose of the current study was to develop a method based on DLLME for the quantitative determination of ENA, ENA_1, ENB, ENB_1 and BEA in samples of water. The applicability of the optimized method was demonstrated by the analysis of different kinds of water.

2. MATERIALS AND METHODS

2.1. Chemicals

Acetonitrile (AcN), methanol (MeOH), dicloromethane (CH_2Cl_2) and carbone tetrachloride (CCl_4) and sodium chloride were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Deionized water was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain) before use. Individual standards of BEA, ENA, ENA_1, ENB and ENB_1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of BEA, ENA, ENA_1, ENB and ENB_1 at a concentration of 1000 μg mL^{-1} were prepared in methanol. These stock solutions were then diluted with pure methanol in order to obtain appropriate working solutions. All solutions were stored in glass-stoppered bottles and darkness in security conditions at -20ºC.

2.2. Sample collection

The applicability of the optimized method was assessed by the analysis of 40 samples of water: 20 samples of commercial bottled water acquired from supermarkets located in Valencia, and 5 samples of surface water, 5 samples of ground water and 10 samples of tap water collected from different areas of Valencia.

The mineralization of the bottled water samples ranged from 131 to 421 mg L^{-1} (according to the bottle label), and the pH ranged from 6.80 to 8.31. The free chlorine content of tap water was of 0.3-0.9 mg L^{-1}. The pH values of

the samples of surface, ground and tap water ranged between 7.20 and 8.00. Measurements of pH were performed employing a GLP21 Crison pH-meter (Crison Instruments, S.A., Barcelona, Spain) with a Hamilton pH electrode (Fisher Scientific, Madrid, Spain). Determination of free chlorine content was carried out with the chlorine test (Merck, Darmstadt, Germany) based on the addition of the N,N´-diethyl-p-phenylenediamine (DPD) reagent to the sample (Standard Methods, 2011).

Moreover, five samples of water from the cooking process of raw pasta were analyzed. The samples of water were collected after 10 min of cooking pasta in boiling tap water.

Method extraction

A mixture of 900 μL of AcN (as disperser solvent) and 100 μL of CCl_4 (as extraction solvent), was rapidly injected into 5.0 mL of water containing 1 g of NaCl. The mixure was shaken on a Vortex for 1 min. Then, the mixture was centrifugated for 15 min at 3500 g (10ºC), and the droplet formed was collected by a 100 μL syringe and it was evaporated to dryness by nitrogen gas at 35ºC using a multi-sample Turbovap LV Evaporator. Finally, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and filtered through 13 mm/0.20 μm nylon filter prior the injection in the LC-MS/MS system.

2.3. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation

was achieved by a Gemini-NX C_{18} (150 mm x 2 mm I.D., 3 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using gradient elution that started at 90% of A (AcN) and 10% of B (10 mM ammonium formate in MeOH), increased linearly to 50% B in 10 min. After, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 mL min^{-1}. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0,5 V; source temperature, 100ºC; desolvation temperature, 300ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L h^{-1}; cone gas 50 L h^{-1} (nitrogen 99.99% purity).

The cone voltage selected was 40 V and the collision energy selected was 35 Ev for all ENs. For BEA, the cone voltage was 40V and the collision energy selected was 40 Ev. 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 99.995% purity) pressure, 3.83 x 10^{-3} mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms.

According with the 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. Mycotoxin quantification was carried out with the primary transition (transition of quantification) and the confirmation with the second transition (transition of confirmation). The follow precursor ion and product ions were selected for each mycotoxin: the precursor ion m/z 681.9 $[M+H]^+$ and the product ions

m/z 228.2 and 210.0 for ENA, the precursor ion m/z 667.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA_1, the precursor ion m/z 639.8 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB, the precursor ion m/z 654.9 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB_1 (Serrano et al. 2013a).

2.4. Method validation

Method validation was performed according to the guidelines established by the European Union (Commission Regulation, 2006). The validation included the determination of linearity, the evaluation of the matrix effect (ME), limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision).

To determine the linearity, calibration curves were constructed for each studied mycotoxin, from standards prepared in methanol and also from standards prepared in extract of blank sample. Two blank samples were selected to develop the experiments: a sample of distilled water and a sample of cooking water from pasta cooking experiments (with no detectable levels of ENs and BEA). The working concentration range was from LOQ to 200 μg L^{-1}.

LODs and LOQs were estimated from two extracts of the above-mentioned samples of water, fortified with decreasing concentrations of the analytes. For 6 days, additions were performed in triplicate from the two samples (n=18 for each sample), to the estimated concentrations for each mycotoxin. The LODs were calculated using a signal-to-noise ratio of 3. The LOQs were calculated using a signal-to-noise ratio of 10.

The ME was evaluated because different constituents present in the matrix can change the instrumental response of the analyte, resulting in a suppression

or enhancement of the signal. ME was calculated by the use of matrix-assisted calibration curves. For ME determination, the slope from the calibration curve prepared with known amounts of standards in extract of blank sample (matrix-matched calibration curve), was compared with the slope from the calibration curve prepared with the same amounts of standards in methanol (standard calibration curve). Two matrix-matched calibration curves were prepared with two different blank samples (distilled water and cooking water). Slopes were compared according to the formula: $\%ME = (slope_{matrix\text{-}matched}/slope_{standard}) \times 100$. ME values higher than 100% reflected an enhancement in the signal intensity. ME values lower than 100% reflected the suppression in the signal intensity. ME values of 100% indicate there were not absolute ME. Values of ME higher than 120% or lower than 80%, were considered as significant signal enhancement or signal suppression, respectively.

Recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision) were evaluated through recovery studies at two concentration levels (10xLOQ and 100xLOQ). Recovery studies were carried out by comparing the area of peaks obtained for the analytes added to the sample before and after the extraction procedure. Intra-day precision was assessed by five determinations at each concentration level in the same day. Inter-day precision was evaluated by one determination at each addition level for five days.

3. RESULTS AND DISCUSSION

3.1. Optimization of the extraction method

As a base, the DLLME method proposed by Berijani et al. (2006) was selected to mycotoxin extraction from water. The method optimization was made by recovery experiments in three replicates using a blank sample of water

spiked at 100 μg L^{-1} of each targeted mycotoxin. The following parameters affecting the extraction efficiency were adjusted: (i) the type of extraction and disperser solvents, (ii) the extraction-disperser solvent ratios, and (iii) the volume of sample. According to other authors, the extraction time is not dependent on the method efficiency (Rezaee et al. 2010; Victor-Ortega et al. 2013).

First, the type of disperser and extraction solvents was optimized. The starting conditions to solvent optimization were selected on the bases of previous studies: 5 mL of sample, 900 μL of disperser solvent and 100 μL of extraction solvent. Besides, it was added 1g of NaCl to the sample, because it has been demonstrated that the addition of the salt give rise to a slight improvement in the extraction efficiency and to facilitate the phase´s separation (Cruz-Vera et al. 2011). AcN and the mixture of AcN/MeOH (50/50, v/v) were selected as disperser solvents, while CCl_4 and CH_2Cl_2 were tested as extraction solvents. The following combinations of disperser-extraction solvents were tested: AcN-CCl_4, AcN-CH_2Cl_2, AcN/MeOH-CCl_4, AcN/MeOH-CH_2Cl_2. Results indicated that the best conditions were acomplished with the mixture of AcN containing CCl_4, with satisfactory recoveries between 88% and 92% (Fig. 1a).

Then, it was studied the effect of the volume on the extraction efficiency. To achieve this prupose, different disperser-extraction solvent ratios were evaluated: 600-200 μL, 900-100 μL, and 1500-500 μL. The disperser-solvent ratios were selected on a basis of previous studies (Antep and Merdivan, 2012; Campone et al. 2012; Lai et al. 2014). Results are showed in Fig. 1b. Comparable recovery values were obtained using 900-100 μL (88-98%) and 1500-500 μL (83-96%). However, it should be noted that with 1500-500 μL,

the ME increased with respect to the other tested ratios. Therefore, it was selected the combination of 900 μL of disperser solvent containing 100 μL of extraction solvent as a good compromise to achieve the best DLLME conditions.

Finally, the volume of sample was optimized by testing 5, 7 and 10 mL of sample. Recoveries lower than 80% were obtained with 7 and 10 mL of sample, while recoveries of 90-100% were achieved with 5 mL. Therefore, a volume of 5 mL was selected as the optimum volume of sample for a reliable and efficient extraction (Fig. 1c).

3.2. Method performance

The method validation was performed in-house for the determination of ENs in distilled water and cooking water from pasta cooking experiments. The analytical results were comparable in both types of water. In order to avoid redundant results, only results relating the blank of cooking water are shown in Table 1. Calibration curves (prepared at six concentration levels from LOQ to 200 μg L^{-1}) showed good linearity with correlation coefficients $r^2 > 0.994$. Considering the data on ME, results were within the acceptable range: %ME between 96%-104% and 98-101% for cooking water and distilled water, respectively. The effects of signal suppression or signal enhancement were not significant. In spite of the small differences between standard and matrix-matched calibration, it was preferred to use the matrix-matched calibration curves in order to improve other validation parameters and for quantification purposes. LODs were of 0.06-0.17 μg L^{-1}, and LOQs were of 0.20-0.58 μg L^{-1}. RSD values ranged between 4.5 and 13.0% for intra-day precision, and between 5.1 and 12.9% for inter-day precision. Recovery ranges at the low

spiked level (10xLOQ) and the high spiked level (100xLOQ) were 78.5-98.9% and 79.9-100.1%, respectively. The validated parameters were in accordance with the limits set in the Commission Decision 2002/657/EC.

Table 1. Analytical parameters for the determination of ENs and BEA in cooking water: correlation coefficient, matrix effect (%), limits of detection and quantification (μg L^{-1}), recoveries (%) and relative standard deviations (%).

Mycotoxin	Correlation coefficient (r^2)	Matrix effect (%) [a]	LOD (μg L^{-1})	LOQ (μg L^{-1})	Intra-day precision [b] Recovery ± RSD (%)		Inter-day precision [c] Recovery ± RSD (%)	
					Low level (10xLOQ)	High level (100xLOQ)	Low level (10xLOQ)	High level (100xLOQ)
ENA	0.999	104	0.06	0.20	79.4 ± 7.2	85.4 ± 7.3	87.6 ± 5.1	79.9 ± 14.1
ENA_1	0.998	101	0.13	0.44	79.6 ± 10.6	81.0 ± 7.8	86.8 ± 9.6	82.5 ± 8.9
ENB	0.994	96	0.17	0.58	78.5 ± 13.0	81.9 ± 5.7	86.8 ± 5.9	83.5 ± 6.3
ENB_1	0.999	98	0.09	0.30	71.6 ± 10.6	85.2 ± 6.8	84.4 ± 6.7	85.3 ± 9.3
BEA	0.996	102	0.06	0.20	98.9 ± 4.5	99.9 ± 7.7	88.9 ± 12.9	100.1 ± 7.6

[a] Matrix effect (%): 100 x (slope of matrix matched water/slope of standard in solvent)
[b] Number of replicates: 5
[c] Different days: 5

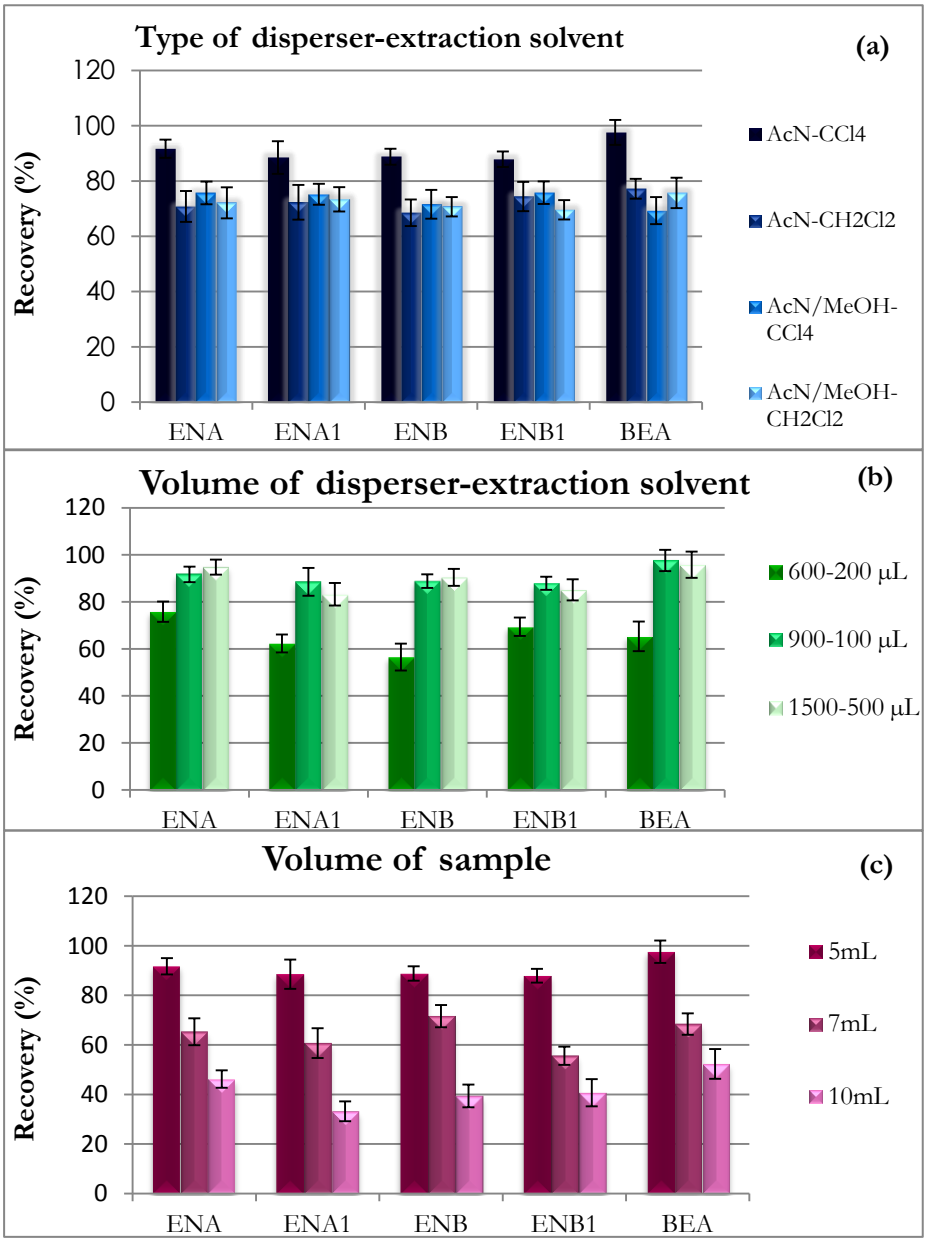

Fig. 1. Recoveries of ENA, ENA_1, ENB, ENB_1 and BEA related to the optimization of different parameters affecting the DLLME efficiency: (a) type of disperser and extraction solvents, (b) volume of disperser-extraction solvent, (c) volume of sample. Error bars represent the standard deviation of the mean recovery for three replicates.

3.3. Application to real samples

The applicability of the method was demonstrated by the analysis of ENs and BEA in different samples of water. As it was expected, the targeted mycotoxins were not detected in the samples of ground, surface, tap and commercial bottled water. To the authors knowledge, no data is available on the presence of emerging *Fusarium* mycotoxins in natural waters. The obtained results are reasonable, since the mycotoxin contamination in water would suggest the fungal growth and mycotoxin production

in different unusual sources, such as the soil of the spring water or water pipeline network (tap water). This fact is not feasible in water for human consumption, since it is necessary an appreciable contamination of organic matter for fungal growth. On the other hand, contradictory results were described by Mata et al. (2015), which surprisingly reported the presence of aflatoxins and ochratoxin A in commercial bottled waters. The authors could not explain these unusual results and they concluded that it should be investigated the origin of mycotoxin contamination in bottled water.

Concerning the results related to the five samples of water collected after the process of cooking pasta, low concentrations of ENs were detected in the samples of water: ENA and BEA were not detected in the samples, ENA_1 levels were between 10 and 50 μg L^{-1}, ENB was detected at levels lower than LOQ, and ENB_1 was detected at concentration levels from 10 to 30 μg L^{-1}.

In Fig. 2 is shown the MRM chromatograms and the daughter ion spectrums of a water sample after the process of cooking pasta, containing 10 μg L^{-1} of ENA_1 and 30 μg L^{-1} of ENB_1.

Initial concentration levels of ENs and BEA in pasta were of 0.21-3.46 mg Kg^{-1}, whereas after the cooking process, the levels in cooked pasta decreased

considerably to 0.04-1.69 mg Kg^{-1}. Considering the low concentration levels detected in the cooking water, it could be concluded that the decreasing levels of ENs perceived in the samples of pasta after the cooking process, cannot be attributed to the leaching of ENs from pasta into the cooking water. According to a previous study conducted by Serrano et al. (2013b), the decreasing levels of ENs achieved during the thermal treatments could be explained by the formation of degradation products structurally related to these mycotoxins.

4. CONCLUSIONS

A new method based on DLLME-LC-MS/MS was developed for the preconcentration and determination of ENs and BEA in water samples. The optimized DLLME to sample preparation offers further advantages, including low operational cost, short extraction time, the use of little laboratory material, and the environmentally friendliness, mainly due to the low required solvent volume. The method was applied to the analysis of different kinds of water. Emerging *Fusarium* mycotoxins were not detected in surface, ground and drinking water, while low concentration levels were detected in water related to the pasta cooking process.

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Conflict of interest

Ana Belén Serrano, Guillermina Font, Jordi Mañes, and Emilia Ferrer declare that they have no conflict of interest.

Ethical approval

This article does not contain any studies with human and animal subjects.

Informed consent

Not applicable

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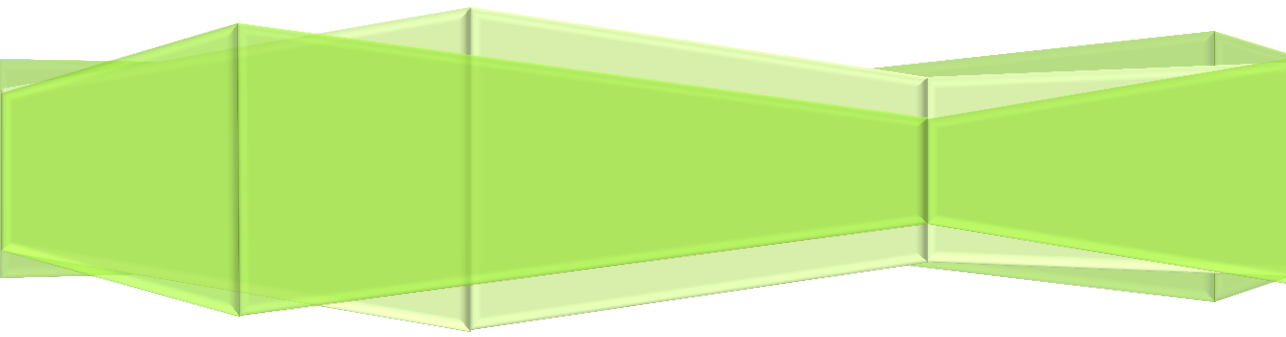
3.3. Development of a rapid liquid chromatography/tandem mass spectrometry method for the simultaneous determination of emerging *Fusarium* mycotoxins enniatins and beauvericin in human biological fluids



Analytical and Bioanalytical Chemistry (Under review)

Development of a rapid liquid chromatography/tandem mass spectrometry method for the simultaneous determination of emerging *Fusarium* mycotoxins enniatins and beauvericin in human biological fluids

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ABSTRACT

A novel method was developed and validated for the simultaneous determination of enniatins A, A1, B and B1 and beauvericin, both in human urine and plasma samples. The method consisted of a simple and easy pretreatment, specific for each matrix, followed by a generic sample extraction and detection by high performance liquid chromatography - tandem mass spectrometry with electrospray ion source. The optimized extraction method was based on a solid phase extraction by graphitized carbon black cartridges after suitable extract dilution, which allowed high mycotoxin absolute recoveries (76-103%) and the removal of the major interferences from the matrix. The method was extensively evaluated for plasma and urine samples separately, providing satisfactory results in terms of linearity (R^2 of 0.991-0.999), process efficiency (>81%), trueness (recoveries between 85 and 120%), intra-day precision (realtive standard deviation, RSD <18%), inter-day precision (RSD<21%) and sensitivity (method quantification limits ranging between 20-40 ng L^{-1} in plasma and between 5-20 ng L^{-1} in urine). Finally, the highly sensitive validated method was applied to some urine and plasma samples from different donors.

Keywords: mycotoxins; enniatins; beauvericin; urine; plasma; liquid chromatography-tandem mass spectrometry

1. INTRODUCTION

Mycotoxins are a group of toxic substances produced by the secondary metabolism of different species of filamentous fungi. Among the genus capable of producing mycotoxins in several commodities [1], *Fusarium* species are probably the most prevalent toxin-producing fungi of the temperate regions of America, Europe and Asia [2]. Mycotoxins and their metabolites present a wide range of adverse effects for the consumer health, including carcinogenic, mutagenic, estrogenic and immunosuppressive effects [3].

Governmental authorities from different nations have general concerns regarding the harmful effects of mycotoxins on human and animal health. Therefore, maximum levels (MLs) have been set in different food products for mycotoxins with recognized advers effects, such as thricotecens A and B, aflatoxins, zearalenone, ochratoxin A, patulin and fumonisins [4]. Furthermore, tolerable daily intake (TDI) or provisional TDI values have been established by the Scientific Committee on Food and the Joint FAO/WHO Expert Committee on Food Additives [5]. In addition to the regulated mycotoxins, currently the attention on the risks posed to human and animal health has also been extended to other potential mycotoxin contaminants, such as the so-called 'emerging' *Fusarium* mycotoxins, especially the structurally related mycotoxins enniatins (ENs) and beauvericin (BEA) [1,6]. The importance of setting legislative measures for ENs and BEAis mainly due to their recently acknowledged toxicity, as proved by *in vitro* studies on several cell lines, including monkey (Vero), rodent (V79), lepidopteran (SF-9) and human cells (Caco-2, Hep-G2, HT-29) [7-9]. For the moment, no reports are available for the toxicity in humans, while *in vivo* preliminary studies on animals showed no observable adverse effects in the treated animals [10,11]. On the other hand,

recent studies concerning ENs and BEA occurrence in a wide range of cereal grains (wheat, barley, rye and oat) and their products have been carried out [12-15]. Results from these studies showed a common mycotoxin co-occurrence and concentrations, ranging from few to several thousands mg kg^{-1}.The actions of these co-occurring mycotoxins within the body represent an interesting subject because synergistic, antagonistic or additive effects could occur. Regarding mycotoxin interactions, Prosperini *et al.* [9] studied the viability of Caco-2 cells evidencing that interactions among different mixtures of ENA, ENA1, ENB and ENB1 could produce a general additive effect.

At present, law limits have not been established for these fusariotoxins, yet. In this regard, the main difficulty is the lack of standardization for the values in the different nations. Current regulations are increasingly based on scientific opinions of authoritative bodies. Recently, the European Food Safety Authority (EFSA) carried out an assessment of the human risk related to the presence of BEA and ENs in food and feed [16]. For this evaluation the combined exposure to ENA, ENA1, ENB and ENB1 was taken into account. Unfortunately, after an exhaustive review of all the available information, EFSA could not perform a reliable risk assessment. This is due to the limited data available regarding human and animal exposure, primarily regarding *in vivo* toxicity. Hence it is important to establish tools for the accurate assessment of human and animal exposure to this group of mycotoxins. Available data indicate that BEA and ENs are absorbed and rapidly metabolised to a range of uncharacterised metabolites [16,17]. Determination of mycotoxins and their main metabolites in body fluids could serve as biomarkers and could facilitate effective exposure assessment, crucial to estimate mycotoxin related disease risk for humans.

For the determination and quantification of mycotoxins in complex matrices, analytical methods based on liquid chromatography- tandem mass spectrometry (LC-MS/MS) have been extensively used [1,18,19]. A wide variety of sample preparations, such as liquid–liquid extraction, solid phase extraction (SPE), accelerated solvent extraction, matrix solid-phase dispersion, and dilute-and-shoot approach have been reported [20]. The effectiveness of the extraction method is always strictly dependent on the nature of the matrix.
To the best of the authors' knowledge, until now, few analytical methodologies have been devoted to the determination and quantification of emerging mycotoxins and their metabolites in biological fluids [1]. Devreese *et al.* [17] validated the first method for the determination of ENA, ENA1, ENB, ENB1 and BEA in pig plasma by LC-MS/MS with satisfactory results. Recently, Juan *et al.* [21] optimized a new method based on a conventional liquid extraction and determination by LC-MS/MS for the quantitative analysis of ENA in serum, urine and feces from Wistar rats. So far, no method on the determination of ENs, BEA and their metabolites in human biological fluids has been reported in the literature. Given the lipophilic nature of ENs and BEA, it could be easier to find the non metabolized form of these mycotoxins in plasma than in urine samples. The determination of the targeted mycotoxins and their main metabolites in urine could be a promising non-invasive alternative. However, currently only *in vitro* phase I metabolites of ENB have been established [22], while no information is available neither for the other ENs and BEA, nor for the phase II and III; for this reason at present only the parent compounds can be determined.

Taking into account the lack of methodology for the extraction of ENs and BEA from human biological fluids, the main aim of this work was the

development of a reliable and sensitive analytical method for the simultaneous determination of ENs and BEA by LC-MS/MS, applicable to human urine and plasma. To achieve this goal, an extraction method specific for the biological fluid, followed by a cleanup based on SPE, was optimized for urine and plasma samples. Finally, after validation, the applicability of the optimized method was demonstrated by the analysis of human samples of urine and plasma.

2. EXPERIMENTAL

2.1. Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), CH_2Cl_2, ammonium formate (99%), HCOOH (> 98%), HCl and $MgSO_4$ were supplied by Sigma-Aldrich (Milan, Italy). All reagents were of analytical reagent grade, solvents were LC-MS grade. Ultrapure water (resistivity 18.2 MΩ cm^{-1}) was obtained using an Arium water purification system (Sartorious, Florence, Italy). In-house Carbograph cartridges were prepared with 500 mg of Carbograph-4 (surface area of 130 m^2 g^{-1} and particle size of 120-400 mesh) purchased by LARA (Rome, Italy) while polypropylene tubes and polyethylene frits were suppied by Supelco (Bellefonte, PA, USA). Carbograph cartridges are similar to Carboprep 200 (Restek, Bellefonte, PA, USA) and Evicarb X (Supelco).

Standards of ENA, ENA1, ENB, ENB1 and BEAwere purchased as powder (Premium Quality Level and/or assay ≥98%) from Sigma-Aldrich (St. Louis, MO, USA). Standard solutions of ENA, ENA1, ENB, ENB1 and BEAwere prepared dissolving 10 mg of each compound in 10 mL of MeOH, obtaining stock solutions with 1mg mL^{-1} concentration. Stock solutions were then diluted with pure MeOH in order to obtain the appropriate working solutions. A composite standard working solution was prepared considering

the intensity response (i.e. sensitivity in the LC-MS/MS measurement) of the target analytes. According to this, the composite standard working solution was prepared by combining aliquots of each individual working solution and diluting with MeOH to obtain the final concentration of 0.02 mg L^{-1} for ENA, ENA1, ENB, ENB1 and BEA. All solutions were stored at −20 °C in amber glass vials and darkness before use.

2.2. Sampling

The applicability of the method was assessed in ten human urine samples and ten human plasma samples. Samples from ten volunteer donors were collected in the early morning. Donors were composed by a group of four men and six women, between 25 and 70 years old, with appropriate physiological conditions. All donors signed an informed consent form before the study. The study was conducted in accordance with the World Medical Association's "Ethical Principles for Medical Research involving human subjects" [23].

Five mL blood and 50 mL urine samples were collected per volunteer. Plasma samples (~2.5 mL) were obtained from whole blood by centrifuging at 1000 g for 5 min to pellet blood cells. The supernatant plasma was removed, split into 250 μL aliquots and stored at −80 °C until further use. Urine samples were centrifuged at 2000 g for 6 min at room temperature to separate sediment. Then, sub-samples of 5 mL per volunteer were aliquoted and stored in a dark and dry place at −20 ºC until analysis. For analysis, all aliquots were thawed at 4 °C and then allowed to warm at room temperature. The samples with undetectable levels of mycotoxins were used for spiking and recovery studies in the method development. For preliminary experiments and

detection/quantification limit evaluation, pools of fresh urine and plasma were used.

2.3. Sample preparation

Sample preparation consisted on an easy sample pretreatment, specific for each fluid, and a mutual cleanup step. A diagram of the extraction method is shown in Fig. 1.

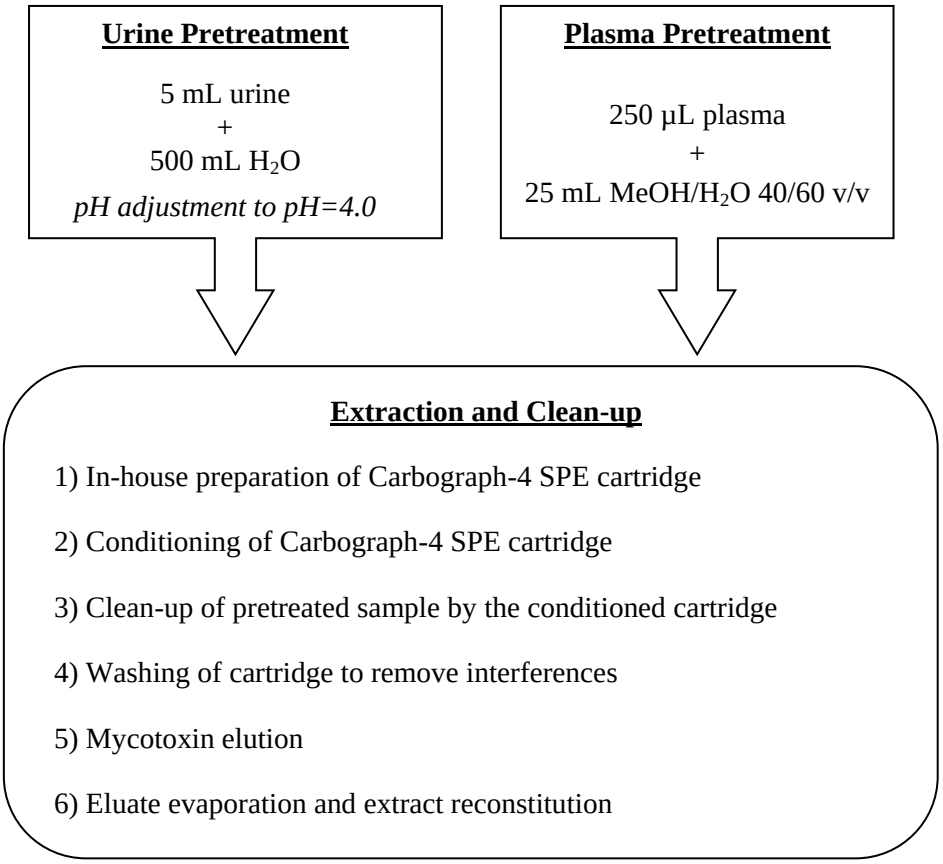

Fig 1. Diagram of the sample preparation process

2.3.1. Sample pretreatments

A 5 mL aliquot of urine was diluted with 500 mL of ultrapure water, and the pH was adjusted to 4 (using a pH-meter) with HCl 1 mol L^{-1} and HCl 0.1 mol L^{-1}. The treated samples were cleaned up using Carbograph-4 cartridges according to the protocol described in the sub-section 'Extraction method'.

A 250 μL aliquot of human plasma was treated with 25 mL of MeOH/water (40/60, *v/v*) to achieve plasma deproteinization. Sample was vortexed for 3 min, centrifuged at 3000 g for 10 min and the supernatant was cleaned up using Carbograph-4 cartridges according to the process described in sub-section 'Extraction method'.

2.3.2. Extraction method

The extraction and cleanup were performed in a single step and the procedure, valid for both fluids,was performed by SPE using a pre-conditioned Carbograph-4 cartridge prepared as reported above. The SPE cartridges were attached onto a vacuum manifold apparatus (Supelco, Bellefonte, PA, USA). First, the cartridges were washed sequentially with 5 mL of CH_2Cl_2/MeOH (80/20, *v/v*) containing 0.2% of HCOOH, 3 mL of MeOH, 10 mL of 10 mmol L^{-1}HCl solution and 5 mL of ultrapure water (at flow rate about 2 mL min^{-1}). Then, the pretreated sample was loaded with a flow rate of 20-25mL min^{-1} for urine samples, and 2-3mL min^{-1} for plasma samples, respectively. The bottle containing the pretreated urine or plasma sample was washed with 100 mL or 10 mL of ultrapure water, respectively, and the washing was passed through the cartridge. Then, 2 mL of MeOH were passed slowly (flow rate of 1 mL min^{-1}) through the cartridge to remove possible interferences without eluting the targeted analytes. Finally, the mycotoxin elution step was performed by

passing through the cartridge 10 mL of CH_2Cl_2/MeOH (80/20, *v*/*v*) containing 0.2% of HCOOH. Vacuum was adjusted to provide a flow rate of 2-3 mL min^{-1}.The eluate was collected into a 1.4 cm i.d. round-bottom glass vial and evaporated to dryness by a gentle nitrogen stream at 37 ºC. The residue was reconstituted with a ACN/water (80/20, *v*/*v*) solution by adding 500 μL for urine and 250 μL for plasma sample, respectively; samples were filtered through 13 mm/0.20 μm nylon membrane syringe filter (Pall Corp., MI, USA) prior to injection into the LC-MS/MS instrumentation.

2.4. LC-MS/MS analysis

For LC-MS/MS analysis, an Ultimate 3000 LC system (Thermo Fisher Scientific, Bremen, Germany) and a TSQ Vantage™ triple-stage quadrupole mass spectrometer (Thermo Fisher Scientific) connected via an electrospray (ESI) source operating in positive ionization mode were used for determination of the analytes. The LC-MS/MS system was managed by the Xcalibur software (v.2.1, Thermo Fisher Scientific).

The LC system consisted of a binary pump connected to a degasser, a thermostatted microwell-plate autosampler set at 14°C, and a thermostatted column oven maintained at 30 °C. The injection volume was 5 μL. The separation was achieved by a Hypersil Gold analytical column (150 mm × 2.1 mm i.d., 3 μm particle size) preceded by a SecurityGuard Hypersil Gold pre-column (4 mm × 2.1 mm i.d., 5 μm particle size), both supplied by Thermo Fisher Scientific. The mobile phase consisted of water (A) and MeOH (B), both containing 5 mmol L^{-1}ammonium formate and 0.1% (*v*/*v*) HCOOH. Gradient elution was started isocratically with 50% B for 1 min. Then B was linearly increased to 99.5% within 6.5 min and kept constant for 3 min. Finally

B was decreased linearly to 50% in 0.5 min and equilibrated for 5 min. Flow rate was set at 300 μL min^{-1}. Retention times of each analyte are reported in Table 1.

Table 1. Retention time, precursor ion, product ions and mass spectrometry optimized parameters for targeted mycotoxins.

Mycotoxin (abbreviation)	Retention time (min)	Precursor ion $[M+NH_4]^+$ (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	S-lens
Enniatin A (ENA)	8.48	699.4	209.7 228.0	35 36	148
Enniatin A1 (ENA1)	8.34	685.2	210.0 228.0	33 33	139
Enniatin B (ENB)	7.97	657.4	196.0 214.0	32 33	137
Enniatin B1 (ENB1)	8.16	671.3	196.0 214.0	33 34	148
Beauvericin (BEA)	8.17	801.3	244.0 262.0	36 34	172

Mass calibrations and resolution adjustments on the resolving lens and quadrupoles were automatically performed using the manufacturer solution introduced by infusion pump at 5 μL min^{-1} flow-rate. In order to optimize tuning parameters for each mycotoxin, 1 ng μL^{-1} of individual standard solutions in MeOH was infused into the instrument. The $[M+NH_4]^+$ ions were selected by the first quadrupole and fragmented in the collision cell with the appropriate collision energy (CE). According to the European Union criteria established for contaminants in food [24], from the MS/MS full-scan spectra, two suitable transitions were selected for acquisition in selected reaction monitoring (SRM) mode. The selected precursor ion, the two most intense

product ions and the optimized SRM parameters (CE and S-lens) of each analyte are presented in Table 1.

Regarding the optimization of general mass spectrometric parameters, the source settings were as follows: 3.2 kV for spray voltages, 280 °C for vaporizer temperature, 220 °C for capillary temperature, 50, 1 and 25 (arbitrary units) for sheat gas pressure, ion sweep gas pressure, and auxiliary gas pressure, respectively.

2.5. Method performance

Performance characteristics of the method included the evaluation of linearity, recovery (yield), process efficiency (PE), sensitivity, trueness, precision (intra- and inter-day precision), method detection limits (MDLs), and method quantification limits (MQLs).

2.5.1. Linearity

Linearity was evaluated by preparing three sets of calibration curves (standard calibration curve, urine and plasma matrix-matched calibration curves) at six concentration levels. Solutions for the standard calibration curve were prepared by diluting composite standard working solution into the solvent over the range 0.02-2.0 ng L^{-1} (0.1-10 pg injected). Plasma and urine matrix-matched calibration curves were prepared by spiking the blank samples with the composite standard working solution in the range of 20-200 ng L^{-1}, although linearity was evaluated in the range of 20-20000 ng L^{-1}. These samples were treated according to the extraction procedure described in the sub-section 'Sample preparation'. Each calibration curve was constructed in triplicate during three consecutive days and results were averaged.

For each analyte the combined ion current profile for the selected transitions was extracted from the LC-SRM dataset, the resulting traces were smoothed by applying the automatic processing smoothing method (Xcalibur) using Gaussian type (7 points). Calibration curves were constructed by plotting the peak area sum of the two transitions versus the mycotoxin concentration. Unweighted regression lines for standard and matrix-matched calibration curves were calculated. The latter calibration curves were used for quantification.

2.5.2. Recovery and process efficiency

Recovery was assessed by comparing the area of peaks obtained for the analytes added to the sample before and after the extraction procedure.

The combined effects of signal suppression/enhancement (matrix effect, ME) and recovery, i.e. PE, was determinated by comparing the slopes of the standard calibration line ($a_{standard}$) with that of the matrix-matched calibration line (a_{matrix}) [25]. The calculation of the PE was performed according to the formula: PE (%)=($a_{matrix}/a_{standard}$)×100.

2.5.3. Method sensitivity

Instrumental limit of detection (ILOD) and instrumental limit of quantification (ILOQ) were calculated to evaluate the sensivity of the optimized LC-MS/MS method. Method sensitivity was evaluated by calculation of MDLs and MQLs for each matrix. For the calculation of detection limits, the standard deviation of the response (σ) was divided by the slope of the calibration curve (S), via the formula: detection limit = 3 σ/S. In the same way, quantification limits were estimated according to the formula:

quantification limit = 10 σ/S. ILOQ and ILOD, MDLs and MQLs were calculated employing data generated from regression statistic performed using standard calibration and matrix-matched calibration, respectively. These values were verified by adding to the samples the concentration obtained by the reported procedure and adjusted by direct sample injection. The sum of the ion currentsof the SRM transitions was considered to determine quantification limits, while the less intense transition was considered to evaluate detection limits.

2.5.4. Trueness and precision

Trueness and precision were evaluated for each matrix at three concentration levels: MQL, 2.5 times MQL and 10 times MQL. To achieve this goal, blank samples of urine and plasma were fortified with an appropriate volume of the composite working standard solution to obtain the above-mentioned concentration levels. Trueness was assessed by measuring the peak area of the spiked samples and comparing this result with that obtained from the matrix-matched calibration at the same analyte concentration; the result was expresses as percentage. The precision of the method was determinated as within laboratory precision using the average trueness. Six replicates for each concentration level were analysed in one day to evaluate intra-day precision. Another three replicates for each concentration level were prepared and analysed on five additional days to estimate the inter-day precision. The method precision was expressed as the relative standard deviation (RSD) of replicate measurements.

Statistical comparisons were performed by ANOVA ($p=0.05$).

3. RESULTS AND DISCUSSION

3.1. LC-MS/MS optimization

A preliminary study was performed to obtain the best instrumental conditions affording high resolution and short analysis time with an optimal analyte separation. Positive and negative ionization modes were tested for all compounds but all the mycotoxins gave better response in positive ionization mode. Sodiated adducts $[M+Na]^+$ exhibited higher signal intensities than protonated adducts $[M+H]^+$ for all mycotoxins. Sodiated adducts were formed because ENs and BEA are ionophoric compounds capable of forming complexes with monovalent and divalent cations through interactions with carbonyl groups oriented within the molecule. Low amount of Na^+ may result from the analytical procedure, mainly from the solvents. In general during the process of fragmentation the sodiated adducts provide low yields in charged fragments different from Na^+, therefore, they are ussually not employed for quantitative purpose. Several authors have reported that sodiated adduct ions can be greatly reduced by adding to the mobile phase modifiers suitable to promote NH_4^+ adduct formation [1,26-28]. In this sense, in the present work it was evaluated the addition of ammonium formate and HCOOH to both mobile phases. The results indicated that the addition of above-mentioned modifiers resulted in an enhancement on the abundance of $[M+NH_4]^+$ and $[M+H]^+$ ions. All mycotoxins gave the highest signal intensity employing a LC mobile phase with water (A) and MeOH (B), both with 5 mmol L^{-1} ammonium formate and 0.1% (*v*/*v*) HCOOH. In these conditions, $[M+NH_4]^+$ adduct prevailed on $[M+H]^+$ formation, and thus the MS/MS parameters were optimized for each compound in order to select the two most intense

transitions of the $[M+NH_4]^+$ adducts. Table 1 shows the list of precursor and product ions of all analytes as well as the optimized S-Lens and CE. Mycotoxin quantification was performed summing the transitions.

Apart from the mobile phases used for the separation, other chromatographic parameters were optimized, such as injection volume and column temperature. Injection volume was set to 5 μL, because larger injection volumes increased the ME. Furthermore, analyte separation was improved by testing different column temperatures (25, 30 and 40 ºC). Thermostated column at 30 ºC resulted in a better mycotoxin separation, but ENB1 was not separed from BEA. This fact does not represent an actual problem, as the two compounds have different MW and the expected concentrations should not cause each other any ME.

3.2. Optimization of the extraction method

Although LC-MS/MS is a powerful technique and direct analysis or methods with a little sample pretreatment are possible in some cases, results could be affected by a heavy ME, which could lead to a low sensitivity. In addition, the presence in biological samples of isobaric interferents giving the same transictions may sometime cause inaccuracy of the final results. Moreover, dirty extracts can result in progressive column detereoration, as well as signal weakening. Starting from these points, the effect of different factors on the extraction method was examined to develop suitable sample preparation procedures for urine and plasma, according to the individual features of each biological fluid. All parameters were tested by recovery experiments in six replicates at the 50 ng mL^{-1} level for each mycotoxin. Recovery was considered acceptable in the range of 70-120%.

3.2.1. Urine

A modification of the SPE procedure described earlier by Capriotti *et al.* [14] for the cleanup of a biscuit extract was initially evaluated for the extraction of ENs and BEA from urine. The rationale for the sample dilution and pH adjustment is explained in that reference and references therein. Briefly, one of the main advantages of using GCB sorbents is the ability to retain organic analytes from large volumes of water or aqueous samples without breakthrough. However, the presence of other organic substances in the sample at relatively high concentration can cause the displacement of some low abundance compounds. Sample dilution and relatively low acidic pH values provide an attenuation of this phenomenon, increasing recovery of some analytes in many cases. In the present work, recovery of the selected analytes still increased about 10-15% by increasing the urine sample dilution with water from 250 to 500 mL. However, in this way some interfering compounds that increased blank background and ME were retained and recovered as well. Suitable modifications which could be tested to tackle this problem and reduce the presence of interferences are the introduction of a washing step and the tuning of elution volumes. Therefore we started with the introduction and optimization of a washing step. On the bases of previous experiences [29] MeOH was selected as the best washing solvent to remove interferences without eluting analytes of interest and was tested at three different volumes (2, 4 and 5 mL) while the solvent volume for analyte elution was fixed to 10 mL. A washing volume of 2 mL significantly decreased the ME, while larger washing volumes resulted in a significant loss of all the analytes (recoveries from 63 to 82% for 4 mL washing). After optimization of the washing step, we moved on to the elution one. In fact, another critical point of SPE was the

elution step, during which the retained analytes were eluted from the sorbent. ENs and BEA were eluted from GCB cartridges using CH_2Cl_2/MeOH (80/20, *v*/*v*) containing 0.2% HCOOH [14]. The solvent volume for elution was optimized by testing 5, 10 and 15 mL. Recoveries of the analytes increased with increasing eluent volume. However, it was found that with 15 mL elution volume recoveries were in the range of 90-99%, but the ME significantly increased. Therefore, a 10 mL eluent volume was chosen as the best compromise.

After method optimization, the recoveries were compared with those obtained by a recently published method for the extraction of other mycotoxins in pig urine, based on the technique salting-out assisted liquid/liquid extraction (SALLE) [30]. In both cases the concentration in the samples was 50 ng $L^{-1}$and results are shown in Table 2. As can be see, for all the compounds recoveries obtained with this method were significantly higher than the ones obtained with SALLE.

3.2.2. Plasma

In early experiments we tried to prepare the plasma sample by the simplest available approach, according to Devreese *et al.* [17]. Plasma samples, spiked at 50 ng L^{-1}, were deproteinized with ACN, employed in a 3:1 (*v*/*v*) ratio to plasma. Then, after centrifugation, the supernatant was withdrawn, diluted with 25 mL of water and cleaned up with the GCB cartridge as already described for urine. However, recoveries were not satisfactory for all the analytes. In order to evaluate the effect of the precipitation step, we carried out a test by adding the analytes to the sample after protein precipitation, obtaining quantitative recoveries (data not shown). A possible explanation for this observation could

have been that ENs and BEA could be adsorbed by plasma proteins. Following this reasoning, another deproteinization mixture was tested, formed by 25 mL of MeOH/H_2O, 40:60 *v*/*v*. The supernatant recovered after centrifugation was then cleaned up without further manipulations. As shown in Table 2, the 250µL plasma treatment with 25 mL of MeOH/H_2O followed by Carbograph cleanup gave better recoveries (76-103%) than those obtained using the simple ACN/plasma 3:1 (*v*/*v*) protein precipitation (62-92%).

Table 2. Comparison of recovery (yield) of the proposed method with two published methods for urine and plasma pretreatment. Samples were spiked at 50 ng L^{-1}.

Mycotoxin	**Recovery ± RSD[a] (%)**			
	URINE		**PLASMA**	
	This method	SALLE[b] [30]	This method	Deproteinization with ACN [17]
ENA	92 ± 6	85 ± 7	99 ± 7	77 ± 15
ENA1	80 ± 10	64 ± 4	90 ± 3	73 ± 14
ENB	82 ± 1	60 ± 9	97 ± 8	92 ± 7
ENB1	95 ± 4	75 ± 5	76 ± 3	88 ± 6
BEA	87 ± 4	73 ± 11	103 ± 12	62 ± 13

[a]Relative Standard Deviation.

[b]Salting-out Assisted Liquid/Liquid Extraction; the extraction was not tested for the reported mycotoxins in the original work.

3.3. Method performance

3.3.1. Linearity, recovery and matrix effect

Linearity was tested by evaluation of determination coefficients (R^2). The linear range was estimated for both standard and matrix-matched calibration curves over the range reported in Experimental section. Results are summarized in Table 3. Mycotoxin calibration regression lines prepared in solvent, blank urine and blank plasma samples showed excellent R^2 in the range 0.991-0.999, 0.991-0.999 and 0.993-0.999, respectively.

As determined by us, the ratio of the slope between matrix matched and standard calibration lines include the effects of recovery and the ME, while the RSD represents the effect of the individual to individual sample variability.

Very often, when a suitable IS is not available, the presence of ME is a major drawback of method performance, because different constituents of biological fluids can lead to a significant suppression or enhancement on the analyte response. Moreover, within the same sample typology, ME variations can be observed from sample to sample. Considering the data shown in Tables 2 and 3, the product of the two effects (PEs) were within the acceptable range (± 20%) both for urine and plasma samples, although the ME is in one case +28%. In spite of the small differences between standard and matrix-matched calibration, we preferred to use the matrix-matched calibration in order to improve trueness and precision.

Table 3. Linearity reported as determination coefficient (R^2) and matrix effect (%) for targeted mycotoxins in urine and plasma samples.

Mycotoxin	R^2			Slope of regresion line (relative standard deviation, %)			Process Efficiency (%)[a]	
	Solvent	Blank urine	Blank plasma	Solvent	Blank urine	Blank plasma	Urine	Plasma
ENA	0.998	0.999	0.993	112.4 (1.7)	112.5 (2.3)	116.3 (5.7)	100.0	103.5
ENA1	0.995	0.991	0.994	25.6 (1.4)	26.7 (3.2)	27.9 (5.6)	108 .2	109.0
ENB	0.998	0.993	0.994	77.8 (2.0)	77.7 (2.1)	77.6 (4.8)	99.9	99.7
ENB1	0.991	0.995	0.997	65.7 (1.7)	53.7 (2.1)	67.0 (3.8)	81.7	102.0
BEA	0.999	0.998	0.999	118.2 (1.0)	100.0 (2.4)	129.4 (2.4)	84.6	109.5

3.3.2. Detection and quantification limits

When operating in SRM mode with the last generation triple quadrupole mass spectrometers, it is quite common to obtain SRM signals without noise. Being so, the calculation of LODs and LOQs becomes quite challenging. They might be estimated from regression statistics using the standard errors on the intercept coefficient via the formulas: LOD = 3 σ/S, and LOQ =10 σ/S, as described in Experimental section. As this method presupposes the homoscedasticity of variance, the extrapolated concentrations have to be used to prepare spiked samples which are then analyzed to verify the conformity to the extrapolated value.

Moreover, following the indication and the intendment of the 2002/657/CE, three conditions have to be satisfied: first, two SRM transitions have to be considered for identification; second, the relative intensity of the

detected ion shall correspond to those of the calibration standard, under certain conditions set, within set tolerances; third, it makes no scientific sense to quantify a compound that has not been confirmed. The second condition is rarely taken into account, though, in our experience, sometimes it becomes the limiting factor. The third condition, not reported explicitly in the 2002/657/CE, is mostly ignored at all and LODs are calculated on the most intense transition.

Another question arises from the fact that many authors differentiate between the quantifier (most intense) and qualifier (less intense) transitions. Once more, in our experience, especially at concentrations near the quantification limits, the RSD of the transition sum is lower than the RSD of the most intense one (unless the second transition is much less intense than the first one). This fact has a logical explanation and originates from the smoothing process necessary to obtain a measurable peak for very low concentrations. In addition, as the limiting factor could be the transitions intensity ratio, both transitions have to be studied also for detection limits.

Therefore, for each analyte, the limits of detection and quantification were extrapolated as reported in the 'Method sensitivity' sub-section, considering the second most intense transition area or the sum of the transition areas, respectively. Then, standard solutions and samples fortified at the extrapolated level were prepared, processed and injected six times. Finally, dataset was evaluated in terms of RSD of the areas and mean transitions ratio. An acceptable value of 20% of the RSD for quantification limits and 50% for the detection limits was arbitrarily set, whereas the acceptable differences established by 2002/657/EC were considered for the ratio between the areas. When both conditions were respected a more diluted sample was prepared,

whereas a more concentrated sample was tested if not. To avoid to repeat the operation too many times, concentration variations were 50-100%. Results are shown in Table 4. Fig. 2 shows the extract masses for the two transitions of the analytes at 0.1 ng injected in standard solution, whereas in Fig. 3 and Fig. 4 the same profiles are reported for plasma and urine, respectively, fortified at 20 ng L^{-1}. As can be seen, the experimental limits were of the same order of magnitude of the extrapolated ones. In addition, by adding to the matrix matched calibration line for urine the concentration corresponding to experimental verified MDL the R^2 did not change significantly. These facts might be due to the restricted concentration range within which calibration lines were considered for expected concentration quantification (the range of linearity covers about 5 orders of magnitude). In some cases detection limits and quantification limits were very similar or even the same: these cases were not in compliance with the provision established by 2002/657/EC regarding the mean value variation of the area ratio between the two transitions. Looking at Fig.s 2, 3 and 4 this evaluation of detection limits and quantification limits could sound highly conservative with respect to other procedures reported in the literature in which the ratio between the two transitions were not considered, nevertheless they appears to be adequate for the assessment of human exposition to these mycotoxins and five times lower than that reported for plasma [17] as far as 500 times lower than that reported for ENA in urine [21].

3.3.3. Trueness and precision

Trueness of the method was evaluated as mean observed concentration/spiked concentration x 100 at three concentration levels, i.e. MQL, 2.5 MQL and 10 MQL, whereas the RSD of average recovery was employed to evaluate method precision (intra- and inter-day; n=5). Recovery and RSD values regarding urine and plasma samples are shown in Table 5. Following the criteria relied on the 2002/657/EC, when trueness and precision are assessed by analyte addition to the matrix, an average recovery ≥90% and repeatability (RSD) <20% should be obtained. As can be seen, although few recoveries were <90%, none of them was significantly different from the required values.

Table 4. Instrumental limit of quantification (ILOQ) and instrumental limit of detection (ILOD), method detection limit (MDL) and method quantification limit (MQL) for urine and plasma, extrapolated (Ext) and experimental (Exp) values.

Mycotoxin	Instrumental				Urine				Plasma			
	ILOQ (pg)		ILOD (pg)		MQL (ng L^{-1})		MDL (ng L^{-1})		MQL (ng L^{-1})		MDL (ng L^{-1})	
	Ext	Exp	Ext	Exp	Ext	Exp	Ext	Exp	Ext	Exp	Ext	Exp
ENA	0.1	0.2	0.03	0.2	25	10	8	10	65	40	20	40
ENA1	0.5	0.1	0.20	0.05	35	10	10	5	65	20	20	10
ENB	0.1	0.05	0.04	0.1	15	5	5	2.5	55	20	15	10
ENB1	0.1	0.05	0.03	0.05	15	20	5	20	45	20	15	20
BEA	0.3	0.2	0.10	0.05	30	10	8	5	30	40	10	20

Table 5. Trueness and precision in urine and plasma samples. Trueness was assessed by measuring the peak area of the spiked samples and comparing this result with that obtained from the matrix-matched calibratiaton at the same analyte concentration; the result was expresses as percentage.

Mycotoxin	Urine						Plasma					
	Trueness % (RSD%)						Trueness % (RSD%)					
	1 × MQL		2.5 × MQL		10 × MQL		1 × MQL		2.5 × MQL		10 × MQL	
	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day	Intra-day	Inter-day
ENA	92±12	88±15	109±8	90±9	102±1	96±14	99±17	101±15	120±6	112±11	95±4	99±11
ENA1	85±10	98±8	96±7	96±11	91±6	94±8	90±13	92±16	114±10	118±14	94±6	93±10
ENB	87±7	110±17	89±13	90±9	98±1	102±10	97±18	95±21	109±12	117±14	110±8	105±9
ENB1	101±14	102±11	89±6	95±8	103±4	90±12	106±13	87±14	112±9	115±9	95±9	98±9
BEA	93±12	97±14	105±10	98±10	101±3	87±12	103±12	91±12	106±10	114±12	96±10	92±11

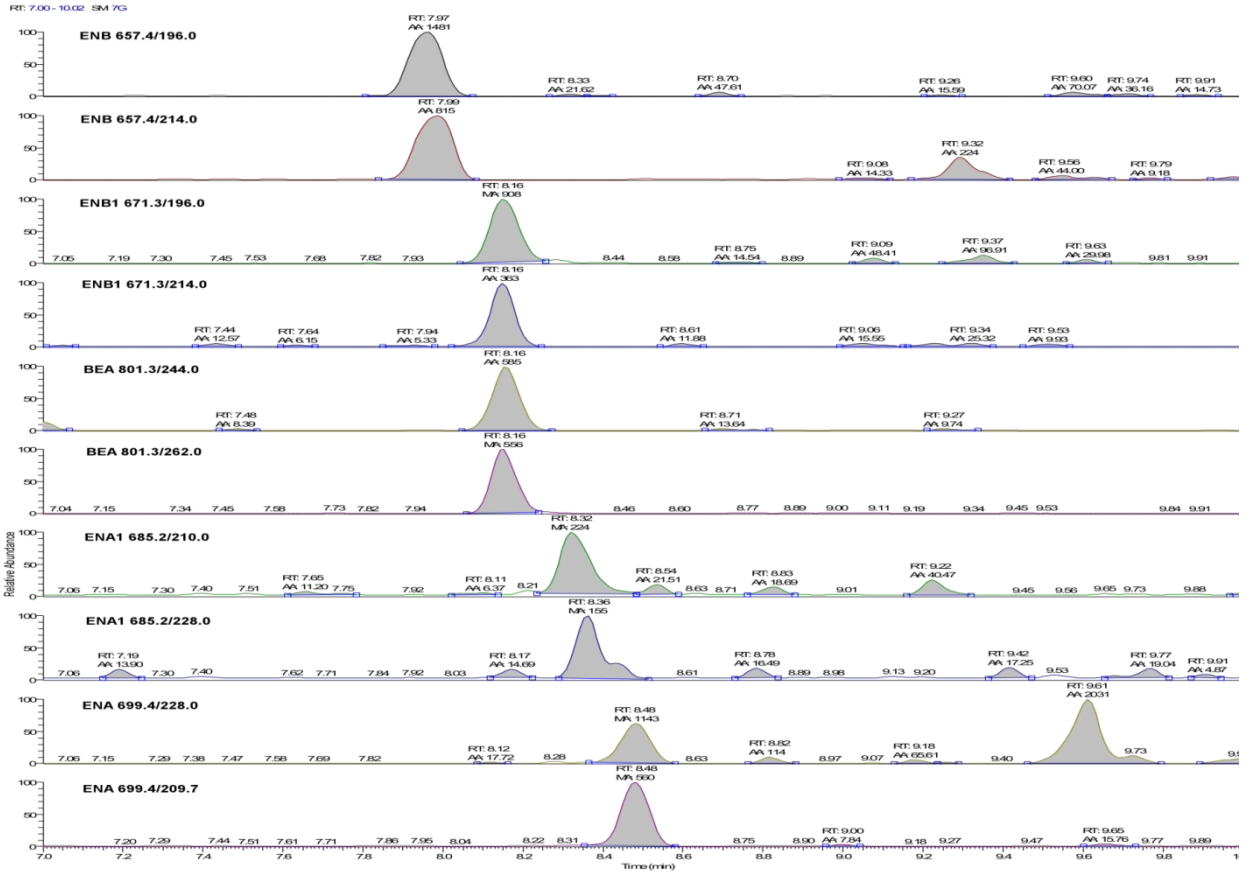

Figure 2. Extract masses for the two transitions of the analytes, 0.1 ng injected in standard solution.

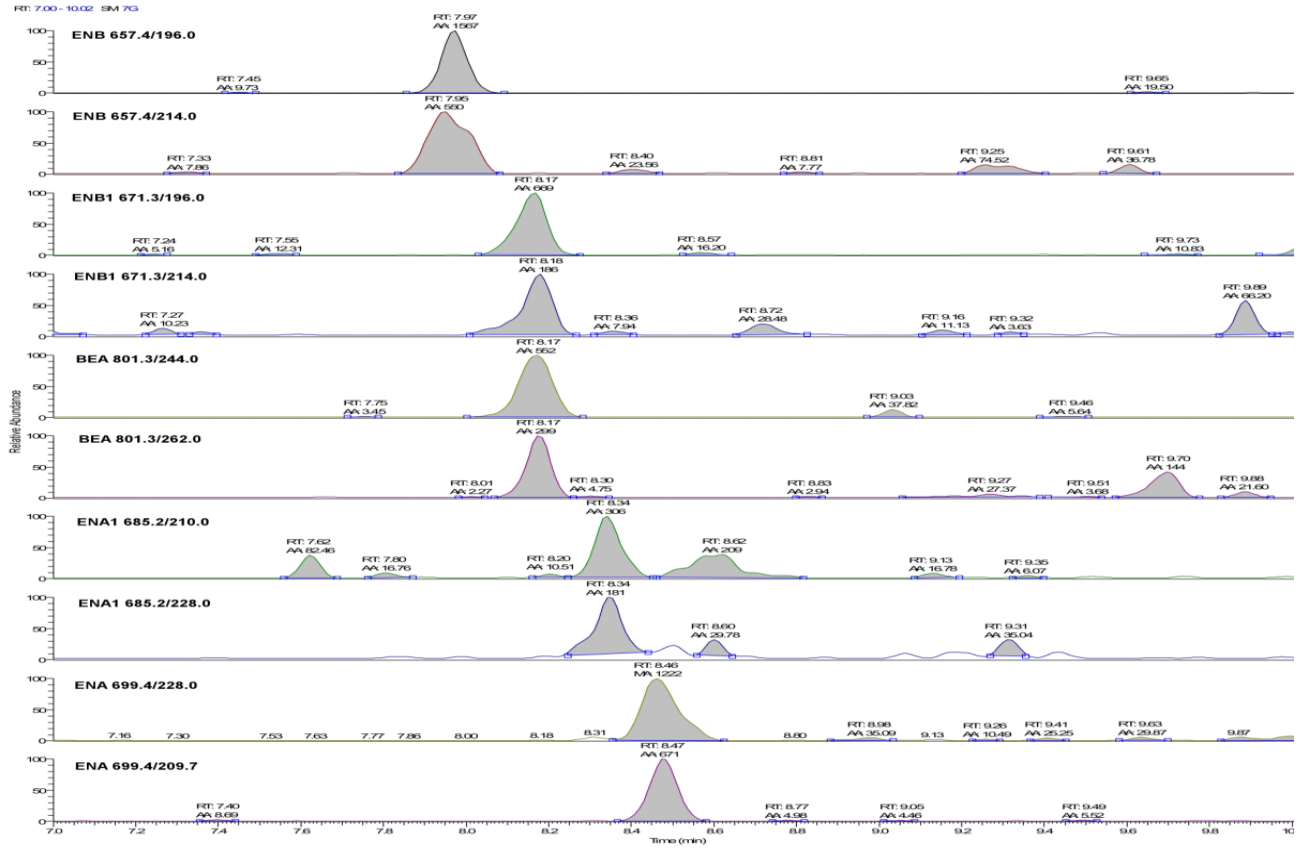

Figure 3. Extract masses for the two transitions of the analytes for a plasma sample fortified at 20 ng L^{-1}.

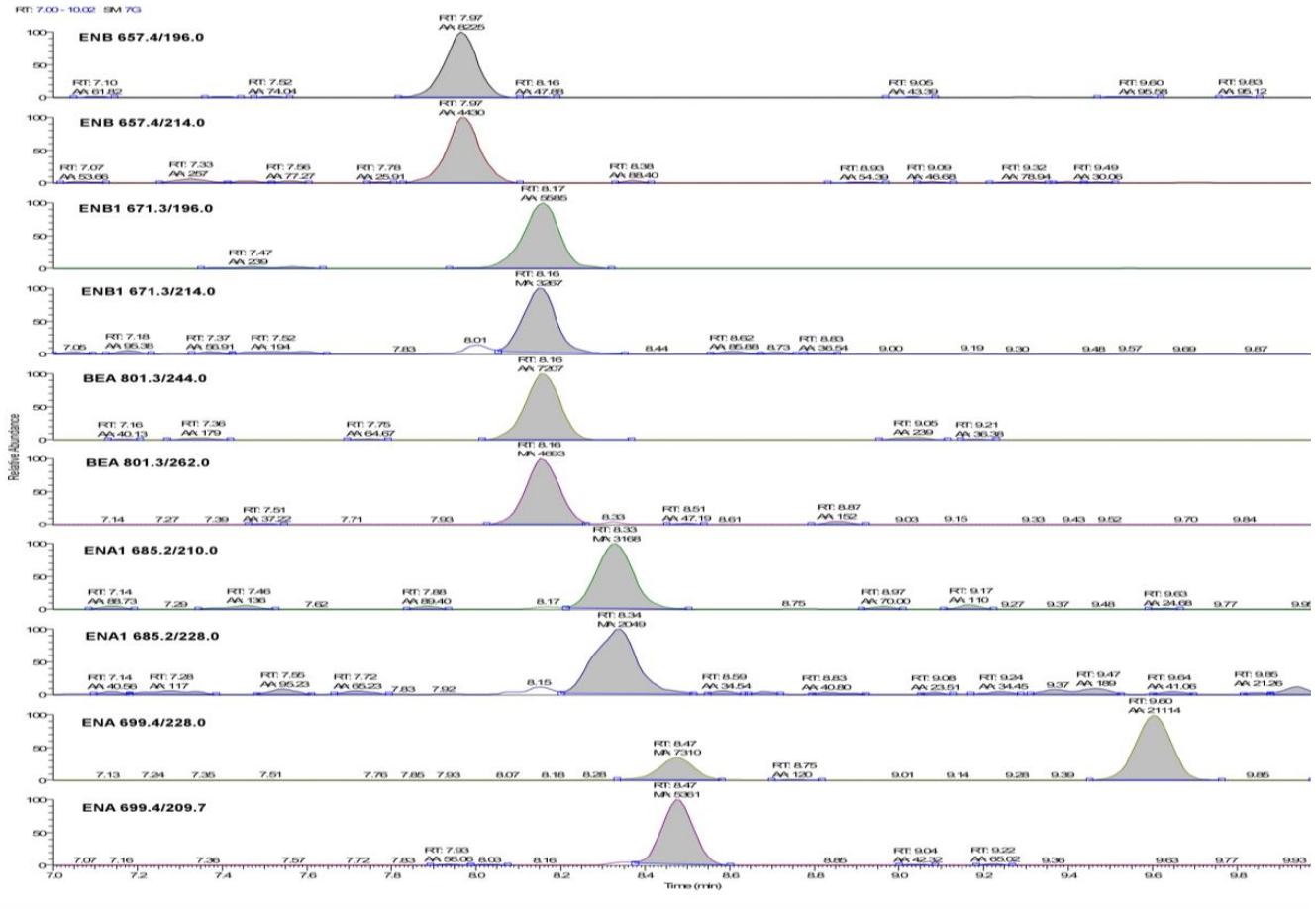

Figure 4. Extract masses for the two transitions of the analytes for An urine sample fortified at 20 ng L^{-1}.

3.4. Application to samples

The suitability of the method was finally tested by analyzing ten samples of human urine and ten samples of human plasma according to the optimized methods. ENs and BEA were not detected in 9 analyzed samples of both plasma and urine. Fig. 5 and Fig. 6 show the chromatograms of a sample of plasma and urine, respectively, taken from the same subject in which some traces of ENB, under MDLs, might be present but were not confirmed. This fact could be the consequence of a rapid absorption, distribution and biotransformation of ENs and BEA to a range of mainly uncharacterized metabolites [16,22]. Until now, the mycotoxin occurrence in biological fluids of different animals has been evaluated only in two studies, which were conducted on animals previously treated with a known, relatively large amount of target mycotoxins [17, 21]. Therefore, it is obvious that results from the present study related to human fluids were not comparable to those obtained in the above-mentioned studies. However, the present study was in agreement with the studies on animals which recognized that ENs show a rapid elimination rate. On the other hand, the *in vitro* metabolism of emerging *Fusarium* mycotoxins has been rarely studied. To the moment only phase I metabolism of ENB has been characterized [22]. In this study, the authors reached to identify a total of 12 biotransformation compounds, being the oxidation and *N*-demethylation of ENB the major metabolic pathways. According to [17], there is a vast difference in oral absorption as well as in the metabolization routes between the different ENs and BEA, although they are structurally similar compounds. Interestingly, ENB1 resulted the most absorbed after oral administration to a pig [17].

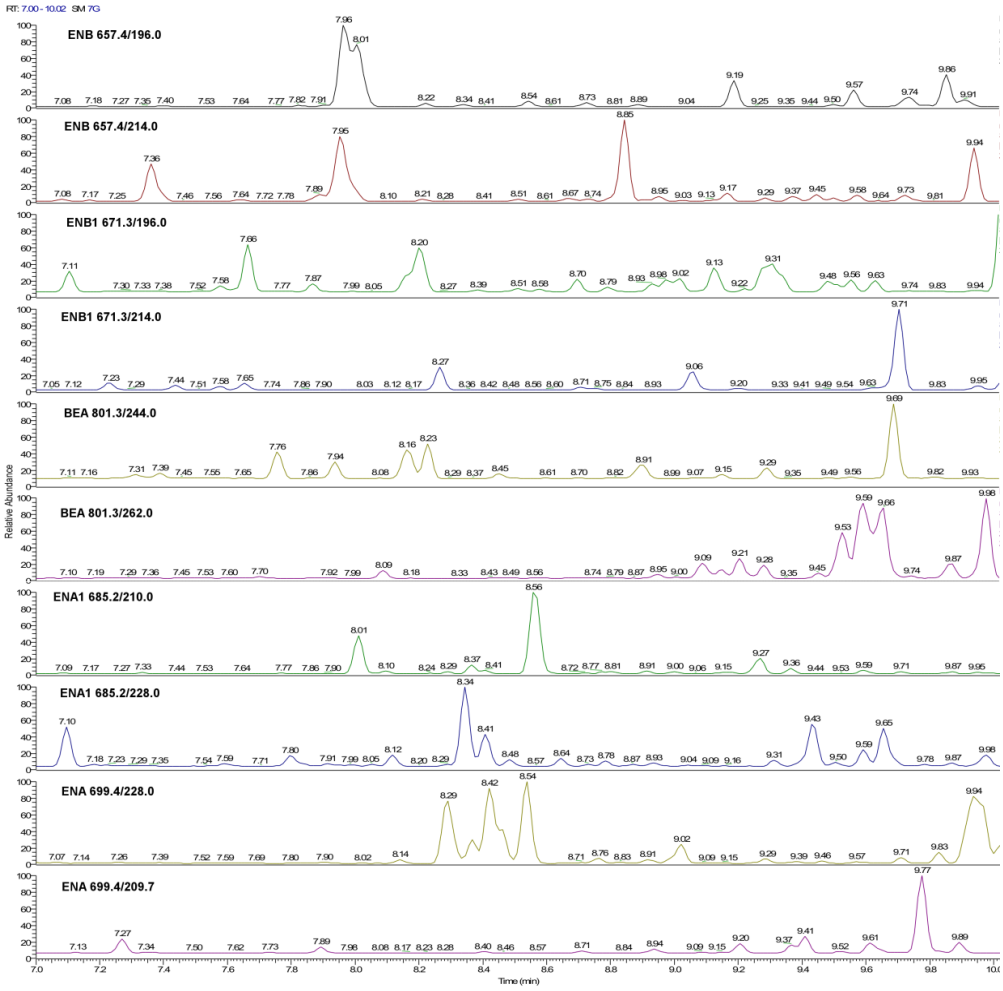

Figure 5. Extract masses for the two transitions of the analytes in blank plasma sample

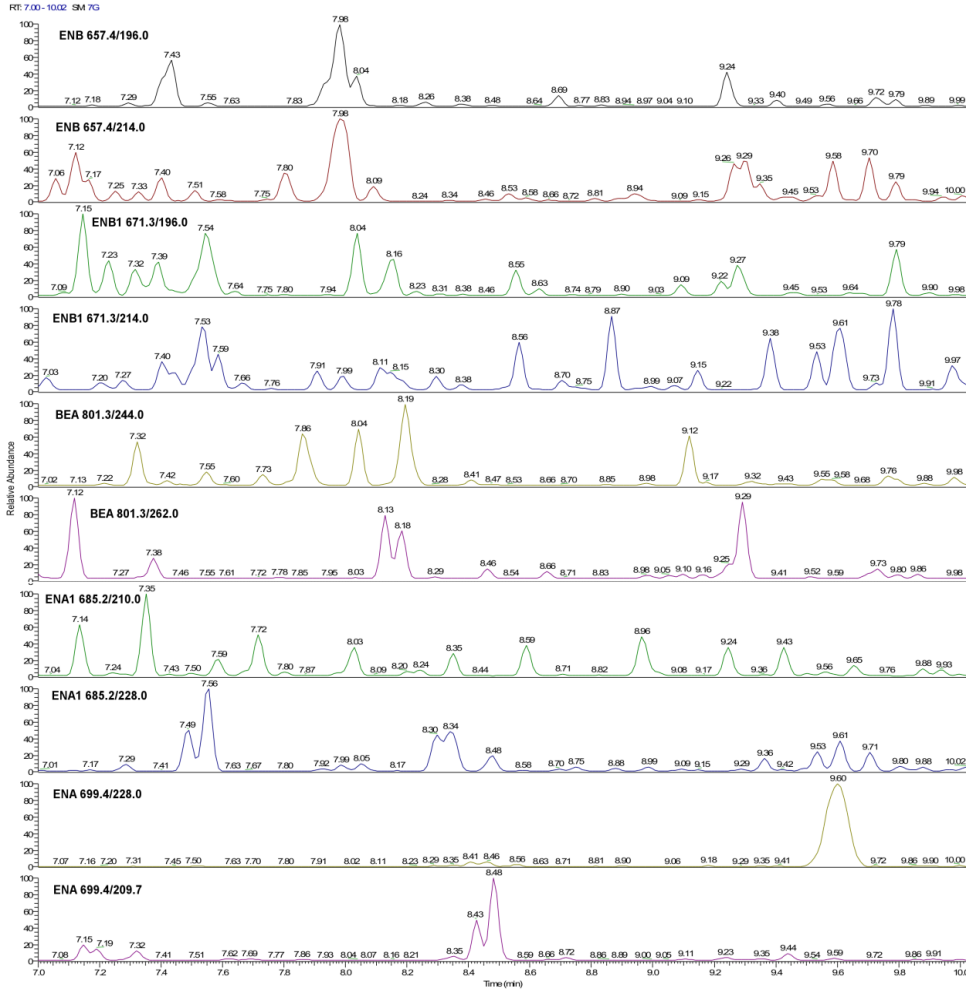

Figure 6. Extract masses for the two transitions of the analytes in blank urine sample.

4. CONCLUSIONS

In this study, rapid and sensitive methods for the determination of ENs and BEA in human biological fluids were developed and fully validated. The two methods differ only for the pretreatment step, which is sample-type specific. Regarding to plasma matrix, the MDLs of the present method were 5-10 times lower than those reported in a previous work for the determination of ENs and BEA in pig plasma [17].

The analysis of 10 subjects, who followed their normal diet, basically a Mediterranean diet, rich in cereals, with no particular indications, did not show any parent mycotoxin either in plasma or urine. This fact does not mean that ENs and BEA cannot present a problem for human exposure, but only that this problem is of minor impact in countries where mycotoxin level controls are extensive.

In any case, the proposed extraction coupled to LC-MS/MS method offered a reliable quantitative analysis of target mycotoxins. This is the first report on the presence of emerging *Fusarium* mycotoxins in urine and plasma of humans. Owing to the fact that target mycotoxins were not detected in any sample, it would be of great interest to characterize the routes of metabolization of each emerging *Fusarium* mycotoxin for a better screening of human exposure in future biomonitoring studies, which would therefore also include metabolite determination.

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3.4. Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area

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Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area

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ABSTRACT

The contents of fourteen mycotoxins were studied in samples of different representative cereals and cereal products from four countries of the Mediterranean region. Two hundred and sixty five samples from Spain, Italy, Morocco and Tunisia were analysed. Samples were extracted with Matrix Solid-Phase Dispersion (MSPD) and determinated by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a triple quadrupole (QqQ) mass analyzer. The percentage of total samples contaminated was 53%. The frequency of contaminated samples from Spain, Italy, Tunisia and Morocco was of 33%, 52%, 96% and 50%, respectively. Nivalenol and beauvericin were the most predominant mycotoxins. This is the first international report that has studied the simultaneous presence of multimycotoxins in different types of cereal (rice, wheat, maize, rye, barley, oat, spelt and sorghum) and cereal products (snacks, pasta, soup, biscuits and flour) from the Mediterranean area, and has estimated the intake of mycotoxins and evaluated the risk assessment.

Keywords: mycotoxins, cereals, LC-MS/MS, risk assessment

1. INTRODUCTION

Mycotoxins are common contaminants of many grains like wheat, barley, maize and rice, and they can evoke a broad range of toxic properties including carcinogenicity, neurotoxicity, as well as reproductive and developmental toxicity. The total number of mycotoxins is not known, but the number of potential toxic metabolites of fungi has been estimated to be in the thousands, although to date only about 300 different mycotoxins have been identified (Krska et al., 2008).

To ensure food safety, the European Union has established maximum levels (MLs) for some mycotoxins in human foods (zearalenone, ochratoxin A, deoxynivalenol, aflatoxins and fumonisins) (EC 401/2006), whereas for others (e.g. nivalenol and beauvericin) maximum tolerable levels have not yet been proposed. For the performance of the current legislation, it is fundamental to have reliable and accurate mycotoxin analytical methods. An important and critical step is sample preparation and sample clean/up. Different strategies have been performed according to the sample and analyte properties, including solid phase extraction (SPE), matrix solid-phase dispersion (MSPD), liquid-liquid and solid-liquid partitioning, accelerated solvent extraction (ASE), multifunctional columns (MFC) and immunoaffinity columns (IAC) (Krska et al., 2008; Rubert, Soler, & Mañes, 2011). Several analytical methods were used for determined mycotoxins, including enzyme-linked immunosorbent assay (ELISA) and gas chromatography (GC) with mass spectrometric detection (MS). Nowadays, liquid chromatography (LC) based methods are the most frequently applied, with several analysers such as fluorescence detector (FLD), single-quadrupole mass spectrometer (MS), time of flight mass spectrometer (TOFMS), electrospray ionization tandem mass spectrometer (ESI-MS/MS),

triple-quadrupole tandem mass spectrometer (MS/MS QqQ) and ion trap tandem mass spectrometer (MS/MS QTrap) (Krska et al., 2008). In the last few years, increased efforts have been made to develop analytical methods for the detection of very low concentrations of mycotoxins in cereal samples and for the simultaneous determination of different classes of mycotoxins using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Recently, in our laboratory, a new rapid, sensitive and reproducible analytical strategy has been developed to determine 14 mycotoxins in cereal products using a MSPD and LC–MS/MS with a triple quadrupole (QqQ) mass analyzer (Rubert, Soler, & Mañes, 2011).

Several studies reported high contamination in a wide variety of important agricultural products world-wide: usually fumonisins (FBs), trichothecenes (TCs), zearalenone (ZEN), aflatoxins (AFs), ochratoxin A (OTA) and beauvericin (BEA) have been found to contaminate grains and cereal-based products (corn, rice, sorghum, wheat, barley and oats) (Gaumy, Bailly, Burgat, & Guerre, 2001; Soriano & Dragacci, 2004; Araguás, González-Peñas, & López De Cerain, 2005; Adejumo, Hettwer, & Karlovsky, 2007; González-Osnaya, Cortés, Soriano, Moltó, & Mañes, 2011). Specifically, the Mediterranean area has a climate characterized by high humidity and high temperature which favor natural occurrence of mycotoxins in the pre-harvest and/or during transport, processing or storage (SCF, 2002).

In 2001 the Joint FAO/WHO Expert Committee on Food Additives (JECFA), established the provisional maximum tolerable daily intake (PMTDI) for certain mycotoxins (deoxynivalenol, sum of toxin T-2 and toxin HT-2, ZEN, FBs), and the provisional maximum tolerable weekly intake (PMTWI) for OTA in food (FAO/WHO, 2001). In 2002 the Scientific Committee on

food (SCF) evaluated Fusarium toxins and established a tolerable daily intake (TDI) for deoxynivalenol (DON), whereas for nivalenol (NIV) and the sum of T-2 toxin (T-2) and HT-2 toxin (HT-2) proposed a temporary tolerable daily intake (t-TDI) (SCF, 2002). Since that time, there have been several other comprehensive reports summarising exposure of these mycotoxins (Reddy et al., 2010; González-Osnaya, Cortés, Soriano, Moltó, & Mañes, 2011).

As far as we know, presently non data have been published for the simultaneous presence of aflatoxins B1, B2, G1 and G2 (AFB1, AFB2, AFG1 and AFG2), OTA, fumonisins B1 and B2 (FB1 and FB2), DON, NIV, diacetoxyscirpenol (DAS), T-2, HT-2, ZEN and BEA in a representative group of different cereal samples from the characteristic geographic Mediterranean area. The aim of this work was to study the contamination levels of mentioned mycotoxins, because of their potential health risk, in different cereal and cereal-based products representatives of consumption in the Mediterranean region with a multiresidual LC-MS/MS QqQ method, which would subsequently enable us to determine the dietary exposure and to assess the potencial health risk resulting from the intake of mycotoxins.

2. MATERIAL AND METHODS

2.1. Chemical and reagents

The standards of AFB1, AFB2, AFG1, AFG2, OTA, FB1, FB2, DON, NIV, DAS, ZEN and BEA were purchased from Sigma-Aldrich (Madrid, Spain). T-2 and HT-2 toxin stock solution (in acetonitrile) were provided by Biopure (Tulln, Austria). Acetonitrile and methanol were provided by Merck (Darmstadt, Germany). Ammonium formate (HCO_2NH_4, 97%) were supplied by Sigma-Aldrich (Madrid, Spain). Solid-phase used for MSPD was

octadecysilica (C_{18}) (MFE-Pack 50μm) from Analisis Vinicos (Tomelloso, Spain). Deionized water (<8MΩ cm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP Reagent Water System (Millipore, Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

Individual stock solutions of AFs and OTA with concentration of 500 μg/ml were prepared in acetonitrile and ZEN, NIV, DON, FB1, FB2, BEA were prepared of the same concentration in methanol. Stock solutions of DAS, T-2 and HT-2 with concentration of 100 μg/ml were prepared in acetonitrile. They were stored in glass-stoppered bottles and in darkness in security conditions at -20 ºC. These stock solutions were then diluted with pure methanol in order to obtain the appropiate working solutions and were stored in darkness at 4 ºC until the LC-MS/MS analysis.

2.2. Samples

A total of two hundred and sixty five samples formed by 130 different cereal-based products (24 snacks, 48 pasta, 38 soup, 8 flour and 12 biscuits) and by 135 cereal samples (grain) were collected during May, June and July of 2010 from supermarkets and food stores located in different Mediterranean countries. The location of samples is described as follows:

Samples from Morocco (n=70): brown rice grain (n=35) and white rice grain (n=35).

Samples from Italy (n=48): products from maize (n=13), oat (n=1), rice (n=11), rye (n=1), spelt (n=2) and wheat (n=20).

Samples from Spain (n= 95): products from maize (n=9), oat (n=5), rice (n=10), rye (n=2), spelt (n=3) and wheat (n=15); and grain from maize (n=11), rice (n=30) and wheat (n=10).

Samples from Tunisia (n=52): products from barley (n=4), sorghum (n=4) and wheat (n=30) and grain from maize n (n=3) and wheat (n=11).

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 analysed within 3 days.

2.3. Extraction Procedure

The method of extraction used for mycotoxins analysis in cereal samples, was reported previously by Rubert, Soler, and Mañes (2011). Samples (200 g) were grounded before mycotoxin extraction. Portions of 1g of ground samples were weighed and placed into a glass mortar (50ml) and blended with 1g of reversed-phase silica C_{18} for 5 min using a pestle. The homogeneous mixture was introduced into a 100 mm x 9mm i.d. glass column, and eluted with 25 ml of acetonitrile/methanol (50/50 v/v) in 1mM ammonium formate 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 methanol and filtered through 13 mm/0.22 μm nylon filter (Membrane Solutions, Texas, USA) prior to injection into the LC-MS/MS system.

2.4. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, and a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Linx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 4.6 mm I.D., 5 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using a gradient that started at 100% of A (5 mM ammonium formate in water) and 0% of B (5 mM ammonium formate in methanol), increased linearly to 100% B in 10 min. After, it was decreased linearly to 80% of B in 5 min and it was gradually decreased to 70% B in 10 min. Afterwards, the initial conditions were maintained for 5 min. Flow rate was maintained at 0.3 ml min^{-1}. Mass spectrometric parameters of studied mycotoxins are described by Rubert, Soler and Mañes (2011).

3. RESULTS AND DISCUSSION

3.1. Method performance

The detection limit (LOD) and the limit of quantification (LOQ) values were calculated from blank cereal extracts (spiked with decreasing concentrations of the mycotoxins), according to signal/noise ratios (S/N) using the criterion of S/N≥3 and S/N≥10 for LOD and LOQ, respectively (Rubert, Soler, & Mañes, 2011) (table 1). For legislated mycotoxins (AFs, FBs, DON, ZEN and OTA), the LOQs were lower than the MLs established by the

European Union (EC 401/2006). Recoveries of fortified cereal samples at two spiked levels (LOQ and 10 times LOQ, table 1), ranged between 68.7-89.6% and 72.6-87.6% for LOQ and 10 times LOQ respectively, with relative standard deviations from 3 to 14%. These values are according to EU criteria (Commission Decision, 2002). Matrix effect in the different matrices was different for each mycotoxin: there was as much signal enhancement as signal suppression. Therefore, to minimize these matrix effects and for a selective and reliable mycotoxin quantification in different cereal samples, the employment of LC-MS/MS required matrix-matched calibration standards prepared with different types of cereal-based products and grain cereal samples. 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.

3.2. Occurrence of AFs, BEA, FBs, OTA, TCs and ZEN

The occurrence and concentration samples obtained from the multimycotoxin analysis in positive samples are presented in table 1, and figure 1 shows the contamination levels of mycotoxins in 265 cereal samples from each country. Mycotoxins object of this study were presented in some of the contaminated samples, except ZEN. The mycotoxin frequency was 53% of the total analysed samples. The frequency of samples from Spain, Italy, Tunisia and Morocco was of 33%, 52%, 96% and 50%, respectively. The mycotoxin incidence with regard to cereal type for wheat, sorghum, spelt, maize, barley, rye, oat and rice was of 59%, 75%, 40%, 39%, 100%, 67%, 17% and 48% respectively, and the mycotoxin incidence for snacks, pasta, soup, flour, biscuits, grain wheat, grain maize and grain rice was 50%, 46%, 53%, 63%,

67%, 52%, 36% and 52% respectively. These results were similar to those obtained by other studies (Schollenberger et al., 2005; Zinedine et al., 2007; Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, & Hedili, 2008; Zaied et al., 2009). According to Rubert, Soler, and Mañes (2011), NIV and BEA were the mycotoxins presented in a highest percentage of flour samples analysed.

3.2.1. Aflatoxins and Ochratoxin A

There were 27 samples contaminated by some aflatoxin (19% of the total positive samples): 20 from Tunisia, 6 from Morocco and 1 from Spain. All type of foods analysed were contaminated, excluding rye, oat, spelt and wheat snacks. AFB1 was present in the great majority of the samples: it was detected in 14 samples (levels from 5.5-66.7 μg/kg). Concentrations of total aflatoxins (AFB1, AFB2, AFG1 and AFG2) ranged from 4.2-66.7 μg/kg. The maximum level (66,7 μg/kg) was found in a wheat pasta sample from Tunisia; this sample was contaminated with AFG2 (7.2 μg/kg) and AFB1 (66.7 μg/kg). In maize products aflatoxins were not detected, while Zinedine et al. (2007) obtained higher concentrations, specially for AFB1. In food wheat samples the values obtained for AFB1, AFB2 and AFG2 were higher than those obtained by other studies (Zinedine et al., 2007; Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, & Hedili, 2008; Klaric, Cvetnic, Pepeljnjak, & Kosalec, 2009). According to the other similar studies, no AFG1 was detected in wheat products, and AFG1 and AFG2 in rice products (Zinedine et al., 2007; Reiter, Vouk, Böhm, & Razzazi-Fazeli, 2010). These authors also detected AFB1 and AFB2. The levels obtained for aflatoxins in rice grain samples were similar or lower than those obtained by Weidenboerner (2000). In oat products aflatoxins were not detected. Nonaka, Saito, Hanioka, Narimatsu, and Kataoka (2009) only

detected AFG2 in one oat meal sample. In barley samples, the levels obtained for AFB1 were the same or similar to that obtained by Ghali et al. (2009). For these products, further data were not available. In samples of sorghum, according to Ghali et al. (2009), AFG2 was not detected. The levels obtained for AFB1 and AFB2 were lower, and for AFG1 were higher in respect to the values obtained by Ghali et al. (2009).

OTA was present in 2% of the positive samples (2 from Tunisia and 1 from Morocco) at levels from 75.0-112 μg/kg. OTA was detected in 2 wheat samples (1 of pasta and 1 of soup) and in 1 grain rice sample. The maximum level of OTA (112 μg/kg), was found in wheat pasta from Tunisia. These results were higher than those obtained by Juan, Moltó, Lino, and Mañes (2008) in wheat products from Spain and Portugal and lower than those obtained by Zaied et al. (2009) in rice grain and wheat products from Tunisia. OTA was not detected in wheat and maize grain, rye, barley, sorghum and oat products, while other studies detected OTA in different cereal samples (Blesa, Berrada, Soriano, Moltó, & Mañes, 2004; Juan, Moltó, Lino, & Mañes, 2008; Zaied et al., 2009; Klaric, Cvetnic, Pepeljnjak, & Kosalec, 2009; Duarte, Pena, & Lino, 2010).

3.2.2. Beauvericin

BEA was present in 27% of the positive samples, at levels from 2.1-844 μg/kg: 19 from Tunisia, 1 from Morocco and 4 from Spain. BEA was detected in all matrices and products to exclude the spelt, rye and oat. The maximum level (844 μg/kg) was found in a sample of wheat pasta from Tunisia. The values obtained for wheat, maize, barley and rice were lower than those obtained by other studies (Jurjevic, Solfrizzo, Cvjetkovic, De Girolamo, &

Visconti, 2002; Meca, Zinedine, Blesa, Font, & Mañes, 2010; Sifou et al., 2011). Some authors detected BEA in rye, oat and spelt (Logrieco, Rizzo, Ferracane, & Ritieni, 2002; Meca, Zinedine, Blesa, Font, & Mañes, 2010; Sifou et al., 2011).

3.2.3. Fumonisins

FBs were present in 4% of the positive samples: FB1 was detected in two wheat samples from Tunisia (pasta and soup), and FB2 was detected in two wheat grain samples from Tunisia and one rice grain sample from Morocco. The levels ranged from <LOQ-184 μg/kg for FB1, and from 121.0-176.0 μg/kg for FB2. The maximum FB1 value (184 μg/kg) was found in a wheat pasta sample from Tunisia, and the maximum FB2 value (176.0 μg/kg) was found in a rice grain sample from Morocco. These results were lower than those obtained in other studies for maize, wheat, rice and barley products (Castella, Bragulat, & Cabanes, 1999; Weidenboerner, 2000; Cavaliere et al., 2007). FBs were not detected in maize samples, while other studies found a high incidence and concentration levels of FBs in maize and maize products (Lino, Silva, Pena, Fernández, & Mañes, 2007; Silva, Lino, Pena, & Moltó, 2007; Martins, Almeida, Marques, & Guerra, 2008; Silva et al., 2009). Martos, Thompson, and Diaz (2010) and Klaric, Cvetnic, Pepeljnjak, and Kosalec (2009) did not detect FBs in wheat grain samples, but in the present study FB2 was detected. On the other hand, in rice grain samples FB1 was not detected, and FB2 was detected ten times lower that obtained by Weidenboerner (2000).

Table 1. Occurrence and concentration range of mycotoxins in positive cereal samples.

Mycotoxin (LOQ in μg kg^{-1})	Concentration range of positive samples (μg kg^{-1})	Sample (number of positive samples/number of total samples)
NIV (85.24)	117-961	Wheat-based products (40/65)
	339-679	Wheat grain (11/21)
	116-422	Maize-based products (9/22)
	150-903	Maize grain (4/14)
	100-356	Rice-based products (6/21)
	114-632	Rice grain (52/100)
	418-667	Sorghum-based products (4/4)
	105-286	Spelt-based products (2/10)
	451-813	Barley-based products (4/4)
	129-482	Rye-based products (2/3)
	472	Oat-based products (1/6)
DON (31.25)	63.2-296	Wheat-based products (4/65)
	265	Maize grain (1/14)
	71.2-176	Rice grain (3/100)
AFB1 (0.25)	5.5-66.7	Wheat-based products (10/65)
	26.0-33.0	Rice grain (2/100)
	6,4	Sorghum-based products (1/4)
	24	Barley-based products (1/4)
AFB2 (1.50)	5.6-7.6	Wheat-based products (2/65)
	6.7-26.0	Wheat grain (3/21)
	7.5	Rice grain (1/100)
AFG1 (0.25)	25.0	Maize grain (1/14)
	35.4-46.5	Rice grain (2/100)
	62.2	Sorghum-based products (1/4)
AFG2 (0.75)	4.2-18.7	Wheat-based products (6/65)
	7,5	Maize grain (1/14)
	4,4-18.2	Rice grain (4/100)

Table 1. cont.

Mycotoxin (LOQ in μg kg^{-1})	Concentration range of positive samples (μg kg^{-1})	Sample (number of positive samples/number of total samples)
DAS (5.00)	65.0-84.0	Wheat-based products (2/65)
	6,4	Maize grain (1/14)
	83.0-97.0	Rice grain (2/100)
FB1 (83.33)	<LOQ-184	Wheat-based products (2/65)
FB2 (83.75)	121.0-158.0	Wheat grain (2/21)
	<LOQ-176.0	Rice grain (2/100)
HT-2 (35.50)	<LOQ-83.0	Wheat-based products (3/65)
	64.0	Maize grain (1/14)
	43.0-87.0	Rice grain (4/100)
T-2 (12.50)	33.8	Wheat-based products (1/65)
	12.9-78.4	Rice grain (7/100)
OTA (3.00)	99.6-112	Wheat-based products (2/65)
	75.0	Rice grain (1/100)
BEA (1.00)	6.2-844	Wheat-based products (12/65)
	2.4-61.4	Wheat grain (3/21)
	5.1	Maize-based products (1/22)
	2,1-73.9	Maize grain (2/14)
	5.3-57.4	Rice grain (3/100)
	4.4	Sorghum-based products (1/4)
	5.6-82	Barley-based products (2/4)

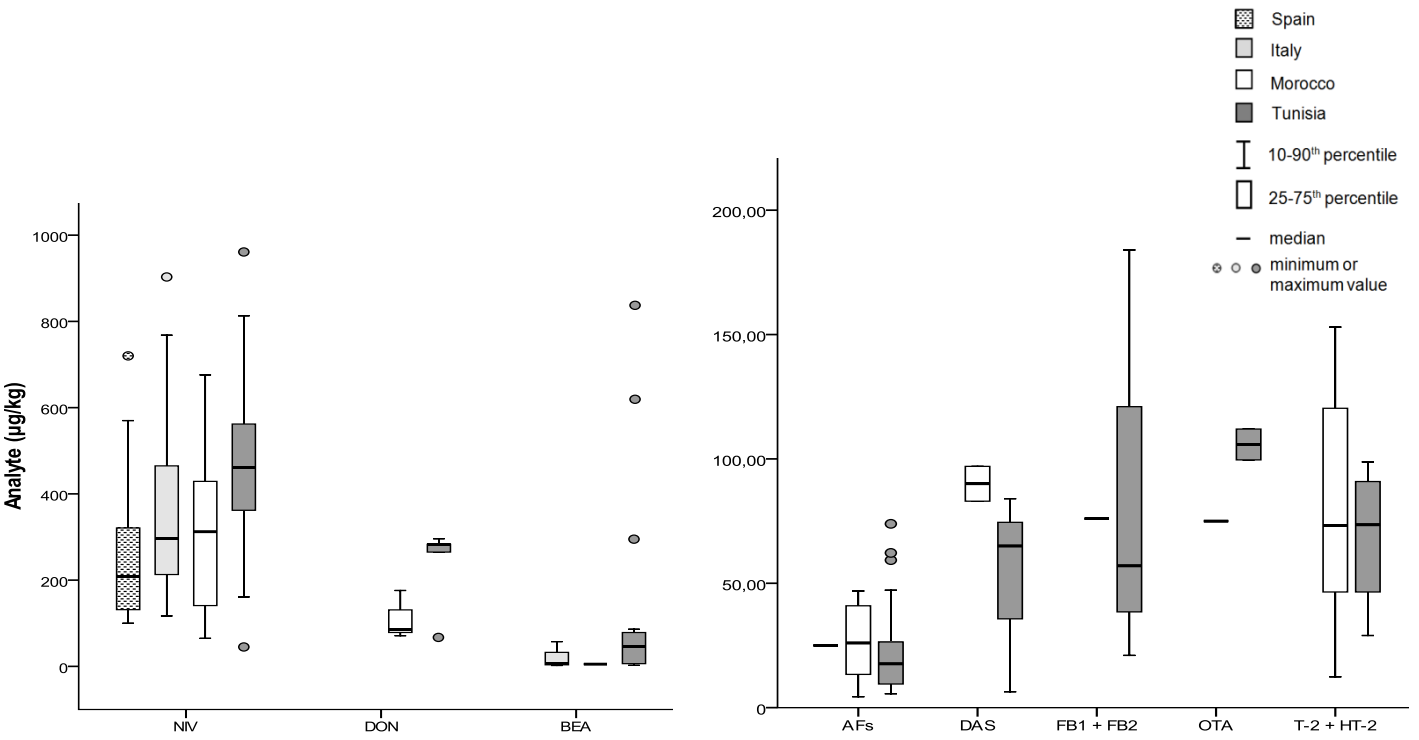

Figure 1. Contamination levels of mycotoxins in 265 cereal samples from Spain, Italy, Morocco and Tunisia.

3.2.4. Trichothecenes

NIV was the most abundant mycotoxin: it was detected in 135 samples (96% of the total positive samples) at levels from 100-961 μg/kg: 46 samples from Tunisia, 24 from Italy, 35 from Morocco and 30 from Spain. This mycotoxin was detected in all foods, and the maximum value (961 μg/kg) was found in wheat biscuits from Tunisia. The values obtained for the samples of rice grain, maize and wheat were similar or superior to the maximum values obtained in other studies (Dall´Asta, Sforza, Galaverna, Dossena, & Marcheli, 2004; Milanez, Valente-Soares, & Baptista, 2006). Other authors had not detected NIV in rice, oat and spelt products (Weidenboerner, 2000; Schollenberger et al., 2005; Lattanzio, Solfrizzo, & Visconti, 2008).

DON was present in 6% of the positive samples, at levels from 63.2-296 μg/kg. DON was detected in 5 samples from Tunisia and 3 from Morocco: 4 wheat samples (3 of pasta and 1 of flour), 1 maize grain sample and 3 grain rice samples. The maximum contamination level of DON (296 μg/kg) was found in wheat pasta from Tunisia. For maize, wheat and their products, our results were similar or lower than those obtained by other autors (Dall´Asta, Sforza, Galaverna, Dossena, & Marcheli, 2004; Schollenberger et al., 2005; Adejumo, Hettwer, & Karlovsky, 2007; Cerveró, Castillo, Montes, & Hernández, 2007; Martins, Almeida, Marques, & Guerra, 2008; González-Osnaya, Cortés, Soriano, Moltó, & Mañes, 2011), and similar or superior than those obtained by Weidenboerner (2000) for samples of rice grain. In rice, spelt and oat DON was not detected, while other autors (Schollenberger et al., 2005; Lattanzio, Solfrizzo, & Visconti, 2008) had detected DON at levels of 10.0-100 μg/kg.

T-2 was present in 6% of the positive samples (1 from Tunisia and 7 from Morocco) and HT-2 was present in 6% of the positive samples (4 from Tunisia

and 4 from Morocco), at levels from 33.8-78.4 and <LOQ-87,0 μg/kg, respectively. There were 3 wheat pasta samples, 4 maize grain samples and 7 rice grain samples contaminated by HT-2. T-2 was detected in 1 wheat pasta sample and in 7 rice grain samples. The maximum contamination level of HT-2 (87.0 μg/kg) and of T-2 (78.4 μg/kg) were found in different samples of rice grain from Morocco. In maize, rice, spelt and oat samples, T-2 and HT-2 were not detected, while other studies detected high levels concentrations in maize, rice and oat products (Schollenberger et al., 2005; Milanez, Valente-Soares, & Baptista, 2006; Lattanzio, Solfrizzo, & Visconti, 2008). However, according to our study, T-2 and HT-2 were not detected in spelt products (Schollenberger et al., 2005). In grain maize samples, only one sample was positive at lower concentration than those obtained by other authors (Milanez, Valente-Soares, & Baptista, 2006). The levels obtained for wheat products were similar or superior to those obtained from other studies (Schollenberger et al., 2005; Martos, Thompson, & Diaz, 2010). In wheat grain samples these mycotoxins were not detected; no other studies are available for this.

DAS (levels from 6.4-97.0 μg/kg) was present in 4% of the positive samples: 3 samples from Tunisia and 2 from Italy. This mycotoxin was detected in 2 wheat flour samples, 1 maize grain sample and 2 rice grain samples, and the maximum NIV value (97.0 μg/kg) was found in a rice grain sample from Morocco. The values obtained for maize grain samples were lower than those obtained by other studies (Adejumo, Hettwer, & Karlovsky, 2007). Schollenberger et al. (2005) indicated similar or lower concentrations in wheat samples, while Dall´Asta, Sforza, Galaverna, Dossena, and Marcheli (2004) obtained similar or superior concentrations. According to some authors

(Schollenberger et al., 2005), the presence of DAS in maize, oat and spelt products were not detected.

3.2.5. Zearalenone

According to Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, and Hedili (2008), in their study, ZEN was not detected. However, different authors detected higher levels in cereal grain and cereal products (Weidenboerner, 2000; Schollenberger et al., 2005; Klaric, Cvetnic, Pepeljnjak, & Kosalec, 2009).

3.3. Co-occurrence of mycotoxins

Concerning the co-occurrence of mycotoxins which are analysed in this study: the majority of the positive samples were contaminated by one mycotoxin (NIV or BEA), while 14 % of the analysed samples were contaminated with at least two mycotoxins and 18 % of the analysed samples were contaminated by more than two mycotoxins simultaneously. With regard to the samples contaminated with at least two mycotoxins simultaneously, NIV and BEA were detected in 11 samples, in 6 samples we detected NIV with some of the four aflatoxins (AFB1, AFB2, AFG1, AFG2) and the others contained NIV and OTA, NIV and FB2 or NIV and T-2. For the samples contaminated with more than two mycotoxins simultaneously, one sample contained seven mycotoxins, two samples contained six mycotoxins, four samples contained five mycotoxins, three samples contained four mycotoxins, and fourteen samples contained three mycotoxins. Besides, for the samples with three mycotoxins simultaneously, in 8 of the samples we detected NIV, BEA and some of the four aflatoxins (AFB1, AFB2, AFG1, AFG2).

Sanchis, Marín, and Ramos (2007) suggested that on the same food was a heterogeneous population of filamentous fungi and its competence affects at the different synthesis and co-ocurrence of mycotoxins. In addition, due to presence of several mycotoxins in the same food (32% of total of positive samples) more toxicity studies are needed to evaluate possible synergism and additive effects.

3.4. Comparison with the mycotoxin regulation

In the positive samples, the results obtained for FBs and DON (table 1) were lower than European Union MLs, according most of the authors (Dall´Asta, Sforza, Galaverna, Dossena, & Marcheli, 2004; Schollenberger et al., 2005; Adejumo, Hettwer, & Karlovsky, 2007; Cavaliere et al., 2007; Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, & Hedili, 2008). All concentrations obtained for the OTA, AFB1 and sum of aflatoxins (table 1) were superiors to the MLs established by the European Union. Although, the information available from the literature, indicate that, according to these results, in most studies was superated the European Union MLs: Ghali, Hmaissia-khlifa, Ghorbel, Maaroufi, and Hedili (2008) found in sorghum from Tunisia maximum concentrations of 67.0 μg/kg, 41.4 μg/kg and 36.4 μg/kg for the sum of aflatoxins, for AFB1 and for OTA, repectively; Weinderboerner (2000) found in rice from India maximum concentrations of 317 μg/kg and 98.0 μg/kg for AFB1 and for the sum of aflatoxins, respectively; Blesa, Berrada, Soriano, Moltó, and Mañes (2004) found in breakfast cereals from Spain, a maximum concentration of 1.9 μg/kg for OTA. For BEA, NIV, DAS, T-2 and HT-2, MLs have not been established.

3.5. Estimated daily intake of mycotoxins

In order to determine the risk assessment of mycotoxins in food, JECFA has had mainly pooled data on mycotoxins available. Estimated total intake for each mycotoxin, were based on a combination of mean food consumption levels with weighted-mean contamination levels of the five GEMS/Food (Global Environment Monitoring System/Food contaminants Monitoring and Assessment Programme) regional diets (African, European, Far Eastern, Latin American and Middle Eastern). Dietary intakes including information on consumption of raw but processed foods were not used to estimate dietary intake. To determine the risk assessment of the Spanish, Moroccan, Tunisian and Italian population, in this study the obtained results are considered as the total exposure to mycotoxins.

Table 2 shows the estimated total intake for each mycotoxin in European and African regions and the TDI (PMTDI or t-TDI) established for some mycotoxins by the JECFA and by the SCF (SCF, 2002; FAO/WHO, 2001). In the European region, the estimated total intake for DON, the sum of HT-2 and T-2, and FBs was available on the concentrations of ten (barley, maize, popcorn, oats, rice, rye, triticale, wheat, and other cereals), six (barley, maize, oats, rice, rye and wheat, and other cereals) and maize commodities, respectively. In the African region, the estimated total intake by JECFA for DON, the sum of HT-2 and T-2, and FBs is available on the concentrations of maize. As regard to OTA, as it occurs mainly in Europe, the estimated total intake was available on coffee, beer, wine, or fruit juices, and information is not available about estimated total intake from Africa. On the basis of current knowledge, the JECFA does not provide the estimated total intake data of AFs, NIV, DAS and BEA in the European and African regions. The JECFA

and the SCF determined a TDI or PMTDI for NIV, DON, FB1 and FB2, T-2 and HT-2, and OTA, but could not establish a TDI value for AFs (these are genotoxic compounds and have carcinogenic properties) recommended that the exposure to these mycotoxins should be reduced to As Low As Reasonably Achievable (ALARA). Moreover, there are gaps in the toxicological information of some mycotoxins and they have not yet TDI value, such as DAS and BEA, so levels of them in food should also be ALARA.

In order to obtain the accurate information on the exposure to mycotoxins in Europe and Africa, through the occurrence data in food products and consumption data, to estimated total intake of the products of this study, an approach was followed. According to FAO (2007), the intake estimates were calculated in Spain, Tunisia and Italy by mean cereals (excluding beer) consumption, whereas in Morocco, only rice consumption was considered due to analysed products were only rice samples. The consumption of cereals (excluding beer) was 97.50 Kg/person/year (Spain), 210.80 Kg/person/year (Tunisia) and 156.90 Kg/person/year (Italy). The consumption of rice in Moroccan people was 1.00 Kg/person/year. However, it is important to note that the estimated intake calculated is based on the daily per capita dietary exposure of cereals, and a wide variety of products made from different cereals taken from different points of the four countries selected. Moreover, these commodities are highly consumed by the European and African population. Taking into account all these points of view, the results indicate that the values obtained would provide a real estimation of the total intake. These results are shown in table 2, which also shows the estimated daily intake obtained on the concentration of each mycotoxin studied in cereals (European countries) and rice (African countries), and the relationship within

the TDI level proposed by the JECFA and the estimated total intake obtained by the results of this study. However, the total intake for DON, T-2, HT-2, OTA, DAS, FB1 and FB2 for the Spanish and Italian population, and AFs and BEA for the Italian population were not estimated because these mycotoxins were not detected in cereals and cereal products from these countries.

Results in table 2 shows that the estimated total intake of DON, and the combined intake of FB1 and FB2 for thr Moroccan and Tunisian population are much lower that the total intake estimated by the JECFA for the African diet. However, the estimate total intakes in Tunisia for DON and the sum of FB1 and FB2 were 1.015-, and 1.205-times higher than those in Morocco, respectively. In the same way, the estimated total intake of the sum of HT-2 and T-2, and OTA for the Moroccan population was considerably lower than the total intake estimated by the JECFA for the European diet (values for the African diet are not estimated). Nevertheless, the estimated total intake for OTA and the sum of T-2 and HT-2 toxin obtained for Tunisian population was 2.56-times higher than that permitted by JECFA for human dietary exposure. In a previous study by Gonzalez-Osnaya, Cortés, Soriano, Moltó, and Mañes (2011), the results obtained for DON and the sum of HT-2 and T-2 for Spanish population were similar to those obtained in this study for the Tunisian population. NIV was found in all samples analysed in this study independently of their origin. The results obtained showed a higher incidence of NIV in Tunisia (5-times) than in Italy (1-times) in samples in respect to the TDI value. Content of NIV in samples from Spain and Morocco were under the TDI by the JECFA.

The relationship between each mycotoxin intake and the TDI value proposed by JECFA has been expressed as a percentage (table 2). The most

mycotoxin intake values found in this study are much lower than the TDI established for each mycotoxin as can be shown in the results obtained from Morocco and Spain (values < 100%). However, the results obtained for NIV in Italian samples were slightly higher (112.95 %) and, the results obtained for NIV (487.23%) and OTA (235.66%) in the Tunisian samples were much higher than expected.

Taking into account the results obtained in this study, to assess whether there is a public health problem for population exposed to mycotoxins is an important aim. For the assessment of intake from the diet, the TDI of all mycotoxins are needed. However, when the TDIs were compared with the estimated total intake in the risk characterisation there were indications that for mycotoxins the TDI may have been exceeded in certain regions or for certain population groups. There are risk groups, such as infants or children who have the highest risk, because they have an exceptionally high intake in relation to their body weight.

Table 2. Estimated of total intake by JECFA and by this study, TDI proposed by JECFA, and relationship between each mycotoxin intake expressed in percentage.

Mycotoxin	Estimated of total intake by jecfa (µg/kg bw/day)		Estimated of total intake (µg/kg bw/day)				TDI(µg/kg bw/day; by jecfa)	Relationship between each mycotoxin intake and the tdi level proposed by jecfa (%)			
	Europe	Africa	Italy	Tunisia	Spain	Morocco [a]		Italy	Tunisia	Spain	Morocco [a]
NIV	No data	No data	0.7907	3.4106	0.4329	0.006121	0.7 [b]	112.95	487.23	61.84	0.87
DON	1.4	0.77	-	0.1888	-	0.000186	1.0	-	18.88	-	0.02
AFB1	No data	No data	-	0.0489	-	0.000033	No data	-	-	-	-
AFB2	No data	No data	-	0.0108	-	0.000004	No data	-	-	-	-
AFG1	No data	No data	-	0.0099	0.0010	0.000046	No data	-	-	-	-
AFG2	No data	No data	-	0.0097	-	0.000021	No data	-	-	-	-
DAS	No data	No data	-	0.0247	-	0.000101	No data	-	-	-	-
Sum of FB1 and FB2	0.2	2.4	-	0.0506	-	0.000042	2.0	-	2.53	-	-
OTA [c]	0.092	No data	-	0.2357	-	0.0003	0.1	-	235.66	-	0.29
Sum of HT-2 and T-2	0.017	No data	-	0.0436	-	0.000361	0.06 [b], [d]	-	72.67	-	0.60
BEA	No data	No data	-	0.3697	0.0029	0.000003	No data	-	-	-	-

[a] Estimated total intake only for rice

[b] t-TDI. The TDI is made temporary (t-TDI) because it is noted that NIV, DON, T-2 and HT-2 toxin belong to the group of several trichothecenes with a common
basic chemical structure, and according to present knowledge they may also share common mechanisms of toxic action

[c] Value expressed in µg/Kg bw/week

[d] Combined t-TDI (T-2 toxin and HT-2 toxin)

4. CONCLUDING REMARKS

The occurrence of fourteen mycotoxins was determinated in different cereal food samples representatives from the Mediterranean area. The percentage of total samples contaminated was 53%. The results obtained for FBs and DON were lower than European Union MLs, while the concentrations obtained for OTA, AFB1 and sum of aflatoxins were higher than the European Commission MLs. The absence of international legislation limits for NIV in foods, its toxicological effects, its high frequency in cereal products and the high levels obtained in this study, indicates that special attention to contamination levels is necessary. Besides, the estimated total intake for NIV for samples from Tunisia and Italy, was higher than the t-TDI established by SCF, the results were higher than expected. Therefore, TDI values for the assessment of mycotoxins in the diet should be determined or re-validated in the light of continuously toxicological data obtained by scientific groups.

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3.5. Emerging *Fusarium* mycotoxins in organic and conventional pasta collected in Spain

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Emerging *Fusarium* mycotoxins in organic and conventional pasta collected in Spain

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ABSTRACT

One of the main sources of emerging *Fusarium* mycotoxins in human nutrition are the cereals and cereal products. In this study, an analytical method to determine enniatins A, A_1, B and B_1 (ENs), beauvericin (BEA) and fusaproliferin (FUS) based on Ultra-Turrax extraction followed by liquid chromatography coupled to triple quadrupole mass spectrometer detector (MS/MS QqQ), was applied for the analysis of pasta. For this purpose, 114 commercial samples of pasta were acquired from supermarkets located in Valencia. The results showed higher frequencies of contamination in organic pasta than in conventional pasta, while the concentration levels were variable for both types of pasta. In positive samples, BEA levels varied from 0.10 to 20.96 μg/kg and FUS levels varied from 0.05 to 8.02 μg/kg. ENs levels ranged from 0.25 to 979.56 μg/kg, though the majority of the values were below 25 μg/kg. Besides, it was observed the simultaneous presence of two or more mycotoxins in a high percentage of the samples. Finally, an evaluation of the dietary exposure of the emerging *Fusarium* mycotoxins was performed in the Spanish population. The prevalence of ENs, BEA and FUS in cereal products suggests that the toxins may pose a health risk to Spanish population.

Keywords: emerging *Fusarium* mycotoxins, dietary exposure, pasta, liquid chromatography-tandem mass spectrometry

1. INTRODUCTION

Wheat is the most consumed cereal worldwide: global consumption of cereals (excluding beer) was 146.60 kg/*per capita* during 2007, and the global consumption for wheat reached about 66 kg/*per capita* (FAO, 2007). The population from Spain is one of the most consuming of wheat in the world: the mean Spanish consumption of wheat (87.40 Kg/*per capita*) was higher respect to the worldwide consumption in the year 2007 (FAO, 2007). Usually, wheat is ground to flour for the production of bread, pasta, biscuits and other products. Nowadays, pasta constitutes one of the most important wheat-based products of the Spanish economy: during the last years global consumption of pasta in Spain has increased around 6% between 2007 and 2011 (MAGRAMA, 2011).

Wheat-based products are one of the main sources of mycotoxins in both the human and animal diets. Surveillance studies have indicated that mycotoxin contamination is a world-wide problem (SCOOP, 2003), since it causes economic losses, both for the grain and for the marketing of foods and feeds, and is a potential threat to animal and human health. In 2006, the European Union proposed the maximum levels (MLs) for some mycotoxins in foodstuffs: aflatoxins, fumonisins and trichothecenes among other (European Commission, 2006). For some *Fusarium* mycotoxins, MLs have been established mainly for cereals and cereal-based products, since usually *Fusarium* species are able to infect cereal crops. The contamination of mycotoxins in cereals is known to be affected by the local climate (rainfall, temperature or relative humidity), agricultural practices, harvest logistics, transport and storage conditions, and processing of products (Bakan et al., 2002). Specially, in the last few years the relationship between the influence of the agricultural

practices (traditional or organic) and mycotoxin contamination have been discussed by many authors. In organic practices, chemical products (fungicides, pesticides, etc.) are no employed increasing the exposure of cereal grains to fungal colonization and to mycotoxin production. Some studies have supported this affirmation (D´Arco et al., 2009; Silva et al., 2009), but other studies have not observed significantly differences between organic and conventional products (González-Osnaya et al., 2007; Ariño et al., 2007). This fact can be due to the crop rotation used in organic and conventional agricultural practises, which prevents the transmission of plant diseases (Jestoi et al., 2004b).

Fusarium genus is probably the most prevalent toxin-producing fungi of the northern temperate region. They are commonly found on cereals grown in the temperate regions of America, Europe and Asia (SCF, 2002). *Fusarium avenacum, Fusarium moniliforme, Fusarium proliferatum* and *Fusarium subglutinans* are the major producers of emerging *Fusarium* mycotoxins enniatins A, A1, B, B1 (ENA, ENA1, ENB and ENB1, respectively), beauvericin (BEA) and fusaproliferin (FUS) in various cereals, especially wheat, barley and maize (Jestoi, 2008). FUS is a bicyclic sesterterpene (Fig. 1) and enniatins (ENs) and BEA possess a cyclic hexadepsipeptide structure (Fig. 2). At present, MLs have not been set for emerging *Fusarium* mycotoxins in spite that most of them represent an important risk for the public health. BEA and ENs have similar toxic actions including the induction of apoptosis, increasing the cytoplasmic calcium concentration and the DNA fragmentation in mammalian cell lines (Dombrink-Kurtzman, 2003; Jow et al., 2004; Lin et al., 2005). Besides, BEA and ENs have cytotoxic and insecticidal properties (Kamyar et al., 2004; Ivanova et al., 2006; Ferrer et al., 2009), and inhibitory effects on acyl-CoA:

cholesterol acyltransferase activity (ACAT) (Tomoda, 1992). Fornelli et al. (2004) showed that on SF-9 cells were in line with earlier results for FUS with IC_{50} (50% inhibitory concentration) and CC_{50} (50% cytotoxic concentration) of >100 μM. FUS caused teratogenicity in a chicken embryotoxicity bioassay (Ritieni et al., 1997), and in the brine shrimp (*Artemia Salina*) larvae bioassay FUS was toxic with an LD_{50} value (dosage leading to death of 50% larvae) of 53,4 μM, toxicity level similar to aflatoxin B1 and deoxynivalenol (Logrieco et al., 1996). Due to the described toxicity, at present emerging *Fusarium* mycotoxins may play a role in the health of consumers. Monitoring studies have revealed the presence of ENs, BEA and FUS in some commodities and foodstuffs, mainly cereals in grain, cereal-based products and eggs (Jestoi, 2008; Garrido et al., 2011). Most of these studies have been performed through the application of the conventional extraction (Ultra-Turrax o el rotatory shaker extraction) (Uhlig et al., 2006; Meca et al., 2010), and posterior determination and quantification by liquid chromatography coupled to diode array ultraviolet-visible detector (Sifou et al., 2011) or triple quadrupole mass spectrometer detector (MS/MS QqQ) (Garrido et al., 2011). In MS/MS detection, the molecules are ionized and the ions are identified according their mass-to-charge (m/z) ratios. Therefore, high selectivity is achieved in the analysis employing LC-MS/MS, which increases confidence in the results of both qualitative and quantitative analyses (Zöllner and Mayer-Helm, 2006).

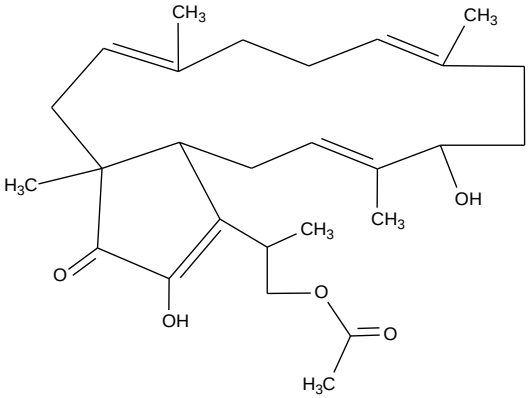
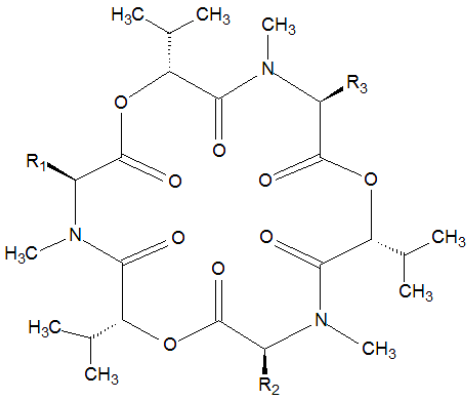


Fig. 1. Chemical structure of fusaproliferin (FUS)



	R_1	R_2	R_3
BEA	Phenilmethyl	Phenilmethyl	Phenilmethyl
ENA	*sec*-butyl	*sec*-butyl	*sec*-butyl
ENA$_1$	*sec*-butyl	*sec*-butyl	*iso*-propyl
ENB	*iso*-propyl	*iso*-propyl	*iso*-propyl
ENB$_1$	*iso*-propyl	*iso*-propyl	*sec*-butyl

Fig. 2. Chemical structures of beauvericin (BEA) and enniatins (ENA, ENA$_1$, ENB and ENB$_1$)

Monitoring studies for emerging *Fusarium* mycotoxins are necessary for legislative purposes, because in the near future an appropriate maximum contamination levels should be set for several mycotoxins by the authorities. Moreover, monitoring studies are required in order to obtain information about of the real exposure of human population to mycotoxins (González-Osnaya et al., 2007; D´Arco et al., 2009). Risk assessment studies are of high interest since they permit the evaluation of the population exposure to toxic substances. Usually, risk assessment studies are carried out by comparison of the mycotoxin levels from the monitoring studies with the corresponding Provisional Maximum Tolerable Daily Intake (PMTDI) established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (D´Arco et al., 2009). At the moment, studies of this type have not been carried out for emerging *Fusarium* mycotoxins in previous works. This is probably due to the absence of established PMTDIs for ENs, BEA and FUS. However, it is possible perform an approach to the risk assessment comparing the levels of emerging *Fusarium* mycotoxins with the PMTDIs established for other *Fusarium* mycotoxins, such as T-2 toxin and HT-2 toxin or deoxynivalenol (JECFA, 2001; Serrano et al., 2012b).

The aim of this study was to assess the exposure of the Spanish population to emerging *Fusarium* mycotoxins present in pasta. Two objectives were proposed: (1) providing data on the natural occurrence of ENs, BEA and FUS in conventional and organic pasta from Spanish supermarkets using LC-MS/MS QqQ determination, and (2) the approach to the risk assessment of ENs, BEA and FUS by evaluation of the dietary exposure.

2. MATERIAL AND METHODS

2.1. Chemical and reagents

Acetonitrile (AcN) and methanol (MeOH) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain) (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain) before use.

The standards of ENA, ENA_1, ENB, ENB_1 and BEA were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard of FUS was kindly given by Professor A. Ritieni (Department of Food Science, University "Federico II" of Naples, Italy). Individual stock solutions of ENA_1, ENB, ENB_1, FUS and BEA with concentration of 1000 μg mL^{-1}, and the solution of ENA with concentration of 500 μg mL^{-1}, were prepared in methanol. They were stored in glass-stoppered bottles and darkness in security conditions at -20 ºC. These stock solutions were then diluted with pure methanol in order to obtain the appropriate working solutions and were stored in darkness at -20 ºC until the LC-MS/MS analysis.

2.2. Sample collection

Commercial samples of pasta (114 samples) were purchased during 2011 from different supermarkets located in Valencia (Spain). The method of sampling was accomplished according to the Commission Regulation (EC) No 401/2006 for the official control of the maximum levels established for aflatoxins, ochratoxin A and *Fusarium* toxins in cereals and cereal products. In this Regulation it has been established that for lots of cereals and cereal

products less than 50 tonnes, the sampling plan shall the used with 10-100 incremental samples, depending on the lot weight, resulting in an aggregate sample of 1 to 10 kg. For very small lots (≤ 0.5 tonnes) a lower number of incremental samples may be taken, but the aggregate sample combining all incremental samples shall be also in that case at least 1 kg.

The samples were divided in 74 samples of conventional pasta (twenty samples of fresh pasta and fifty-four samples of dry pasta) and 40 samples of organic pasta (eighteen of whole-grain dry pasta and twenty-two of white dry pasta). The mentioned samples were produced in different countries: Spain (n=87), Germany (n=6), Portugal (n=4) and Italy (n=17). Table 1 shows a specific description of the samples. In accordance to the Commission Regulation (EC) No 401/2006, before the analysis all samples were grounded, mixed and divided in subsamples of 100 g. The subsamples were stored in a dark and dry place at 4 ºC until analysis.

Table 1. Specific description of the samples.

Agricultural practice (number of samples)	Sample (number of samples)	Main composition (number of samples)	Origin (number of samples)
Organic (40)	Whole-grain dry pasta (18)	Whole durum wheat (18)	Spain (15) Germany (1) Italy (2)
	White dry pasta (22)	Durum wheat (22)	Spain (19) Germany (1) Italy (2)
Conventional (74)	Dry pasta (54)	Durum wheat (54)	Spain (38) Germany (4) Italy (8) Portugal (4)
	Fresh pasta (n=20)	Durum wheat and egg (n=20) [a]	Spain (17) Italy (3)

[a] Percentages of egg in the fresh pasta were: 20% (thirteen Spanish samples), 15% (four Spanish samples and two Italian samples) and 13% (one Italian sample).

2.3. Extraction Procedure

Samples (5 g) were extracted with 50 mL of AcN using a Ika T18 basic Ultra-Turrax (Staufen, Germany) during 3 min. After, centrifugation for 15 min at 3554 g and 5 ºC, the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The extract was dissolved with 5 mL of AcN, and was evaporated to dryness by nitrogen gas at 35 ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and was filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) prior the injection in the LC-MS/MS system.

2.4. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 2 mm I.D., 3 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using gradient elution that started at 90% of A (AcN) and 10% of B (20 mM ammonium formate in MeOH), increased linearly to 50% B in 10 min. After, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 mL min^{-1}. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows:

capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0,5 V; source temperature, 100 ºC; desolvation temperature, 300 ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L h^{-1}; cone gas 50 L h^{-1} (nitrogen 99.99% purity).

Ideal fragmentation conditions were accomplished varying the cone voltage and collision energies for each compound. The cone voltage selected was 40 V for the fragmentation of ENA, ENA_1, ENB, ENB_1 and BEA, and 50 V for the fragmentation of FUS. The collision energy selected was 35 Ev for ENA, ENA_1, ENB and ENB_1, 40 Ev for BEA and 10 Ev for FUS. 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 99.995% purity) pressure, 3.83 x 10^{-3} mbar; interchanel 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. According with the 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 follow precursor ion and product ions was selected for each mycotoxin: the precursor ion m/z 681.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA, the precursor ion m/z 667.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA_1, the precursor ion m/z 639.8 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB, the precursor ion m/z 654.9 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB_1, the precursor ion m/z 784.4 $[M+H]^+$ and the product ions m/z 244.0 and 262.0 for BEA, the precursor ion m/z 445.0 $[M+H]^+$ and the product ions m/z 409.0 and 427.0 for FUS.

2.5. Dietary exposure

The dietary exposure study of Spanish population to ENs, BEA and FUS present in pasta, was performed by the calculation of the Estimated Daily Intakes (EDIs). The EDIs were calculated for each mycotoxin individually and for the sum of all mycotoxins as follows:

EDI (μg/kg bw/day) = mean conc. (μg/kg) * pasta consumption (kg/kg bw/day) (1)

Pasta consumption data are available in the databases of the Spanish Ministry of Agriculture, Food and Environment (MAGRAMA, 2011). Given that the samples were purchased during the year 2011, the consumption data were acquired for the same year. The Spanish consumption of dry and fresh pasta was 3.49 and 0.21 kg/person/year, respectively. Assuming 70 kg as the mean of body weight (bw) for the Spanish population, the daily consumption *per kg of bw* was calculated for dry and fresh pasta. The average levels obtained in our study and the daily consumption of dry and fresh pasta were used to calculate the EDIs (Eq. 1).

3. RESULTS AND DISCUSSION

3.1. Method validation

The analytical method was validated in-house for pasta samples. The evaluation of the matrix effects for each mycotoxin was performed by the use of matrix-assisted calibration curves. According to other authors (Rubert, Soler, & Mañes, 2011), a mixture of extracts of dry and fresh pasta, where none of the studied mycotoxins were detected, was used as a blank sample in order to ensure results representatively. Calibration curves were constructed for each

studied mycotoxin from the standards prepared in methanol and from the standards prepared in extract of blank sample. The standards were prepared at six concentration levels: 0.025-25 μg kg^{-1} for ENA and 0.05-50 μg kg^{-1} for ENA_1, ENB, ENB_1, BEA and FUS. Suppression of the signal (SS) was obtained for all mycotoxins (between 53.0 and 78.6 %). For more certain results, matrix effects were evaluated using blank extracts of fresh pasta and dry pasta separately, since they have different compositions: the principal component of the samples of dried pasta is durum wheat, while samples of fresh pasta are mainly composed of durum wheat, water and eggs (20% approx.). The results indicated that the SS for dry pasta samples and SS for fresh pasta samples were very similar to SS for the mixture of samples. Therefore, there was no difference in the use of standards prepared in extracts of dry pasta, fresh pasta or in a mixture of both types of pasta.

The evaluation of the linearity, calibration curves were constructed at six concentration levels: 0.025-25 μg kg^{-1} for ENA, and 0.05-50 μg kg^{-1} for ENA_1, ENB, ENB_1, BEA and FUS. The results showed good linearity with good correlation coefficients (r^2 > 0.992). The detection limits (LODs) were calculated using a signal-to-noise ratio of 3. The limits of quantification (LOQs) were calculated using a signal-to-noise ratio of 10. The LOQs and LODs obtained for ENs, BEA and FUS are presented in the Table 2. The accuracy was evaluated through recovery studies at two concentration levels, LOQ (low level) and 100xLOQ (high level). Intra-day precision was assessed by five determinations at each addition level in the same day, while inter-day precision was assessed by one determination at each addition level during five days. The mean recoveries and the corresponding relative standard deviations (RSDs) are presented in Table 2. RSD values ranged between 4 and 11% for

intra-day precision, and between 5 and 15% for 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. Therefore, the results were in accordance to the limits set in Commission Decision 2002/657/EC: a mean recovery (n=5) between 70% and 120%, and a RSD lower than 20%.

Table 2. Analytical parameters: recoveries (%), relative standard deviations (%) and limits of detection and quantification (μg/kg).

Mycotoxin	Recovery ± RSD (intra-day precision)[a]		Recovery ± RSD (inter-day precision)[b]		LOQ (μg/kg)	LOD (μg/kg)
	Low level (LOQ)	High level (100 x LOQ)	Low level (LOQ)	High level (100 x LOQ)		
ENA	92±5	91±4	93±8	90±6	0.50	0.15
ENA_1	88±7	86±9	85±11	88±8	0.25	0.08
ENB	109±8	112±6	110±8	109±9	0.50	0.15
ENB_1	99±10	97±11	97±13	95±15	0.50	0.15
BEA	93±6	94±4	96±7	94±5	0.10	0.02
FUS	94±13	91±11	93±14	91±13	0.05	0.03

[a] Number of replicates: 5
[b] Different days: 5

3.2. Monitoring study

3.2.1. Occurrence and concentration levels of ENs, BEA and FUS

The occurrence and levels of concentration for ENs, BEA and FUS in the samples are reported in Table 3. The MRM chromatograms and the daughter ion spectrums corresponding to a sample of dry conventional pasta naturally contaminated with 9.04 μg/kg of BEA, 23.28 μg/kg of ENB and 19.24 μg/kg

of ENB_1, and to a sample of organic white pasta naturally contaminated with 6.43 μg/kg of ENB_1, 14.92 μg/kg of ENA_1, and 8.02 μg/kg of FUS, are presented in fig. 3a and fig. 3b, respectively. In 105 out of 114 total samples (92%) were found some of the studied mycotoxins. The 77% of the samples contained ENA, and the maximum concentration (42.04 μg/kg) was found in one sample of organic white pasta produced in Spain. The maximum concentrations of ENA_1, ENB_1 and BEA (21.89, 979.56 and 20.96 μg/kg, respectively) were found in one sample of conventional dry pasta produced in Germany. The frequency of samples that were found positive for ENA_1, ENB_1 and BEA was 76%, 71% and 18%, respectively. FUS was the mycotoxin less predominant (11%) and the maximum concentration (0.49 μg/kg) was found in a sample of organic white pasta produced in Spain. ENB was the most abundant mycotoxin found in the samples (80% of the samples), and the maximum concentration (122.13 μg/kg) was found in a sample of conventional dry pasta produced in Portugal. Although the ENB was the most common mycotoxin, the differences between the incidences of ENA, ENA_1, ENB and ENB_1 were not significant (between 71 and 80%). However, other authors have obtained incidences of ENB considerably higher than those obtained for ENA, ENA_1 and ENB_1 (Uhlig et al., 2006; Sifou et al., 2011).

Regarding to the type of mycotoxin, the data obtained in our study indicated both a higher incidence of ENs and higher ENs concentrations than those obtained for BEA and FUS. This is in accordance with Mahnine et al. (2011), which concluded that the highest mycotoxin amounts were due to ENs. Uhlig et al. (2006) and Oueslati et al. (2011) have also reported the same findings. The presence and the levels of BEA and FUS have been diversely discussed by some authors. While some have not found BEA and FUS in the

analyzed samples, others state that BEA and FUS are highly prevalent. Jestoi et al. (2004a) studied the presence of FUS in grain cereals (wheat, barley, oat and rye) from Finland, and Zapata et al. (2010) studied the presence of FUS and BEA in wheat grain from Argentina. In both studies, none of the studied mycotoxins were detected in the analyzed samples. However, other published studies have indicated high contamination levels and high incidence of BEA and FUS. Shepard et al. (1999) reported incidences around of 100% in maize from South Africa, and maximum concentrations of 1734 and 62 μg/kg for BEA and FUS, respectively. Logrieco et al. (2002) found high concentration levels of BEA (between 640 and 3500 μg/kg) and the 100% of prevalence in rye from Finland. Sørensen et al. (2008) obtained high levels of BEA in samples of whole maize from Denmark, with a maximum concentration of 988 μg/kg and incidence up to 50% (50 out of 80 total samples). The incidence of BEA and FUS obtained in our study is lower than those evidenced in the studies described above (Shepard et al., 1999; Logrieco et al., 2002; Sørensen et al., 2008). However, our findings concerning BEA and FUS are supported by some researchers from countries with similar climatic conditions, such as Morocco or Italy (Jestoi et al., 2004b; Mahnine et al., 2011).

The findings from our study showed that the concentrations were higher for ENs type B (Table 3). Other authors have also reported similar findings in cereal products. Jestoi et al. (2004b) studied the presence of ENs, BEA and FUS in grain-based products from Finland and Italy, obtaining concentrations varied from <0.6 to 20 μg/kg for ENs type A and concentrations ranging from <3.8 to 170 μg/kg for ENs type B, while BEA levels were below 10 μg/kg and FUS was not detected in the samples. The contamination levels evidenced by Jestoi et al. (2004b) are in the same order of magnitude than those obtained in

our study. Uhlig et al. (2006) analyzed the contents of ENs and BEA in different grain cereals (oat, barley and wheat) from Norway, obtaining maximum concentrations of 59 μg/kg for ENA and 500 μg/kg for ENA_1, while the maximum concentrations for ENB, ENB_1 and BEA were 5800, 1900 and 120 μg/kg, respectively. The results obtained by Uhlig et al. (2006) in cereals from Norway are comparable with those obtained in studies from other Nordic countries, such as Finland (Jestoi et al., 2004a; Yli-Mattila et al., 2006; Sørensen et al., 2008), and higher than those obtained in our study. On the other hand, several studies that analyzed emerging *Fusarium* mycotoxins in cereals from countries located in the Mediterranean area showed highest concentration levels for ENs type A, particularly for ENA_1, which is in disagreement with our results. Meca et al. (2010) studied the levels of ENs, BEA and FUS in Spanish cereals and found high levels of ENA_1 (concentrations between 33.38 and 814.42 mg/kg), ENB (concentrations between 2.23 and 21.37 mg/kg), ENB_1 (concentrations between 4.34 and 45.94 mg/kg), BEA (levels ranged from 0.51 to 11.78) and FUS (levels between 1.01 and 6.63 mg/kg), while ENA was not found in the samples. Sebastiá et al. (2012) evaluated the presence of ENs, BEA and FUS in tiger nuts collected in Spain, evidencing the lowest incidence in the samples, but high concentrations ranged from 32.2 to 4440 mg/kg for ENs type A and from 21.6 to 346 mg/kg for ENs type B. Moreover, other authors which analyzed cereals from countries with similar conditions to Spanish climatology, such as Morocco or Tunisia, also described high levels of emerging *Fusarium* mycotoxins, specially of ENA1 (Mahnine et al., 2011; Oueslati et al., 2011; Sifou et al., 2011). The ENs, BEA and FUS levels found in the studies described above are considerably higher than the levels evidenced in our study.

Table 3. Incidence and concentration range of enniatins, beauvericin and fusaproliferin in organic and conventional pasta.

Mycotoxin	Organic pasta (N=40) [a]				Conventional pasta (N=74) [a]			
	Whole-grain dry pasta		White dry pasta		Dry pasta		Fresh pasta	
	N [a] (percentage of positive samples)	Concentration range of positive samples (μg kg^{-1})	N [a] (percentage of positive samples)	Concentration range of positive samples (μg kg^{-1})	N [a] (percentage of positive samples)	Concentration range of positive samples (μg kg^{-1})	N [a] (percentage of positive samples)	Concentration range of positive samples (μg kg^{-1})
ENA	18 (100%)	0.50-22.04	22 (91%)	0.50-42.04	54 (72%)	0.50-24.79	20 (55%)	0.50-5.09
ENA1	18 (72%)	0.25-11.64	22 (82%)	0.25-14.92	54 (81%)	0.25-21.89	20 (60%)	0.25-6.98
ENB	18 (89%)	0.66-22.10	22 (77%)	0.50-12.61	54 (76%)	0.50-122.13	20 (85%)	0.50-33.13
ENB1	18 (72%)	0.50-24.32	22 (82%)	0.50-9.34	54 (65%)	0.50-979.56	20 (75%)	0.50-13.41
BEA	18 (28%)	0.10-10.14	22 (23%)	0.10-7.69	54 (11%)	0.62-20.96	20 (20%)	0.10-0.12
FUS	18 (22%)	0.05-0.30	22 (9%)	0.49-8.02	54 (11%)	0.05-0.36	20 (10%)	0.05-0.23

[a] N: number of total samples

The results from our investigation could not be compared with the corresponding legislated MLs, since the European Commission has not been set yet the MLs for ENs, BEA and FUS. It could be concluded from this study that the incidence and the contamination levels of ENs were very high comparing to BEA and FUS levels. These findings suggest that the samples of pasta could be produced from infected cereal crops, because the colonization by the genus *Fusarium* in wheat-grain is favored under appropriate environmental conditions. Specially, the characteristic climatic conditions achieved in the Mediterranean area (high humidity and temperature, among others) promote the *Fusarium* colonization and the ENs production in the field.

3.2.2. Simultaneous presence of ENs, BEA and FUS

With regard to the simultaneous presence of four ENs, BEA and FUS in the samples, the 3% of the samples (3 out of 114 total samples) contained all mycotoxins. In 74 out of 114 samples (65%) were found four, three or two ENs. One sample of whole-grain organic pasta from Italy was contaminated simultaneously by ENA, BEA and FUS. The 14% of the samples (16 out of 114 total samples) contained BEA with at least one of the four ENs, and the 8% of the samples (9 out of 114 samples) contained FUS with at least one of the four ENs. Also, in one sample of fresh conventional pasta from Italy only was found ENB, while in one sample of dry conventional pasta from Spain only was found FUS.

The simultaneous presence of BEA or FUS with ENs observed in our study (Fig. 3a and 3b) is in agreement with other published studies: Sifou et al. (2011) obtained a high number of rice samples from Morocco (49%) contaminated with ENs and BEA, while only two samples (3%) were

contaminated with ENs and FUS. Mahnine et al. (2011) concluded that the 12% of breakfast cereals from Morocco were contaminated with at least two groups of mycotoxins (ENs/BEA or ENs/FUS).

Furthermore, the results from our study showed the high percentage of samples contaminated by two or more ENs. These results are in line with the findings of Oueslati et al. (2011) in Tunisia, who reported that the 74% of the analyzed cereal samples (wheat, barley, corn and sorghum) were contaminated by at least two different ENs. Jestoi et al. (2004b) have also demonstrated the high prevalence of cereal samples (barley, oat and wheat) that contained simultaneously two or more ENs.

Given that the simultaneous presence of emerging *Fusarium* mycotoxins is very common and that the combinations of so called traditional *Fusarium* mycotoxins (trichothecenes, fumonisins, etc.) have been found by many authors in foodstuffs (Songsermsakul and Razzazi-Fazeli, 2008), probably both types of mycotoxins (traditional and emerging) could be produced by the genus *Fusarium* mainly in cereals. Therefore, it is necessary to develop multi-toxin methods for the simultaneous analysis of emerging and traditional *Fusarium* mycotoxins. Moreover, the combined intake of mycotoxins represents a possible health risk than the intake of only one mycotoxin alone. The available data show that the different mixtures of trichothecenes (e.g. NIV with T-2, DAS or DON, and DON with T-2 or DAS) caused pronounced additive, antagonistic or synergistic effects (Speijers and Speijers, 2004). Considering the described findings, in the future it would be interesting to include the ENs, BEA and FUS in studies about combined toxic effects of mycotoxins.

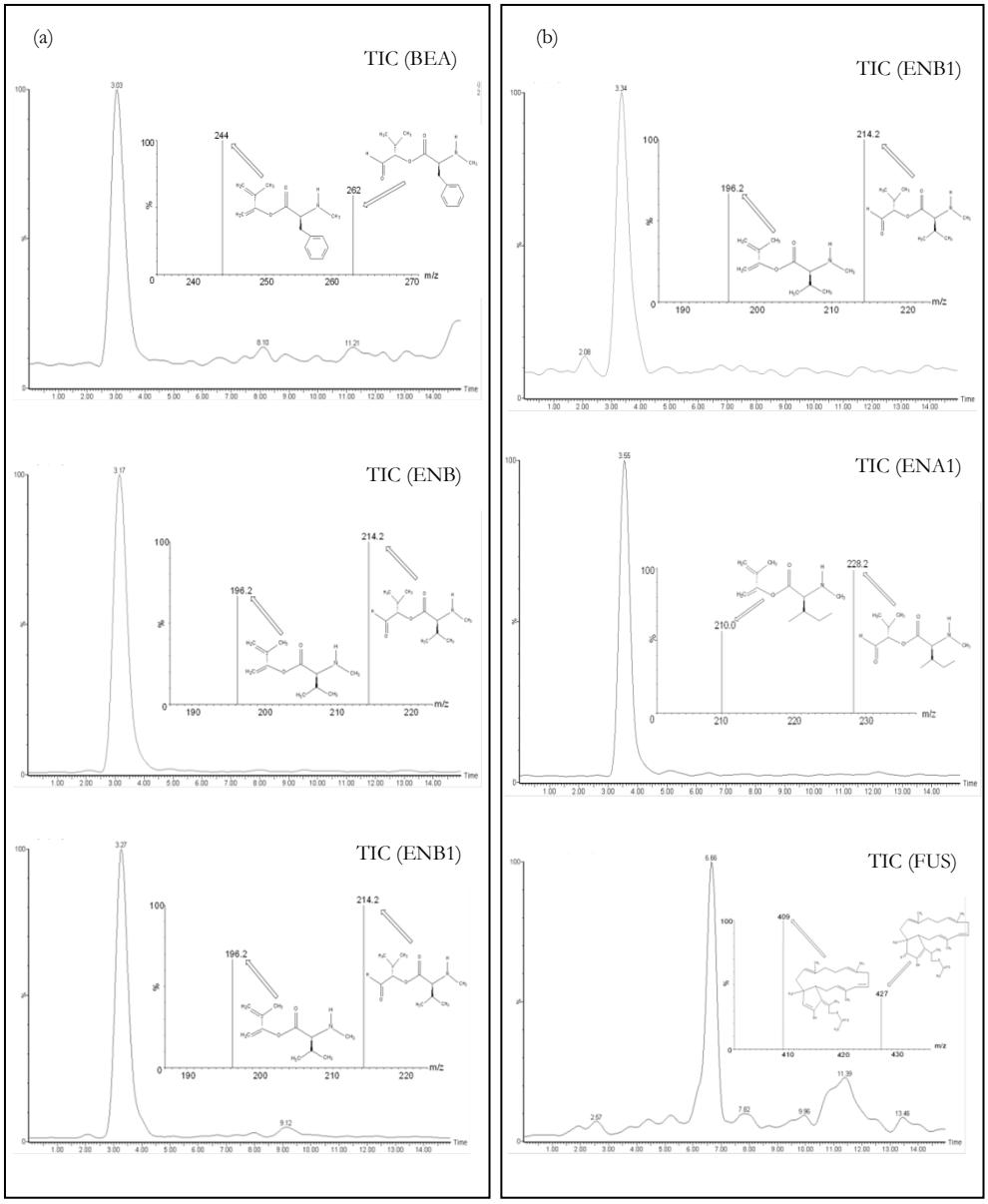


Fig. 3. MRM Chromatograms and daughter ion spectrums corresponding to: (a) sample of dry conventional pasta naturally contaminated with 9.04 μg/kg of BEA, 23.28 μg/kg of ENB and 19.24 μg/kg of ENB1, and (b) sample of organic white pasta naturally contaminated with 6.43 μg/kg of ENB1, 14.92 μg/kg of ENA1, and 8.02 μg/kg of FUS.

3.2.3. Influence of the agricultural practice in organic and conventional pasta

The 100% of the organic samples (40 samples) and the 88% of the conventional samples (65 out of 74 conventional samples) were contaminated by some of the studied mycotoxins. The percentages of incidence of ENA, ENB, ENB1 and BEA (95%, 83%, 78% and 25%, respectively) were higher in samples of organic pasta (Table 3). With respect to ENA1 and FUS, no significantly differences were obtained in organic and conventional samples (Table 3). With regard to the levels of contamination, the mean concentrations of ENA and FUS were higher in organic pasta (7.25 and 0.23 μg/kg, respectively) than in conventional pasta (4.79 and 0.02 μg/kg, respectively), while for ENA1 and BEA no significantly differences were obtained between both practices. However, mean concentrations of ENs type B were significantly higher for conventional samples (12.84 μg/kg of ENB and 18.79 μg/kg of ENB1) than for organic samples (5.93 μg/kg of ENB and 4.97 μg/kg of ENB1). Therefore, the results about the levels of concentration were not conclusive for the two types of pasta.

At the moment, only one published study is available about the occurrence of emerging *Fusarium* mycotoxins in organic and conventional products. Jestoi et al. (2004b) studied the natural presence of ENs, BEA and FUS, among other mycotoxins, in grain-based products from organic and conventional farming. The results from this study showed highest concentrations of BEA, ENB and ENB1 in conventional products, while there were not observed significantly differences in the concentrations of ENA and ENA1. Therefore, the results concerning ENA1, ENB and ENB1 are similar than those obtained in our study.

The influence of the agricultural practice also has been reported for other mycotoxins by many authors. D´Arco et al. (2009) and Silva et al. (2009) described highest contamination levels and highest frequency of fumonisins in organic cereals, while other authors affirmed that the contamination and incidence of trichothecenes were higher in cereals from conventional production (Gottschalk et al., 2007; Ibañez-Vea et al., 2012). Furthermore, most authors have concluded that the farming system is not decisive factor for the fungi colonization and mycotoxin contamination in agricultural products (Ariño et al., 2007; González-Osnaya et al., 2007; Edwards, 2009). In general, an intensive tilling the compliance with crop rotations as basic principles of organic and conventional agriculture practices could be prevent the fungi colonization and the mycotoxin production in the field. Therefore, opposite conclusions have been obtained between the different researchers that compared the mycotoxin levels in conventional and organic cereals.

Moreover, the two types of samples (organic and conventional) were classified in two sub-types. The samples of organic pasta were classified in organic whole-grain pasta and in organic white pasta. The samples of conventional pasta were classified in dry pasta and fresh pasta. If the data are analyzed according to this classification, in our study were obtained the following results. Concerning conventional samples, the mean concentrations of the studied mycotoxins were higher in conventional dry pasta than in conventional fresh pasta (Table 4). The incidence of ENA and ENA1 was considerably higher in samples of dry pasta, while the incidence of ENB, ENB1 and BEA was higher in samples of fresh pasta (Table 3). With regard to the incidence of FUS, significant differences were not obtained for both types of pasta (Table 3). As far as we know, no data have been published about the

presence of mycotoxins in fresh pasta. On this account, it was not possible the comparison between our results and the results from other studies. Concerning organic samples, the mean concentrations of the studied mycotoxins were higher in organic whole-grain pasta than in organic white pasta (Table 4). The prevalence of ENA, ENB, BEA and FUS was higher in samples of whole-grain pasta, while the prevalence of ENA_1 and ENB_1 was higher in white pasta (Table 3). It is in agreement with the majority of the published studies, because the highest levels of mycotoxins are found in the bran and the shell present in whole-grain foods (Duarte et al., 2010).

Generally, in our study was observed that the organic practices lead to higher occurrence of the emerging *Fusarium* mycotoxins in cereal samples, which is due mainly to the fact that fungicides are not used in organic production. Moreover, conclusive results were not obtained about the relationship between agriculture practices and mycotoxin contamination levels from this study. For this reason, it could be of great interest the realization studies about the presence of emerging *Fusarium* mycotoxins comparing other factors than the agricultural practices, such as the temperature, the humidity, the soil type or the physical integrity of the product.

3.3. Evaluation of the dietary exposure

Table 4 shows the mean concentrations and the IDEs of ENs, BEA and FUS individually, and the IDEs for the sum of all mycotoxins in organic samples (whole-grain and white pasta), in conventional samples (dry and fresh pasta), and in total samples. In total samples, it was observed that the EDIs of ENA, ENA_1, ENB and ENB_1 were in the same order (EDIs between 0.00212 and 0.00472 μg/kg bw/day). The EDIs of FUS (0.0001 μg/kg bw/day) and

BEA (0.0005 μg/kg bw/day) were around ten times lower than the EDIs estimated for ENs. Concerning to the type of sample, the dietary exposure of organic pasta (whole-grain and white pasta) was estimated on the order of 0.003 μg/kg bw/day. The highest dietary exposure (sum of total mycotoxins) was estimated in conventional dry pasta (0.0072 μg/kg bw/day), while the lowest dietary exposure was obtained for conventional fresh pasta (0.0001 μg/kg bw/day). It is due to the fact that the intake of fresh pasta by the Spanish population is much lower than the intake of dry pasta (MAGRAMA, 2011). The study of Serrano et al. (2012a) provided data about the dietary exposure of BEA in different cereals from Spain, Italy, Morocco and Tunisia. In that study, BEA was found in several grain cereals and cereal products from Spain, Morocco and Tunisia. The EDI obtained in our study for BEA in total samples (0.0005 μg/kg bw/day) are 10 times lower than those obtained for Spanish population by Serrano et al. (2012a). This fact is due that in our study the EDI was calculated from the pasta consumption, while in the work of Serrano et al. (2012a) the EDI of BEA were calculated considering more cereal products (soup, pasta, biscuits, snacks and flour).

Given that PMTDIs for emerging *Fusarium* mycotoxins have not been established by the authorities, the risk assessment was not possible. However, with the available data it was possible the approximation to the estimation of the risk assessment according to Serrano et al. (2012b). On this account, our results were compared based on the safety guidelines for other *Fusarium* mycotoxins: PMTDIs established for nivalenol, deoxynivalenol, toxins T-2 and HT-2, fumonisin B1 and fumonisin B2 (1, 0.7, 0.1 and 2 μg/kg bw/day, respectively) (JECFA, 2001; SCF, 2002; EFSA, 2011). The EDI estimated in our study for all samples and for the sum of total mycotoxins (0.0135 μg/kg

bw/day) is one or two times lower than the PMTDIs established by other *Fusarium* mycotoxins. According to the results, the intake of emerging *Fusarium* mycotoxins from Spanish pasta does not represent a high risk to the adult population.

4. CONCLUSION

The study about the toxicity and the presence of the emerging *Fusarium* mycotoxins is a prerequisite for legislative purposes, since limited data are available on these mycotoxins and maximum levels have not been established yet by the authorities. The results from this study indicated that more attention should be paid to the contamination by ENs in cereals. In general, the prevalence of emerging *Fusarium* mycotoxins was superior in cereals from organic agriculture. However, the results in terms of concentration levels in organic and conventional pasta were diverse for each mycotoxin, which raises the necessity of more studies depending on the agricultural practice. In addition, other factors influencing the presence of these mycotoxins, such as environmental conditions, should be controlled and studied in the future.

Given the limited studies on the toxicity *in vivo* and *in vitro* of the emerging fusariotoxins, at the moment is not possible to predict if emerging *Fusarium* mycotoxins are a public health problem.

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Table 4. Estimated daily intake (EDI) of ENs, BEA and FUS present in organic and conventional pasta.

Mycotoxin	Organic whole-grain pasta		Organic white pasta		Conventional dry pasta		Conventional fresh pasta		EDI of total samples (µg/kg bw/day)
	Mean conc. (µg/kg)	EDI (µg/kg bw/day)	Mean conc. (µg/kg)	EDI (µg/kg bw/day)	Mean conc. (µg/kg)	EDI (µg/kg bw/day)	Mean conc. (µg/kg)	EDI (µg/kg bw/day)	
EN A	3.20	0.000437	8.14	0.001112	5.37	0.000734	3.28	0.000027	**0.00255**
EN A_1	5.92	0.000809	3.54	0.000484	5.91	0.000807	2.20	0.000018	**0.00212**
EN B	8.01	0.001094	3.98	0.000544	15.47	0.002113	5.73	0.000047	**0.00380**
EN B_1	6.32	0.000863	3.28	0.000448	24.78	0.003385	2.61	0.000021	**0.00472**
FUS	0.02	0.000003	0.40	0.000055	0.02	0.000002	0.02	0.0000002	**0.00010**
BEA	1.79	0.000244	0.38	0.000052	1.25	0.000171	0.02	0.0000001	**0.00050**
Sum of total mycotoxins	*25.26*	*0.003450*	*19.72*	*0.002694*	*52.80*	*0.007212*	*13.86*	*0.000114*	***0.01350***

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3.6. Risk assessment associated to the intake of the emerging *Fusarium* mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin

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Risk assessment associated to the intake of the emerging *Fusarium* mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin

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ABSTRACT

Forty-five samples of Spanish follow-up infant formula with different chemical compositions were analyzed determining the emerging *Fusarium* mycotoxins beauvericin (BEA), enniatins (ENs) (A, A_1, B, B_1), and fusaproliferin (FUS). The samples were extracted three times with ethyl acetate using an Ultra-turrax homogenizer. Mycotoxins were identified and quantified using a liquid chromatography (LC) coupled to a diode array detector (DAD). Results showed that the percentage of the samples contaminated with ENs and FUS were 46.6 and 20.0% respectively, whereas all analyzed samples were free of BEA. The ENs A and B were detected only in one sample with 149.6 and 39.4 mg/Kg respectively. The ENB_1 was the more detected mycotoxin with levels ranging from 11.4 to 41.9 mg/Kg. The ENA_1 was detected at levels ranging from 6.3 to 101.7 mg/Kg. The minor *Fusarium* mycotoxin FUS was detected in a range variable from 0.7 to 1.7 mg/Kg. Finally, dietary exposure of Spanish infants (between 6 and 12 months) to ENs, BEA and FUS, was estimated through the consumption of commercial follow-up infant formula by the calculation of the estimated daily intake (EDI). Considering the sum of the mycotoxins studied, the data evidenced that the EDI was 236.2 μg/Kg bw/day.

Keywords: Enniatins, beauvericin, fusaproliferin, *Fusarium* spp, infant formula, risk assessment

1. INTRODUCTION

Mycotoxins are naturally occurring toxic secondary metabolites produced under appropriate favorable conditions by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. Among the mycotoxins produced by *Fusarium* spp., are included the enniatins A, A_1, B and B_1 (ENA, ENA_1, ENB and ENB_1), beauvericin (BEA) and fusaproliferin (FUS), which are a group of bioactive compounds called emerging mycotoxins or minor *Fusarium* mycotoxins (Meca et al., 2010). FUS is a bicyclic sesterterpene formed by five isoprenoid units, whereas BEA and enniatins (ENs) are cyclic hexadepsipeptides. In particular BEA is composed by of alternated D-α-hydroxyisovaleryl-(2-hydroxy-3-methylbutanoic acid) and N-methyl-L-phenylalanyl residues, whereas ENs are of three D-α-hidroxyisovaleryl and three N-methyl-L-amino acid residues (Jestoi, 2008). BEA and ENs show a similar chemical structures, and therefore can be assumed that present the same toxic dynamic actions: has been demonstrated that have cytotoxic effects in several cell lines (Fornelli, Minervini, & Logrieco, 2004; Kamyar, Rawnduzi, Studenik, Kouri, & Lemmens- Gruber, 2004; Ferrer, Juan-García, Font, & Ruiz, 2009); whereas FUS showed causes teratogenic effects on chicken embryos (Ritieni et al., 1997). Due to these toxic effects, the contamination by mycotoxins causes severe economic losses annually, and depends of several factors including climatic conditions, genetic susceptibility of cultivars to fungal infection, soil type and nutritional factors (Bakan, Melcion, Richard-Molard, & Cahagnier, 2002).

Several analytical methods have been described in the literature for the detection of minor *Fusarium* mycotoxins in several foods. The methods include the common steps applied in mycotoxin analyses: sample preparation using

extraction of the analytes and removal of impurities using different types of columns. Sample analysis is carried out using high performance liquid chromatography (LC) with ultraviolet (UV) or mass spectrometric (MS) detection (Jestoi et al., 2005; Santini, Ferracane, Meca, & Ritieni, 2009). The presence of ENs, BEA and FUS in food commodities has been recently reported during the last decade in some European countries (Finland, Norway, Spain, Slovakia, Croatia, Switzerland and Italy), in USA, in South Africa and in Australia. Recently our research group has reported the contamination of cereals (maize, wheat and barley) and cereal products (breakfast cereals) available in Morocco and Spain (Mahnine et al., 2011; Meca, Zinedine, Blesa, Font, & Mañes, 2010; Zinedine, Meca, Mañes, & Font, 2011; Sifou et al., 2011). Mahnine et al. (2011) studied the presence of ENs, BEA and FUS in breakfast and infant cereals from Morocco, evidencing principally the presence of the ENB_1 and ENA_1.

Infants are considered a vulnerable group of the population considering the mycotoxins intake due to a diet rich in cereals and also to a reduced body weight (bw) compared to the adults. As consequence, maximum limits for mycotoxins in baby foods are much lower than the limits set for other cereal products (European Commission, 2006). In order to prevent infant exposure to mycotoxin contamination, dietary exposure studies are of great interest. Most evaluations have focussed in the called "traditional" mycotoxins such as fumonisins or trichothecenes (D´Arco et al., 2009; González-Osnaya et al., 2011), and Tolerable Daily Intakes (TDIs) have been set by scientific committees such as the Scientific Committee for Food (SCF) for many of *Fusarium* mycotoxins (SCF, 2002). In contrast, no TDIs have been set for minor *Fusarium* mycotoxins, probably due to their late recognition and

therefore, their limited data available on their toxicity, concentration levels and occurrence. Several studies suggest that infants up to the age of 6 months typically lose the capacity to detoxify and eliminate substances (Dourson et al., 2004). In this context, the aims of this study were: a) to determine the presence of the minor *Fusarium* mycotoxins BEA, ENs and FUS in different follow-up infant formula of Spanish origin and b) to evaluate the risk exposure of Spanish infants to these *Fusarium* mycotoxins present in the samples analyzed by the evaluation of the Estimated Daily Intake (EDI).

2. MATERIALS AND METHODS

2.1. Chemical and reagents

Acetonitrile and ethyl acetate, all of HPLC grade, were purchased from Merck (Whitehouse Station, N.J., U.S.A.). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath. The stock standard solutions of BEA and ENA, ENA1, ENB and ENB1 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Standard solution of FUS was gently given by Professor Alberto Ritieni, Department of Food Science, University of Naples "Federico II", Italy. All stock solutions were prepared by dissolving 1 mg of the mycotoxin (BEA, FUS or ENs) in 1 mL of pure methanol, obtaining a 1 mg/ml solution. These stocks solutions were then diluted with pure methanol in order to obtain the appropriated work solutions. All solutions were stored in darkness at 4ºC until the LC analysis.

2.2. Samples description

A total of forty-five samples of infant formula were studied and divided in: nine follow-up infant formulas composed with eight different flours (wheat, corn, rice, oat, barley, rye, sorghum, and millet), seven follow-up infant formulas composed with enriched with fruit juice (8-25%) and eight different cereal flours (wheat, corn, rice, oat, barley, rye, sorghum, and millet), seven follow-up infant formulas composed with enriched with honey (5%) and eight different flours (wheat, corn, rice, oat, barley, rye, sorghum, and millet), five follow-up infant formulas composed with six different flours (wheat, corn, rice, oat, barley, rye), ten follow-up infant formulas whole cereals (corn, wheat and rice), and seven follow-up infant formulas composed with two different flours (rice and corn). A 20-40 g of sample (according to the indication of the package) was diluted in 200 ml of milk infant formulas. A 100 ml subsample was collected in a plastic bag and kept at -20ºC until analysis.

2.3. Mycotoxin extraction procedure

The method used for the analysis of the mycotoxins (BEA, ENs and FUS) was reported by Jestoi (2008). Briefly, 3 g of reconstituted infant formula were extracted with 30 ml of ethyl acetate using an Ultra Ika T18 basic Ultra-turrax (Staufen, Germany) for 5 min. The extract was centrifuged at 4500g for 15 min and then the supernatant evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland) and then re-dissolved in 2 ml of extraction solvent. This final solution was filtered through a 25 mm/0.45 μm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before the injection into the LC-DAD system for analysis.

2.4. LC-DAD analysis

LC analyses of BEA, ENs and FUS were performed using a Shimadzu LC system equipped with LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Gemini (150 x4.6 mm, 5 μm) Phenomenex column was used. LC conditions were set up using a constant flow at 1.0 mL/min and acetonitrile-water (70:30 v/v) as starting eluent system. The starting ratio was kept constant for 5 min and then linearly modified to 90% acetonitrile in 10 min. After 1 min at 90% acetonitrile, the mobile phase was taken back to the starting conditions in 4 min. BEA and ENs were detected at 205 nm, while FUS was detected at 261 nm. All samples were filtered through a 0.22 μm syringe filter Phenomenex prior to injection (20 μl) onto the column. Mycotoxin identification was performed by comparing retention times and UV spectra of purified extracted samples to pure standards. Quantification of mycotoxins was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

2.5. LC-MS/MS confirmation

The presence of ENs, FUS, and BEA in positive samples of cereals was confirmed by LC-MS/MS according to the method described by Sørensen, Nielsen, Rasmussen, and Thrane (2008). Briefly, analysis of BEA, FUS and ENs was carried out with a TQ mass spectrometer Quattro LC from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters, Milford, MA) consisted of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and a Mass Lynx NT software 4.1 were used for data acquisition and processing. The auto

injector was programmed to inject 20 µl into the Luna C18 column (150x4.6 mm, 5 mm) Phenomenex maintained at 30 ºC.

The analytical separation for LC-MS/MS was performed using gradient elution with water as mobile phase A, and acetonitrile as mobile phase B, both containing 0.5% formic acid. After an isocratic step of 65% B for 3 min, it was linearly increased to 75% B in 4 min and held constantly for 3 min. Flow rate was maintained at 0.3 ml/min.

Analysis was performed in positive ion modes. The ESI source values were as follows: capillary voltage, 3.20 kV; source temperature, 125ºC; desolvation temperature, 350ºC; desolvation gas (nitrogen, 99.95% purity) flow, 700 l/h. Ideal fragmentation conditions were accomplished varying the cone voltage and collision energies for each compound. For the detection of FUS and BEA the precursor ion were m/z 445 and 801 being the product ions selected were m/z 427-409 and 784-756 specifically. For ENB the precursor ion was m/z 640, and the product ions were m/z 196 and 527. For ENB_1 the precursor ion was m/z 654 and the product ions m/z were 196 and 228, for ENA the precursor ions was m/z 682, and the product ions were m/z 210 and 555, for ENA_1 the precursor ion was m/z 668, and the product ions were m/z 210 and 541.

2.6. Sample fortification

Recoveries of the extraction method were determined by sample fortification. Samples were artificially contaminated in triplicates at levels of ENs (0.1-50 mg/Kg), FUS (0.1-50 mg/Kg) and BEA (0.1-50 mg/Kg). For this purpose, 3 g of infant formula sample were fortified 1 h before extraction.

After the fortification and homogenization, samples were analyzed as described above.

3. RESULTS AND DISCUSSION

3.1. Method performance

Mean recoveries of fortified infant formula samples (n=5) at levels of ENs (0.1-50 mg/Kg), FUS (0.1-50 mg/Kg) and BEA (0.1-50 mg/Kg) were respectively 84.6%, 70.5%, and 88.6% with a relative standard deviations of 3.5%, 4.6% and 3.2%. Intra-day (n=5) and inter-day (5 different days) variation values were 2.2-8.9 %, 3.0-10.8% and 2.8-8.9%, respectively for ENs, FUS and BEA. These values are below 15% which is the maximum variation for certification exercises for several mycotoxins. The detection limit (LOD) and the limit of quantification (LOQ) values were calculated according to signal/noise (s/n) ratio that were s/n=3 and s/n=10, respectively. The LODs and the LOQs of FUS are 0.07 and 0.30 μg/Kg. The LODs and the LOQs of BEA are 0.19 and 0.55 μg/Kg, respectively. The LODs and the LOQs of ENs are 0.21 and 0.64 μg/Kg for ENA, 0.19 and 0.50 μg/Kg for ENA_1, 0.14 and 0.40 μg/Kg for ENB and finally 0.17 and 0.60 μg/Kg for ENB1.

3.2. Occurrence of ENs, BEA, and FUS in infant formula samples

Results from the natural occurrence of the four ENs in analyzed infant formula samples are summarized in Table 1. As shown, 21 of 45 total samples evidenced at least one of the four ENs. The frequency of contamination of total samples with total ENs (ENA, ENA_1, ENB and/or ENB_1) was 46.6%. Only one sample (infant formula composed by a mix of six cereals) was contaminated by ENA with 149.6 mg/Kg.

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

Samples	Number of samples	Enniatins (mg/Kg)														
		Positive samples and frequency (%)	EN A			Positive samples and frequency (%)	EN A_1			Positive samples and frequency (%)	ENB			Positive samples and frequency (%)	ENB_1		
			Mean	Max. level	Min. level		Mean	Max. level	Min. level		Mean	Max level	Min. level		Mean	Max level	Min. level
Eight cereals and juice	7	0	ND	ND	ND	1 (14.2)	73.2	73.8	ND	0	ND	ND	ND	4 (57.1)	29.1	36.5	20.6
Whole grain	10	0	ND	ND	ND	1 (10)	6.3	6.3	ND	1 (10)	39.4	39.4	ND	1 (10)	35.8	35.8	ND
Eight cereals	9	0	ND	ND	ND	2 (22.2)	38.8	62.6	14.9	0	ND	ND	ND	4 (44.4)	22.9	37.8	11.4
Eight cereals with honey	7	0	ND	ND	ND	0	ND	ND	ND	0	ND	ND	ND	3 (42.8)	29.6	41.9	14.9
Six cereals	5	1 (20)	149.6	149.6	ND	1 (20)	101.7	101.7	ND	0	ND	ND	ND	3 (60)	31.7	34.5	28.9
Rice and corn	7	0	ND	ND	ND	1 (14.2)	23.8	23.8	ND	0	ND	ND	ND	3 (42.8)	31.0	38.6	26.1

ND, not detected

The frequencies of contamination of samples with ENA_1 were 14.2, 10.0, 22.2, 20.0, and 14.2% respectively considering the six classes of samples analyzed. The mean value evidenced was of 47.2 mg/Kg and the highest data (101.7 mg/Kg) was detected in the sample of the infant formula composed by a mix of six cereals. The class of samples that resulted more contaminated by the ENA_1, was the follow up infant formulas composed by a mix of eight cereals, with the 22.2% of frequency, whereas in the samples composed by a mix of eight cereals enriched with honey this bioactive compound was not detected in any of the samples analyzed. The ENB was detected only in one of the samples analyzed, and in particular in the follow up infant formula composed by whole grain flours. The data evidenced was of 39.4 mg/Kg (Table 1). The ENB_1 was the more detected bioactive compound considering the compounds studied and in particular the frequency of contamination evidenced for this mycotoxin in the six samples typologies analyzed was of 57.1, 10.0, 44.4 42.8, 60.0, and 42.8%. The mean value detected was of 28.9 mg/Kg whereas the highest and lowest data were of 41.9 and 11.4 mg/Kg, respectively. The minor *Fusarium* mycotoxin FUS was detected in 9 of the samples analyzed and the frequency of contamination evaluated in the samples typologies ranged from 14.2 to 40.0%. The mean contamination data of this mycotoxin was of 0.8 mg/Kg (Table 2). The co-occurrence of ENs in follow up infant formula samples showed that 4 samples (8.8% of total samples) were co-contaminated with at least two ENs, but any of the samples was found to be co-contaminated with three or more ENs. LC-DAD chromatogram of positive sample of follow up infant formula with eight cereals and juice naturally contaminated with FUS is represented in Fig. 1a. LC-DAD

chromatogram of positive sample of follow-up infant formula with eight cereals naturally contaminated with ENA_1 and ENB_1 is represented in Fig. 1b.

This work can be considered the first study where the minor *Fusarium* mycotoxins were determined in follow-up infant formulas of Spanish origin. In the literature, there are more information about the traditional mycotoxins as ochratoxin A (OTA), aflatoxin B_1 (AFB_1) and deoxinivalenol (DON). This is partly due that BEA, ENs and FUS have been appreciated only during the last two decades (Jestoi, 2008).

Table 2. Presence of FUS in analyzed samples.

Samples	**Number of samples**	**Fusaproliferin (mg/Kg)**			
		Positive samples and frequency (%)	**FUS**		
			Mean	**Max. level**	**Min. level**
Eight cereals and juice	7	1 (14.2)	0.5	0.5	ND
Whole grain	10	3 (30)	1.4	1.7	1.1
Eight cereals	9	1 (11.1)	0.4	0.4	ND
Eight cereals with honey	7	0	ND	ND	ND
Six cereals	5	2 (40)	0.4	0.5	0.3
Rice and corn	7	1 (14.2)	0.5	0.5	ND

ND, not detected

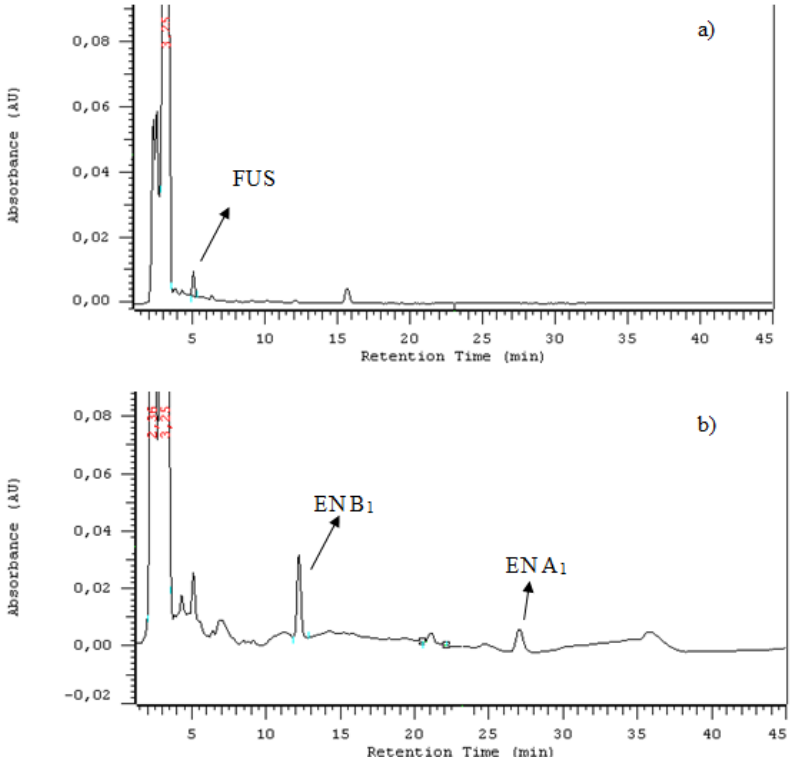

Figure 1. LC-DAD chromatograms of a) follow up infant formula (eight cereals and juice) contaminated with the mycotoxin FUS and b) follow up infant formula (eight cereals) contaminated with the mycotoxins ENB_1 and ENA_1.

Logrieco et al., 2002 evaluated the presence of the minor *Fusarium* mycotoxins BEA and ENs on 13 rye samples from Finland origin evidencing the presence of BEA, together with some ENs, in all the analysed samples in a concentration range variable from 3.5 to 640 mg/Kg.

Castoria et al. 2005 evaluated the presence of the bioactive compound BEA in spelt samples of Italian origin detecting in three samples out of eight this compound in the range variable from 1 to 4 mg/kg (mean values from 0.5 to 11.7 mg/Kg).

Sørensen et al. (2008) studied the contamination of 30 maize Finnish samples collected in autumn of 2005, and 43 Finnish samples collected in the same period of the year 2006. The ENs detected by the authors occurred in the samples analyzed in the relationship of ENB>ENB_1>ENA_1>ENA for both years, evidencing mean concentration data ranging from 4 to 78 mg/Kg in the 2005 and from 10 to 137 mg/Kg in the 2006. The data evidenced by the authors and in particular the maximum value is lower if compared with the data evidenced in our study.

Meca et al. (2010) reported the contamination of cereals samples from Spain with ENs, BEA and FUS with frequencies of 73.4%, 32.8% and 7.8%, respectively. Authors found that ENA_1 was the most mycotoxin found.

Zinedine et al. (2011) evaluated the presence of the mycotoxins BEA, FUS and ENs in raw cereals from Morocco. Analytical results showed that the frequencies of contamination of total samples with ENs, BEA and FUS were 50.0, 26.5 and 7.8%, respectively. ENA_1 was the most common EN found with a percentage of contamination of 39.0%.

Oueslati et al. (2011) evaluated the presence of *Fusarium* mycotoxins ENs, BEA and FUS in cereals and derived products from Tunisia. The percentage of contamination of total samples with ENs was 96.0%. ENA_1 was also the most common EN found with the highest prevalence of 92.1%.

Srobarova et al. (2002) studied the contamination level by FUS of eleven maize samples from Slovakian origin. In this surveillance, FUS was detected and quantify in a concentration variable from 0.025 to 8.2 mg/Kg in 7 out of 11 maize samples.

Mahnine et al. (2011) studied the levels of emerging *Fusarium* mycotoxins ENs, BEA and FUS in breakfast and infant cereals from Morocco. Analytical

results showed that the percentages of analyzed samples contaminated with total ENs, FUS and BEA were 30.8%, 10.3% and 5.8%, respectively. ENA_1 was the mycotoxin most often found: ENA_1 levels ranged between 37.5 and 688 mg/Kg. FUS and BEA were present in levels lower than 7.4 and 10.6 mg/Kg, respectively.

Munkvold et al. (1998) reported high contamination levels of animal feeds of American origin with FUS with levels up to 30 mg/Kg. Logrieco et al. (1996) reported high levels of BEA up to 60 mg/Kg in maize from Poland, while Ritieni et al. (1997) reported high levels of BEA up to 520 mg/Kg in maize from Italy. BEA and ENs have been detected worldwide, and are generally found in grains contaminated with several *Fusarium* species (Logrieco et al., 2002; Plattner and Nelson, 1994).

3.3. Estimation of the daily intake for ENs, BEA and FUS

One of the most important aspects of the risk assessment of mycotoxins is the determination of the degree of human exposure. The risk assessment related to the intake of ENs, BEA and FUS in Spanish infant population was carried out for the first time. The EDI was calculated relating the presence of the ENs, BEA and FUS in the samples (Table 3) with the infant formula consumption by the Spanish infants (0.0106 Kg of infant formula/Kg bw/day) (Fomon and Nelson, 1995). The value of consumption was calculated considering a mean of body weight for infants between 6 and 12 month-old (8.5 Kg) (Fomon and Nelson, 1995), and an intake of 90g of infant formula, according to the recommended daily intake reported on the package label of the infant formula studied, Table 3 shows the mean concentration data for all

samples (positives and negatives), and the EDI for ENs, BEA, FUS and also the sum of ENs.

Table 3. Estimated daily intake (EDI) of ENs and FUS from follow-up infant formulas.

Mycotoxin	Mean levels (μg/kg)	EDI (μg/kg bw/day)
EN A	3326	35.2
EN A_1	6300	66.7
EN B	876	9.3
EN B_1	11660	123.4
Sum of ENs	22162	234.6
FUS	153	1.6

The values of ENs EDI ranged from 9.3 to 123.4 μg/Kg bw/day, and for the sum of ENs the EDI data was of 234.6 μg/Kg bw/day. With the data evidenced in our study is not possible to confirm that the ENs, BEA and FUS represent a risk for infant Spanish consumers, since JECFA have been not established a provisional maximum tolerable daily intake (PMTDI). Considering that for this class of mycotoxins no PMTDI data are available, the approximation to the risk assessment was carried out, according to the safety guidelines established for other *Fusarium* mycotoxins (JECFA 2001; SCF, 2002). PMTDI values established for nivalenol (NIV), deoxynivalenol (DON), toxin T-2 and toxin HT-2 (T-2 and HT-2), fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) are 1, 0.7, 0.06 and 2 μg/Kg bw/day. Considering that these PMTDI

values are calculate for adults population, and that the EDI of infants for other toxic substances is one or two fold higher than the estimated adult dietary exposure (SCF, 2001), EDI values obtained in our study for infants were converted in adult EDI using the equation reported below (Eqn. 1); in order to compare the EDI obtained for the minor *Fusarium* mycotoxins with the PMTDI of the most toxic fusarotoxins.

$$EDI_a = EDI/100 \quad (1)$$

EDI_a calculated for the sum of ENs was 2.35 μg/Kg bw/day, and it was comparable with the PMTDI established for DON and for the sum of FB_1 and FB_2, and one and two fold higher than the PMTDI of NIV and the sum of T-2 and HT-2, respectively. EDI_a calculated for FUS (0.016 μg/Kg bw/day) was lower than PMTDI values established by JECFA for all legislated *Fusarium* mycotoxins. Considered the data presents in the scientific literature only two studies focused on the risk associated to the intake of the *Fusarium* mycotoxin present in infant food of Spanish origin were published.

In particular D´Arco et al. (2009) studied the presence of FB_1, FB_2 and FB_3 in conventional and organic corn products (baby food, corn flour, corn flakes, pasta, cookies and other corn products), and evaluated the risk assessment of fumonisin intake presents in corn products by the EDI estimation in infant and adult population. The mean data of EDI for infants related to conventional and organic samples were of 0.0017μg/Kg bw/day and 0.72 μg/Kg bw/day, respectively. The most contaminated organic samples presented an EDI data of 3.42 μg/Kg bw/day, almost 2-fold higher than those calculated to PMTDI for the sum of FB_1 and FB_2.

Cano-Sancho et al. (2011) studied the exposure of Catalonian population (seniors, adult females, adult males, adolescents, children, infants, ethnics and celiac sufferers) to DON through food consumption (corn flakes, wheat flakes, sweet corn, corn snacks, pasta, beer, sliced bread, gluten-free foods, ethnic foods and baby foods), evidencing that Catalonian population should be expected to be exposed at moderated levels of DON. The population group of infants was the most exposed, with an EDI of 0.90 μg/Kg bw/day, but this value was lower than the PMTDI of 1 μg/Kg bw/day established for DON.

4. CONCLUSIONS

The results obtained in this study showed that an important number of samples of the follow up infant formula were contaminated with the studied emerging *Fusarium* mycotoxins and that the intake of the sum of ENs could represent a risk for the infant Spanish population.

Owing to the lack of available objective information of risk assessment associated to emerging *Fusarium* mycotoxins contamination, more research is needed both adult population and infant population.

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3.7. Risk assessment of beauvericin, enniatins and fusaproliferin present in follow-up infant formula by in vitro evaluation of the duodenal and colonic bioaccessibility

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Risk assessment of beauvericin, enniatins and fusaproliferin present in follow-up infant formula by in vitro evaluation of the duodenal and colonic bioaccessibility

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ABSTRACT

In this study, 72 samples of follow-up infant formula of Spanish origin were analyzed for the presence of the mycotoxins beauvericin (BEA), enniatins (ENs) (A, A1, B, B1), and fusaproliferin (FUS). The samples analyzed were extracted three times with ethyl acetate and then the mycotoxins were identified and quantified using a liquid chromatography (LC) coupled to a diode array detector (DAD).

The positive samples analyzed were digested through a simulated gastrointestinal digestion model, which permit to simulate the physiological condition of the human gastrointestinal tract (duodenal and colonic compartments) in order to assess the bioaccessibility of the bioactive compounds contained in the follow-up infant formula, and to perform the approach to the risk assessment related to the intake of these bioactive compounds. The ENB, was detected in 12 of the totally positive samples with a mean value of 32.3 ± 1.2 mg/kg, whereas the ENA was evidenced only in one sample with a value of 149.62 ± 2.36 mg/kg. The ENA_1 presented a mean contamination of 55.36 ± 2.1 mg/kg. The duodenal bioaccessibility of the bioactive compounds present in the analyzed samples ranged from 0.37 to 22.41%, whereas employing the duodenal + colonic digestion the mean bioaccessibility of the mycotoxins studied ranged from 1.63 to 29.00%.

Keywords: Beauvericin, Enniatins, Fusaproliferin, Infant formula, Bioaccessibility, LC-MS/MS.

1. INTRODUCTION

Mycotoxins are secondary metabolites which are produced by several fungi mainly belonging to the genera: *Aspergillus, Penicil- lium, Fusarium* and *Alternaria* (Sweeney & Dobson, 1998). Until now, approximately 400 secondary toxic metabolites produced by more than 100 molds have been reported.

The enniatins (ENs) are fungal metabolites produced by several *Fusarium* species (Ivanova, Skjerve, Eriksen, & Uhlig, 2006). They are cyclic depsipeptides, which are commonly composed of three D- α-hydroxyisovaleric acid residues linked alternatively to three L-configured N-methyl amino acid residues to give on 18- membered cyclic skeleton. ENs are described as phytotoxins, with antibiotic, herbicidal and insecticidal activities. They also have enzymatic inhibiting and oxidative phosphorylation properties (Tomoda et al., 1992). The cytotoxicity studies of ENs have been studied in vitro test assays in rodent, monkey, porcine, insect and human cell lines (Behm, Degen, & Follmann, 2009; Fornelli, Minervini, & Logrieco, 2004; Hyun, Lee, Lee, & Shin, 2009; Ivanova et al., 2006; Lee et al., 2008; Meca, Font, & Ruiz, 2011; Tonshin, Teplova, Andersson, & Salkinoja-Salonen, 2010; Vongvilai et al., 2004).

Fusaproliferin (FUS) is a recently described mycotoxin that is produced by two closely related species, *Fusarium* subglutinans and Fusarium proliferatum (Ritieni et al., 1995, 1997). Both species are important pathogens of maize (Kommedahl, Sabet, Burnes, & Windels, 1987; Ritieni et al., 1999) and other economically impor- tant crop plants and may be isolated together from the same plant (Moretti, Mulè, Ritieni, & Logrieco, 2007). FUS is toxic to *Artemia salina* (Moretti et al., 2007; Steenkamp, Wingfield, Desjardins, Marasas, & Wingfield, 2002), IARC/LCL 171 human B lymphocytes (Logrieco et al., 1996) and SF-9 insect cells (Ritieni et al., 1997) and has teratogenic and pathogenic effects

on chicken embryos (Logrieco et al., 1996). Recently the occurrence of FUS in several naturally contaminated samples of corn was reported (Jestoi, 2008).

The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body for absorption. The release of contaminant in the gastrointestinal tract, bioaccessibility, from the ingested product is a prerequisite for the absorption in the gastrointestinal tract and thus for the bioavailability of a contaminant (Versantvoort, van de Kamp, & Rompelberg, 2004). *In vivo* experiments using the mouse or rat as biological models are the best approach to the evaluation of the bioaccessibility and bioavailability of several bioactive compounds, but present several ethical problems related with the toxicity of the mycotoxins.

In vitro digestion models simulate in a simplified way the digestion processes in mouth, stomach, small and large intestine, in order to asses investigation the bioaccessibility of bioactive com- pounds presents in food matrices during the transit in the gastro- intestinal tract (GI). The models have to be based considering the human physiology (chemical composition of digestive fluids, pH and residence time periods typical for each compartment) and the compartments used to simulated the different organs of the GI have to simulate the digestion and the absorption of the components present in food (Versantvoort, Oomen, van de Kamp, Rompelberg, & Sips, 2005).

The study of the bioaccessibility and bioavailability of different mycotoxins was evaluated by many authors, whereas no data are available on the bioaccessibility of the minor *Fusarium* mycotoxins present in follow-up infant formula.

In particular, Avantaggiato, Havenaar, and Visconti (2003, 2004) studied the intestinal absorption of zearalenone (ZEA), fumonisin B1 (FB1), fumonisin B2 (FB2), ochratoxin A (OTA), deoxynivalenol (DON) and aflatoxin B1 (AFB1) by using a laboratory system that simulate the metabolic processes of the gastrointestinal tract of healthy pigs. Kabak, Brandon, Var, Blokland, and Sips (2009) and Versantvoort et al. (2005) describe the applicability of an in vitro digestion model to measure of the bioaccessibility of the AFB1 and of the OTA ingested from peanut slurry, buckwheat and infant formulas as an indicator of oral bioavailability.

Burkhardt, Pfeiffer, and Metzler (2009) and Videmann, Mazallon, Tep, and Lecoeur (2008), studied the bioavailability and the transepithelial transport of ZEA, alternariol (AOH) and alter- nariol-9-methyl ether (AME), on the Caco-2 cell system.

The aims of this study were: a) to study the presence of the minor *Fusarium* mycotoxins in 72 samples of follow-up infant formula and b) to evaluate in the positive samples the bioaccessibility of the minor Fusarium mycotoxins using an *in vitro* simulated gastrointestinal digestion. This article can be considered the first where the risk assessment of mycotoxins present in follow-up infant formula was carried out associating two different techniques as the analytical contamination data and the bioaccessibility data of the mycotoxins present in the food products analyzed as complementary strategies to the risk evaluation.

2. MATERIALS AND METHODS

2.1. Materials

Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, a-amylase, hydrochloric acid (HCl), pepsin, pancreatin, bile salts, ENs A, A_1, B, B_1, and BEA were obtained from Sigma Aldrich (Madrid, Spain). Acetonitrile, methanol and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultra- sonic Corp., CT, USA) ultrasonic bath.

The mycotoxin FUS used in this study was gently provided by the Prof. Alberto Ritieni of the University of Naples "Federico II".

2.3. Samples description

Totally 72 samples of infant formula were studied and divided in:

- 10 follow-up infant formulas composed with eight different flours (Wheat, corn, rice, oat, barley, rye, sorghum, and millet).
- 15 follow-up infant formulas composed with eight different cereal flours (Wheat, corn, rice, oat, barley, rye, sorghum, and millet) and enriched with fruit juice (8-25%).
- 15 follow-up infant formulas composed with eight different flours (Wheat, corn, rice, oat, barley, rye, sorghum, and millet) and enriched with honey (5%).
- 15 follow-up infant formulas composed with six different flours (wheat, corn, rice, oat, barley, rye).

- 10 follow-up infant formulas composed with whole cereals (corn, wheat and rice).
- Seven follow-up infant formulas composed with two different flours (rice and corn).

A 20-40 g of sample (according to the indication of the package) was diluted in 200 mL of milk infant formulas free by the contamination of mycotoxins. A 100 mL subsample was collected in a plastic bag and kept at -20 ºC until analysis.

2.3. Mycotoxin extraction procedure

The follow-up infant formulas were suspended in distillated water to obtain a suspension at 12% (w/v). 20 mL of each mixture were introduced in a separatory funnel and extracted three times with 40 mL of ethyl acetate during 3 min. The organic phases were completely evaporated by a rotary evaporator (Buchi, Switzerland)

operating at 30 ºC and 30 mbar pressure, resuspended in 1 mL of methanol filtered with a 0.22 mM filter (Phenomenex, Madrid, Spain) and injected in the LC-DAD apparatus.

2.4. LC-DAD analysis

LC analyses of BEA, ENs and FUS were performed using a Shimadzu LC system equipped with LC-10AD pumps and a diode array detector (DAD) from Shimadzu (Japan). A Gemini (150 x 4.6 mm, 5 μm) Phenomenex column was used. LC conditions were set up using a constant flow at 1.0 mL/min and acetonitrile-water (70:30 v/v) as starting eluent system. The starting ratio was kept constant for 5 min and then linearly modified to 90% acetonitrile in 10 min. After 1 min at 90% acetonitrile, the mobile phase was taken back to the starting conditions in 4 min. BEA

and ENs were detected at 205 nm, while FUS was detected at 261 nm. All samples were filtered through a 0.22 μm syringe filter Phenomenex prior to injection (20 μl) onto the column. Mycotoxin identification was performed by comparing retention times and UV spectra of purified extracted samples to pure standards. Quantification of mycotoxins was carried out by comparing peak areas of investigated samples to the calibration curve of authentic standards.

2.5. LC-MS/MS confirmation

The presence of ENs, FUS, and BEA in positive samples of cereals was confirmed by LC-MS/MS according to the method described by Sørensen, Nielsen, Rasmussen, and Thrane (2008). Briefly, analysis of BEA, FUS and ENs was carried out with a TQ mass spectrometer Quattro LC from Micromass (Manchester, UK), equipped with an LC Alliance 2690 system (Waters, Milford, MA) consisted of an autosampler and a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface, and a Mass Lynx NT software 4.1 were used for data acquisition and processing. The auto injector was programmed to inject 20 μl into the Luna C18 column (150x4.6 mm, 5 mm) Phenomenex maintained at 30 ºC.

The analytical separation for LC-MS/MS was performed using gradient elution with water as mobile phase A, and acetonitrile as mobile phase B, both containing 0.5% formic acid. After an isocratic step of 65% B for 3 min, it was linearly increased to 75% B in 4 min and held constantly for 3 min. Flow rate was maintained at 0.3 ml/min.

Analysis was performed in positive ion modes. The ESI source values were as follows: capillary voltage, 3.20 kV; source temperature, 125ºC; desolvation temperature, 350ºC; desolvation gas (nitrogen, 99.95% purity) flow, 700 l/h. Ideal fragmentation conditions were accomplished varying the cone voltage and collision energies for each compound. For the detection of FUS and BEA the precursor ion were m/z 445 and 801 being the product ions selected were m/z 427-409 and 784-756 specifically. For ENB the precursor ion was m/z 640, and the product ions were

m/z 196 and 527. For ENB_1 the precursor ion was m/z 654 and the product ions m/z were 196 and 228, for ENA the precursor ions was m/z 682, and the product ions were m/z 210 and 555, for ENA_1 the precursor ion was m/z 668, and the product ions were m/z 210 and 541.

2.6. Bacterial strains and growth condition

13 commercial probiotic strains were used for the in vitro system that simulates the physiological condition of the colonic intestinal compartment. In particular *Lactobacillus animalis* CECT 4060T, *Lactobacillus casei* CECT 4180, *L. casei rhamnosus* CECT 278T, *Lb. plantarum* CECT 220, *Lactobacillus. rhuminis* CECT 4061T, *L. casei casei* CECT 277, *Bifidobacterium breve* CECT 4839T, *Bifidobacterium adolescentes* CECT 5781T and *Bifidobacterium bifidum* CECT 870T, *Corynebacterium vitaeruminis* CECT 537, *Streptococcus faecalis* CECT 407, *Eubacterium crispatus* CECT 4840, *Saccharomyces cerevisiae* CECT 1324 were obtained at the Spanish Type Culture Collection (CECT Valencia, Spain), in sterile 18% glycerol.

For longer survival and higher quantitative retrieval of the cul- tures, they were stored at -80 ^{O}C. When needed, recovery of strains was undertaken by two consecutive subcultures in appropriate media prior to use.

2.7. In vitro digestion model

The procedure was adapted from the method outlined by Gil-Izquierdo, Zafrilla, and Tomás-Barberá (2002), with slightly mod- ifications. The method consists of three sequential steps; an initial saliva/pepsin/HCl digestion for 2 h at 37 ^{O}C, to simulate the mouth and the gastric conditions, followed by a digestion with bile salts/ pancreatin for 2 h at 37 ^{O}C to simulate duodenal digestion (Fig. 1). The colonic

conditions were simulated adding to the duodenal simulated fluid some bacteria representative of the gastrointestinal tract.

For the saliva/pepsin/HCl digestion, 10 mL of follow-up infant formula suspension, were mixed with 6 mL of artificial saliva composed by: KCl 89.6 g/L, KSCN 20 g/L, NaH_2PO_4 88.8 g/L, $NaSO_4$ 57 g/L, NaCl 175.3 g/L, $NaHCO_3$ 84.7 g/L, urea 25 g/L, 290 mg of a-amylase. The pH of this solution was corrected at 6.8 with NaOH 0.1N.

The mixture containing the artificial saliva was putted in plastic bags, containing 40 mL of water and homogenized by a Stomacher IUL Instruments (Barcelona, Spain) during 30 s.

At this mixture, 0.5 g of pepsin (14,800 U) dissolved in 25 mL of HCl 0.1 N was added. The pH of the mixture was corrected at a value of 2 with HCl 6 N, and then incubated in a 37 ºC orbital shaker (250 rpm) (Infors AG CH-4103, Bottmingen, Switzerland) for 2 h.

After the gastric digestion, the pancreatic digestion was simulated. The pH was increased to 6.5 with $NaHCO_3$ (0.5 N) and then 5 mL (1:1; v/v) of pancreatin (8 mg/mL)-bile salts (50 mg/mL), dissolved in 20 mL of water were added and incubated in a 37 ºC shaking orbital (250 rpm) for 2 h. A 5 mL of the duodenal simulated fluid was aliquoted for the extraction of the BEA and the determi- nation of the duodenal bioaccessibility.

To simulate the colonic compartment *Bacterial* strains (described above) were grown in a sterile plastic centrifuge tube overnight at 37ºC in MRS broth (Oxoid, Madrid, Spain) under anaerobic conditions (5% CO_2/95% air). After that the tubes were centrifuged at 4000 rpm during 5 min at 23 ºC and the bacteria were resuspended in sterile PBS. A 500 µL of a mixture of the bacterial suspensions at

concentrations of 10^{14} CFU/mL were added to duodenal simulate intestinal fluid and incubated at 37 ºC in 5% CO_2/95% air during 48 h (Laparra & Sanz, 2009).

After this last digestion, 5 mL of the mixture were centrifuged at 4000 rpm during 10 min at 4ºC, and extracted for the determination of the BEA and the estimation of the duodenal + colonic bioavailability.

2.8. Mycotoxins extraction from the simulated intestinal fluids

BEA, ENs and FUS contained in the duodenal and duodenal + colonic simulated fluids were extracted as follows (Jestoi, 2008). 5 mL of each mixture previously described were putted in a 20 mL test tube, and extracted three times with 5 mL of ethyl acetate utilizing a vortex VWR international (Barcelona, Spain) for 1 min. After that the mixtures were centrifuged (Centrifuge 5810R, Eppendorf, Germany) at 4000 rpm and 4ºC for 10 min. The organic phases were completely evaporated by a rotary evaporator (Buchi, Switzerland) operating at 30ºC and 30 mbar pressure, resuspended in 1 mL of methanol filtered with a 0.22 μM filter (Phenomenex, Madrid, Spain) and analyzed by LC-MS/MS.

2.9. Method performance

The method validation was reported previously by Serrano, Font, Mañes, and Ferrer (2013b). The detection limits (LODs) were calculated using a signal-to-noise ratio of 3. The limits of quantifi- cation (LOQs) were calculated using a signal-to-noise ratio of 10. The LOQ and LOD for ENA1 were 0.25 μg/kg and 0.08 μg/kg, respectively. LOQ was 0.80 μg/kg for ENA, ENB and ENB_1. LOD was 0.15 μg/kg for ENA, ENB and

ENB_1. ENs exhibited good linearity over the working range (from 0.05 μg/kg to 20 mg/kg), and the regression coefficient of calibration curves was higher than 0.993. The accuracy was evaluated through recovery studies at two concentration levels (LOQ and 100 x LOQ). Intra-day precision was assessed by five determinations at each addition level in the same day. Inter-day precision was assessed by one determination at each addition level during five days.

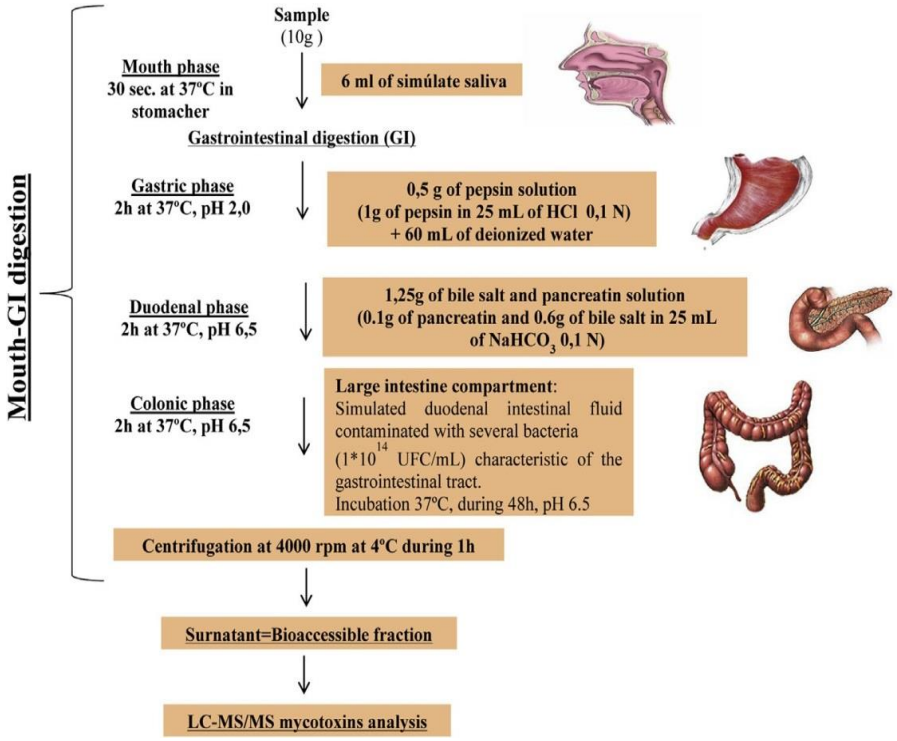

Fig. 1. Schematic representation of the *in vitro* digestion model. The in vitro digestion model describes a four-step procedure simulating the digestive processes, considering the mouth, stomach, small and large intestine. In each compartment, the matrix was incubated at 37 ºC and the pH was modified according to the physiologic conditions. This procedure permit to simulate in vitro the physiological condition of the human gastrointestinal tract and to study how some components present in the diet can influence the bioaccessibility of the bioactive compounds.

3. RESULTS AND DISCUSSION

3.1. Occurrence of BEA, ENs and FUS in follow-up infant formula

The minor *Fusarium* mycotoxins BEA, ENs and FUS were determined in 72 samples of follow-up infant formula of Spanish origin employing the technique of the LC-DAD (Fig. 2). As evidenced in Table 1 only the ENs B_1, A and A_1 were detected in the samples analyzed. The samples positive to the presence of the bioactive compounds detected were 14 that represent the 19.4% of the total samples analyzed. As is shown in Table 1, the most detected mycotoxin was the EN B_1, present in 12 of the totally positive samples with a mean value of 32.3 ± 1.2 mg/kg. The highest data were evidenced in the samples 71 (follow-up infant formula whole cereal) with 41.94 ± 2.21 mg/kg whereas the lowest was detected in the sample 72 (follow-up infant formula whole cereal) with 20.67 ± 1.22 mg/kg. The ENA was detected only in one sample with a value of 149.62 ± 2.36 mg/kg that represents the highest contamination value evidenced in all the samples analyzed. The ENA_1 was detected in 5 samples with a mean contamination of 55.36 ± 2.1 mg/kg. The highest and the lowest contamination values were of 14.99 ± 0.69 and 101.73 ± 2.36 mg/kg respectively. All the samples analyzed were free by the contamination of ENB, BEA and FUS. This study can be considered the first where the presence of the minor *Fusarium* mycotoxins ENs BEA and FUS has been evaluated in follow-up infant formula of Spanish origin but the presence of these classes of contaminants in other kind of samples has been evaluated by other authors. In particular Meca, Zinedine, Blesa, Font, and Mañes (2010) evaluated the contamination level by minor Fusarium mycotoxins BEA, ENs and FUS of cereals samples of Spanish origin, evidencing that the ENA1 was the most mycotoxin found with levels ranged from 33.38 to 814.42

mg/kg. The contamination level by ENB ranged from 2.23 to 21.37 mg/kg, whereas for the EN B1 the quantities evidenced in the samples analyzed varied from 4.34 to 45.94 mg/kg. All samples analyzed by the authors were free of ENA. BEA levels ranged from 0.51 to 11.78 mg/kg and FUS levels varied between 1.01 and 6.63 mg/kg.

Zinedine, Meca, Mañes, and Font (2011) evidenced the presence of the mycotoxins BEA, FUS and ENs in rice of Morocco origin (Rabat, Casablanca, Kénitra, Mohammadia, Tanger and Errachidia). ENA_1 was the most common EN detected with a percentage of contamination of 39%, and with contamination levels ranged between 14 and 445 mg/kg. ENB was detected in 14 samples (21.8%) with levels ranged from 5 to 100 mg/kg. The ENB_1 was present in four samples (6.2%) with a contamination level ranged from 8 to 32 mg/kg whereas ENA was detected in only one sample with 34 mg/kg. Serrano et al. (2013b) determined the ENs A, A_1, B and B_1, BEA and FUS in pasta samples. For this purpose, 114 commercial samples of pasta were acquired from supermarkets located in Valencia city. The results showed higher frequencies of contamination in organic pasta than in conventional pasta, while the concentration levels were variable for both types of pasta. In positive samples, BEA levels varied from 0.10 to 20.96 mg/kg and FUS levels varied from 0.05 to 8.02 mg/kg. ENs levels ranged from 0.25 to 979.56 mg/kg, though the majority of the values was below 25 mg/kg. Besides, it was observed the simultaneous presence of two or more mycotoxins in a high percentage of the samples.

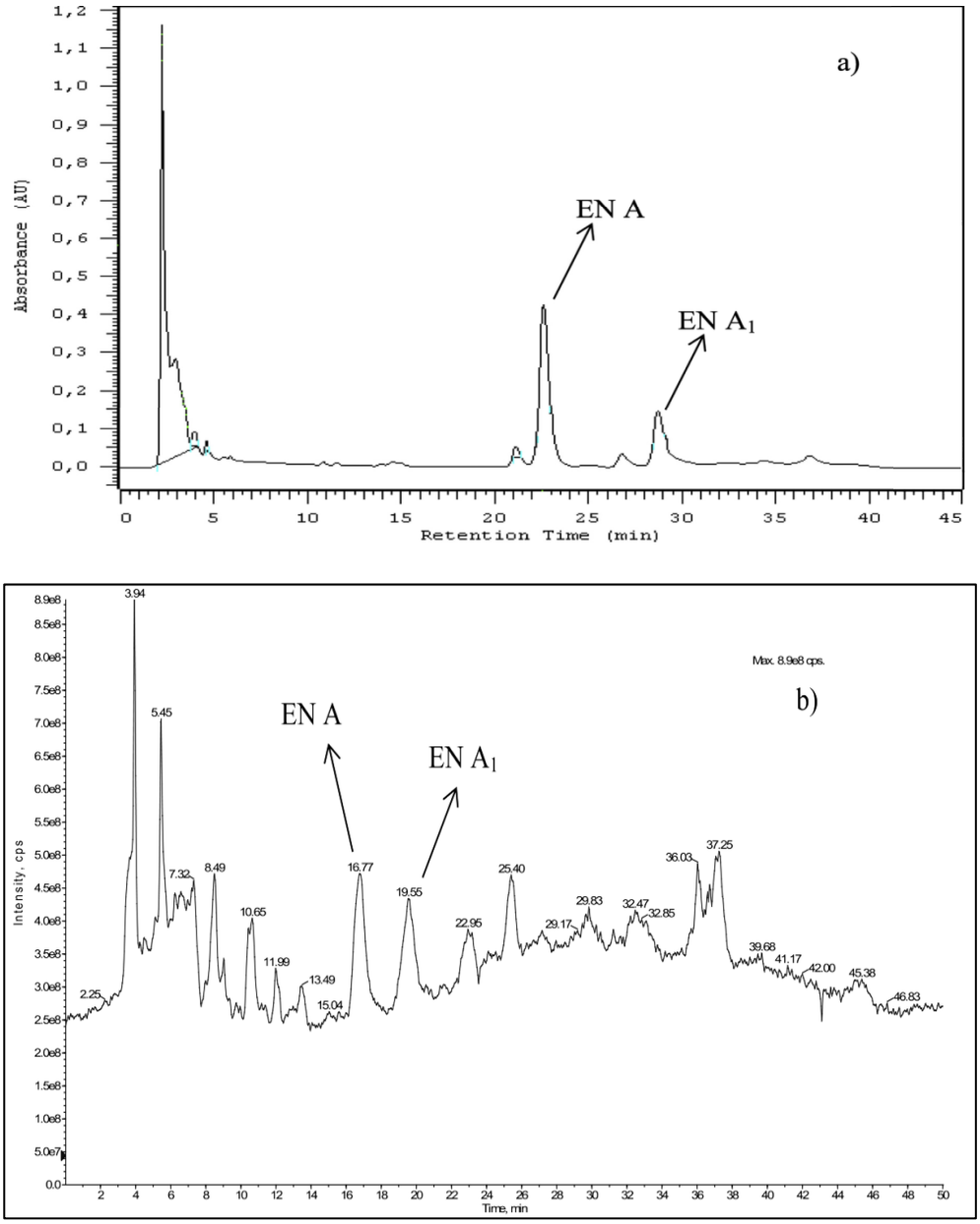

Fig. 2. a) LC-DAD chromatogram of the ENs A and A_1 present in the follow up infant formula 48 and b) confirmation LC-MS/MS chromatogram.

Table 1. Positive samples to the presence of the enniatins B_1, A and A_1 in the analyzed follow up infant formula.

n° samples	Enniatins (mg/kg)		
	EN B_1	EN A	EN A_1
31	nd	nd	62.62 ± 2.01
35	27.40 ± 1.21	nd	nd
48	nd	149.62 ± 2.36	101.73 ± 2.36
57	34.54 ± 1.31	nd	nd
60	37.82 ± 2.52	nd	14.99 ± 0.69
61	36.51 ± 2.34	nd	73.82 ± 3.21
62	32.19 ± 1.55	nd	nd
63	38.63 ± 2.36	nd	23.84 ± 1.33
64	26.12 ± 1.63	nd	nd
66	32.12 ± 1.27	nd	nd
67	28.45 ± 1.56	nd	nd
69	31.58 ± 1.36	nd	nd
71	41.94 ± 2.42	nd	nd
72	20.67 ± 1.22	nd	nd

3.2. Duodenal and colonic ENs bioaccessibility

The term bioaccessibility has been defined as the fraction of a bioactive compound present in a food matrix that passes unmodified the complex of the biochemical reactions related to the gastrointestinal digestion and thus become available for intestinal absorption (Fernández-García, Carvajal-Lérida, & Pérez-Gálvez, 2009).

The positive samples analyzed in this study were digested through simulated gastrointestinal digestion model, which permit to simulate the physiological condition of the human gastrointestinal tract (duodenal and colonic compartments) in order to assess the bioaccessibility of the bioactive compounds studied and con- tained in the follow-up infant formula. The determination of the bioaccessibility permits to understand as the food composition can influence the

presence of a bioactive compound in the intestinal fluid and also to have an exactly idea of the real concentration of a bioactive compound that can be absorbed by the intestinal epithelium (Fig. 3).

In Tables 2 and 3, are evidenced the data related to the duodenal bioaccessibility of the mycotoxins ENs present in the samples studied. In particular the mean bioaccessibility data for the three compounds detected were of 1.43±0.1, 0.37±0.02 and 22.41±1.10% respectively, whereas employing the combined process of the duodenal + colonic digestion the mean data evidenced were of 3.35±0.25, 1.63±0.01 and 29.41±1.22% respectively. Considering the duodenal bioaccessibility the highest bio- accessibility data were evidenced by the ENA1 present in the sample 63 with 54.54 ± 2.15%, whereas the lowest was detected by the ENB1, present in the sample 69 with 0.09±0.01%. During the combined process of the simulated digestion employing the duodenal þ colonic condition the highest bioaccessibility data were evidenced by the ENA1 present in the samples 60 and 63 with 45.17±2.48 and 60.53±2.64% respectively, whereas the lowest bioaccessibility data were observed by the ENB_1 with 1.49±0.04 and 1.56±0.05% respectively. Comparing the data obtained in the two different process it's possible to observe that the bioactive compound detected in the samples analyzed are more bioaccessible during the simulated digestion process where we applied the combined condition of duodenum þ colon than the bioaccessibility evidenced only using the simulated digestion process until the duodenal compartment. In particular the ENs B1 and A presented meanly the 53.32±1.21 and 77.34±2.12% (Table 3) of bio- accessibility treating the samples with the combined duodenum þ colon process respect to the samples treated only with the duodenal digestion, whereas the bioaccessibility data evidenced by the ENA_1, was 22.73±0.25% higher

than the duodenal digestion. This phenomenon can be related to different factors:

(a) *Food composition:* In particular, the mycotoxins that are spiked in a food or that naturally contaminate the food are complexes to the food matrix. The formation of this complex is dependent of the amount of the micro and macronutrients contained in the food, such as fibers, sugars, proteins, etc (Kabak et al., 2009). In particular the alimentary fiber is one of the com- pounds that more affinity has with the bioactive compounds presents in food. Different bioavailability values were also re- ported by several authors (Versantvoort et al., 2005), comparing the bioavailability of the same compounds contained in a liquid or solid food (Meca, Mañes, Font, & Ruiz, 2012b).

(b) *Fermentation of macronutrients by colon microflora:* the macronutrients present in food like the proteins or the alimentary fibers have the capacity to bind the bioactive compounds present in food like the polyphenols or the mycotoxins until the treatment with the colonic bacteria that hydrolyze the prebiotic compounds increasing partially the bioaccessibility of the mycotoxins. Considering that the products analyzed contained in the majority several different cereals with a percentage of fibers ranged from 2% and 5% this factor could influence the increase of the bioaccessibility using the combined simulated gastrointestinal digestion (Meca et al., 2012a; Meca et al., 2012c).

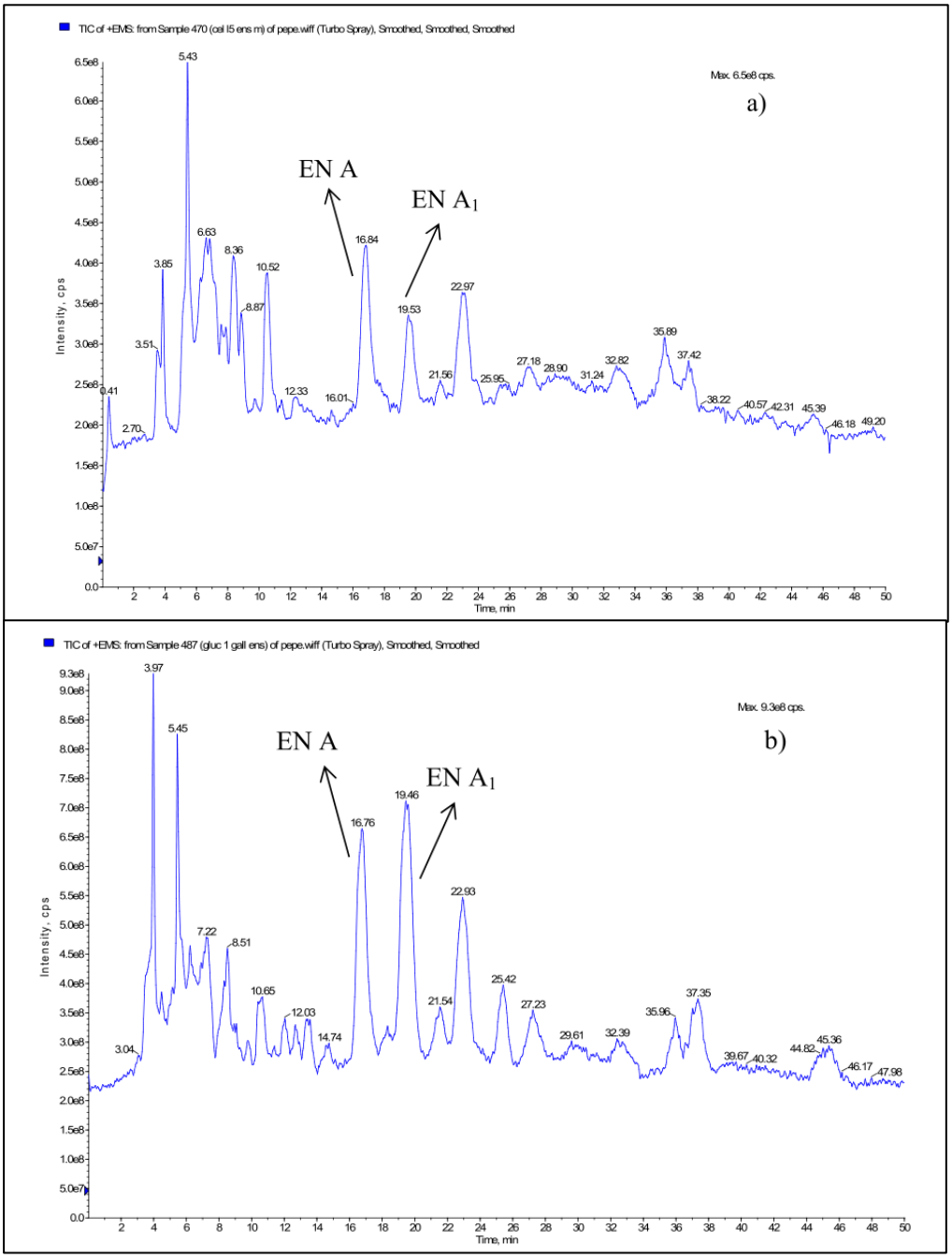

Fig. 3. LC-MS chromatograms of the ENs A and A_1 present in the a) duodenal and b) duodenal + colonic simulated fluids.

Table 2. Duodenal bioaccessibility of the minor *Fusarium* mycotoxins ENs presents in the follow up infant formula studied.

n° samples	% Duodenal bioaccessibility		
	EN B_1	EN A	EN A_1
31	nd	nd	23.84 ± 1.11
35	1.07 ± 0.08	nd	nd
48	nd	0.37 ± 0.02	0.56 ± 0.24
57	0.62 ± 0.12	nd	nd
60	0.72 ± 0.14	nd	32.44 ± 1.62
61	0.80 ± 0.08	nd	0.68 ± 0.04
62	1.65 ± 0.17	nd	nd
63	1.80 ± 0.23	nd	54.54 ± 2.15
64	3.47 ± 0.52	nd	nd
66	5.51 ± 0.46	nd	nd
67	0.10 ± 0.02	nd	nd
69	0.09 ± 0.01	nd	nd
71	0.75 ± 0.10	nd	nd
72	0.63 ± 0.04	nd	nd

Table 3. Duodenal + colonic bioaccessibility of the minor *Fusarium* mycotoxins ENs presents in the follow up infant formula samples.

n° samples	% Colonic + Duodenal bioaccessibility		
	EN B_1	EN A	EN A_1
31	nd	nd	32.47 ± 1.24
35	3.45 ± 0.31	nd	nd
48	nd	1.63 ± 0.01	4.36 ± 0.61
57	2.36 ± 0.22	nd	nd
60	1.56 ± 0.54	nd	45.17 ± 2.48
61	5.36 ± 0.38	nd	2.45
62	2.47 ± 0.37	nd	nd
63	3.21 ± 0.41	nd	60.53 ± 2.64
64	5.47 ± 0.55	nd	nd
66	8.43 ± 0.64	nd	nd
67	1.49 ± 0.04	nd	nd
69	1.56 ± 0.05	nd	nd
71	2.36 ± 0.03	nd	nd
72	2.47 ± 0.04	nd	nd

In the scientific literature only few studies are available on the influence of the food composition on the bioaccessibility and bioavailability of the minor Fusarium mycotoxins BEA ENs and FUS. In particular Meca et al. (2012b) studied the ENs bioaccessibility, spiked in commercial wheat crispy bread at 1.5 and 3.0 mmol/g concentrations, their transepithelial transport and bioavailability using Caco-2 cells as a model of the human intestinal epithelium. The content (%) of the four ENs contained in the gastric fluid was variable from 69 to 91%, considering the two concentrations assayed. The mean bioaccessibility data for the compounds studied, resulted of 80%. The compounds that evidenced the highest ab- sorption, using the in vitro model which simulated the trans- epithelial transport, were the EN A (70.8±1.3% of absorption) and A1 (73.8±0.9%) at 1.5 and 3.0 mmol/g concentrations, respectively. The compound with the lowest transport value (50.7±1.3%) was the EN A at 3.0 mmol/g concentration. The bioavailability data evidenced by the other ENs employed ranged from 55.2 ±1.1 to 66.1 ± 1.0%.

4. CONCLUSIONS

The minor *Fusarium* mycotoxins ENs B_1, A and A_1 were detected in several samples of the Spanish follow-up infant formula analyzed. The result of the duodenal bioaccessibility of the positive samples demonstrated that only a part of the bioactive compounds present in the sample was able to the absorption by the cells of the intestinal epithelium, and that the bioaccessibility increase adding to the duodenal digestion also the colonic digestion. The complexes that the bioactive compounds present in food products can form with their structural macronutrients can reduce the risk associated to the intake of these compounds.

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3.8. Degradation study of enniatins by liquid chromatography-triple quadrupole linear ion trap mass spectrometry

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Degradation study of enniatins by liquid chromatography-triple quadrupole linear ion trap mass spectrometry

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ABSTRACT

Enniatins A, A_1, B and B_1 (ENs) are mycotoxins produced by *Fusarium* spp. and are normal contaminants of cereals and derivate products. In this study, the stability of ENs was evaluated during food processing by simulation of pasta cooking. Thermal treatments at different incubation times (5, 10 and 15 min) and different pH (4, 7 and 10) were applied in an aqueous system and pasta resembling system (PRS). The concentrations of the targeted mycotoxins were determined using liquid chromatography coupled to tandem mass spectrometry. High percentages of ENs reduction (81-100%) were evidenced in the PRS after the treatments at 5, 10 and 15 min of incubation. In contrast to the PRS, an important reduction of the ENs was obtained in the aqueous system after 15 min of incubation (82-100%). In general, no significantly differences were observed between acid, neutral and basic solutions. Finally, several ENs degradation products were identified using the technique of liquid chromatography-triple quadrupole linear ion trap mass spectrometry.

Keywords: Enniatins, reduction, thermal treatments, degradation products, LC-MS/MS, LC-MS-LIT.

1. INTRODUCTION

The *Fusarium* genus is the most prevalent toxin-producing fungi of the Northem temperate region (SCF, 2002). Several *Fusarium species as avenacum, moniliforme, proliferatum* and *subglutinans* are producers of some minor *Fusarium* mycotoxins called enniatins (ENs). These bioactive compounds are cyclic hexadepsipeptides formed by the alternating of the D-α-hydroxy-isovaleric acid (HyLv) and different N-methylamino acid residues as valine (Val) and isoleucine (Ile). The ENs are classified as ionophoric compounds, forming stable molecules with a "sandwich" structure with alkali metals or alkaline earth metals, across human cell membranes (Jestoi, 2008).

In vitro studies have demonstrated that ENs evidenced cytotoxic activity in different cell lines, including rodent (V79), lepidopteran (SF-9), monkey (Vero) and human cells (Caco-2, Hep-G2, HT-29) (Fornelli, Minervini & Logrieco, 2004; Behm, Degen & Föllmann, 2009). Generally, the contamination levels by ENs evidenced in cereals collected in Mediterranean area is higher than the data evidenced in Central and Northern European region, due probably to the different clime condition of these two different parts of the continent (Santini, Meca, Uhlig & Ritieni, 2012). ENs have been detected in processed products containing essential cereals for adult and infant nutrition, such as breakfast cereals, rice, pasta, infant formula, bread mill and other derived products (Jestoi, 2008; Serrano, Font, Mañes & Ferrer, 2013a).

Several studies have been published related to the mitigation strategies of mycotoxins in food, focalized principally on the reduction of the trichothecenes, fumonisins (FBs), aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT) and zearalenone (ZEA) during food processing (Ryu, Hanna, Eskridge & Bullerman, 2003; Park, Scott, Lau & Lewis, 2004; Abramson,

House & Nyachoti, 2005; Bullerman & Bianchini, 2007; Cramer, Königs & Humpf, 2008; Kushiro, 2008). At the moment, only two studies are available in the scientific literature on the thermal degradation of the minor *Fusarium* mycotoxins. In particular, Meca, Ritieni and Mañes (2012b) studied beauvericin (BEA) stability during several heat treatments, in a model system and also in homemade crispy bread, obtaining percentages of degradation variables from 20 to 90%. Vaclavikova, Malachova, Veprikova, Dzuman, Zachariasova and Hajslova (2013) determined ENs levels during beer and bread production, concluding that ENs concentrations were reduced during breadmaking (from 71% to 79% in milling and from 50% to 60% in baking), whereas these mycotoxins were not detected in the final beer.

Nevertheless, other studies have been focused in the identification of the mycotoxins degradation products formed after the treatments, as well as in the evaluation of the toxicity of these new identified compounds. Meca et al. (2012a) studied the stability of BEA in a solution model and in wheat flour using allyl isothiocyanate (AITC) as a reactant. Two reaction products between the bioactive compounds employed in this study were identified by LC-MS-LIT, corresponding to BEA conjugates containing one or two AITC molecules. Bretz et al. (2006) studied the DON stability in a food system elaborated with different macronutrients as sugar, starch and proteins, at temperatures ranging from 150 to 200 ºC using incubation times variables from 5 to 20 min. The DON reduction was temperature and time dependent. Also three DON degradation products were identified in commercial samples. The cytotoxicity of the degradation products was compared to DON by cell culture experiments. The results evidenced that DON degradation products were less cytotoxic than DON, and the heat treatments employed reduced the

risk associated to DON intake. The reduction of the fumonisins has been evidenced by many studies during food processing and cooking (baking, frying, roasting, extrusion and heating). The studies demonstrated that FBs stability depends of several factors, such as temperature, time or sugar and water content. Moreover, different FBs degradation products have been identified during food treatments (Humpf et al., 2004).

Considering the lack of data related to the degradation of ENs during food processing, the aims of the study were: a) to study the thermal stability of EN A, EN A_1, EN B and EN B_1 at different pH, in an aqueous system and in a food model simulating pasta composition (pasta resembling system) by liquid chromatography coupled to a triple quadrupole mass spectrometer detector (LC-MS/MS QqQ), b) to identify and characterize ENs degradation products produced during heat treatments by liquid chromatography coupled to the mass spectrometry-linear ion trap (LC-MS-LIT).

2. MATERIALS AND METHODS

2.1. Materials

Acetonitrile (AcN) and methanol (MeOH) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) and formic acid (> 98%) were supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain) before use.

Gluten from wheat (≥ 80% of protein), starch from potato (PhEur) and albumin from bovine serum (≥ 98%, lyophilized powder) used for the preparation of the pasta resembling system were purchased from Sigma-Aldrich (Oakville, ON, Canada). The standards of ENA, ENA_1, ENB and ENB_1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Standard solutions of ENs A, A_1, B and B_1 were prepared dissolving 1 mg of each compound in 1mL of MeOH, obtaining stock solutions of the minor *Fusarium* mycotoxins of 1000 mg L^{-1}. The stock solutions were then diluted with pure MeOH in order to obtain the appropriate working solutions. All the solutions were stored in glass-stoppered bottles in darkness at -20ºC.

2.2. Sample treatment

2.2.1. Aqueous system

The model solutions were prepared in 100 mL Erlenmeyers at three different pH values (pH 4, 7 and 9). In order to adapt the experiments to the real cooking processes conditions, lemon juice and sodium bicarbonate marketed in Valencia were employed to reach the required pH. The acid (pH 4) and basic (pH 9) solutions were prepared adding 1.5 mL of lemon juice and 2 g of sodium bicarbonate to 50 mL of deionized water under continuous stirring. The experiments in the neutral solution (pH 7) were carried out using deionized water. The pH measurements were performed employing a GLP21 Crison pH-meter (Crison Instruments, S.A., Barcelona, Spain) with a Hamilton pH electrode (Fisher Scientific, Madrid, Spain). The model solutions were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain), and then 980 μL of each solution was contaminated with 20 μL of each EN (1000 mg L^{-1}) to obtain a final concentration of 20 mg L^{-1}. ENs reduction

experiments were performed at 100ºC in a water bath SS40-2 (Gran Instruments, Cambridge, United Kingdom) at different boiling times (5, 10 and 15 min). Aliquots of each treatment were filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) and injected into the LC-MS/MS and LC-MS-LIT systems.

2.2.2. Pasta resembling system (PRS)

The PRS was formulated by mixing 65 g of starch, 8 g of gluten and 2 g of albumin to obtain homogenous flour. The experiments were performed simulating the boiling process of pasta (100 g of pasta and 1L of water) employing three aqueous solutions: acid, basic and neutral (see preparation in sub-section 2.2.1.). For this purpose, 0.075 g of PRS was contaminated with 20 μL of each EN (1000 mg L^{-1}) individually, and 1000 μL of aqueous solution was added to the vial. The final concentration of each EN in the vials was of 20 mg L^{-1}. Thermal experiments were performed at 100ºC in a water bath at different times (5, 10 and 15 min). Then, mycotoxin extraction was carried out as pointed out in next paragraph.

2.3. Mycotoxin extraction

Treated samples from PRS, were extracted with 10 mL of AcN using a Ika T10 basic Ultra-Turrax (Staufen, Germany) for 3 min. The supernatant was evaporated to dryness by nitrogen at 35ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH 50/50 v/v, and filtered through 13 mm/0.20 μm nylon filter until the analysis in the LC-MS/MS and LC-MS-LIT systems.

2.4. Analysis

2.4.1. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 2 mm I.D., 3 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using gradient elution that started at 90% of A (AcN) and 10% of B (20 mM ammonium formate in MeOH), increased linearly to 50% B in 10 min. After, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 mL min^{-1}. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0,5 V; source temperature, 100ºC; desolvation temperature, 300ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L h^{-1}; cone gas 50 L h^{-1} (nitrogen 99.99% purity).

The cone voltage selected was 40 V and the collision energy selected was 35 Ev for all ENs. 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 99.995% purity) pressure, 3.83 x 10^{-3} mbar; interchanel 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. According with the 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 follow precursor ion and product ions were selected for each mycotoxin: the precursor ion m/z 681.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA, the precursor ion m/z 667.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA_1, the precursor ion m/z 639.8 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB, the precursor ion m/z 654.9 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB_1 (Serrano, Font, Mañes, Ferrer, 2013b).

2.4.2 LC-MS-LIT identification of the ENs degradation products

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. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole can also be operated as a linear ion trap (QqLIT) with improved performance. In the QqLIT configuration the Q TRAPTM can also operate in Enhanced Resolution (ER) scan and in enhanced product ion scan (EPI) modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing. A Gemini (150 x 2.0 mm, 5 μm) Phenomenex column was used for separation. The mobile phase (AcN/water 70/30 v/v with 0.1 % of HCOOH) was delivered in an isocratic manner at a constant flow rate of 0.3 mL min^{-1}. The MS was operated in the positive ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350 ºC, desolvation temperature 270 ºC and collision gas energy 5 eV. The analyses were carried out using: 1) the ER mode for newly formed compounds in the

mass range from 200 to 800 Da; 2) the EPI mode to obtain a MS^2 scan of a fragment of the adducts. The mass spectrometry data obtained from these two scan modes has permitted the elucidation of the compounds of interest. Its absolute molecular structure still needs to be confirmed with other analytical methods including NMR and IR (Meca, Luciano, Zhou, Tsao & Mañes, 2012a).

2.5. Statistical Analysis

The experiments were carried out in triplicates. Statistical analysis were carried out using analysis of variance (ANOVA), followed by Dunnet's multiple comparison tests. Differences were considered significant if $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Method performance

The method validation was reported previously by Serrano et al. (2013b). The detection limits (LODs) were calculated using a signal-to-noise ratio of 3. The limits of quantification (LOQs) were calculated using a signal-to-noise ratio of 10. The LOQ and LOD for ENA_1 were 0.25 μg Kg^{-1} and 0.08 μg Kg^{-1}, respec-tively. LOQ was 0.50 μg Kg^{-1} for ENA, ENB and ENB_1. LOD was 0.15 μg Kg^{-1} for ENA, ENB and ENB_1. ENs exhibited good linearity over the working range (from 0.05 μg Kg^{-1} to 20 mg Kg^{-1}), and the regression coefficient of calibration curves was higher than 0.993. The accuracy was evaluated through recovery studies at two concentration levels (LOQ and 100 x LOQ). Intra-day precision was assessed by five determinations at each addition level in the same day. Inter-day precision was assessed by one determination at each addition level during five days. The mean recoveries and

the corresponding relative standard deviations (RSDs) are presented in Table 1 Supplementary data. RSD values ranged between 3% and 9% for intra-day precision, and between 4% and 11% for inter-day precision. Recovery ranges for the low spiked level (LOQ) and the high spiked level (100xLOQ) were 85–95% and 88–97%, respectively. Therefore, the results were in accordance to the limits set in Commission Decision, 2002 /657/EC: a mean recovery (n=5) between 70% and 120%, and a RSD lower than 20%.

3.2. ENs reduction in the model systems

3.2.1. Aqueous system

Figure 1 shows the percentages of ENs reduction in the aqueous solutions (acid, neutral and basic) after heat treatments at 5, 10 and 15 min. The degradation of the tested ENs in this study was time dependent. The degradation trend of ENB was similar in the three tested solutions. The degradation trend of ENA, ENA_1 and ENB_1 using the acid solution (figure 1a) was comparable with the data observed using the neutral solution (figure 1b), whereas the degradation was different in the basic solution (figure 1c).

As is possible to observe in figures 1a and 1b, percentages of degradation of ENB and ENA_1 in the acid and neutral solutions after 5 min of treatment (between 74.1 and 98.4%) were higher than those obtained for ENB_1 and ENA (between 16.8 and 27.7%). Percentages of reduction after 10 min of treatment were similar for all ENs using acid and neutral solutions (76.0-100.0%). After 15 min of treatment, ENs reductions were close to 100%; only the ENA reduction in the acid solution was of 80%.

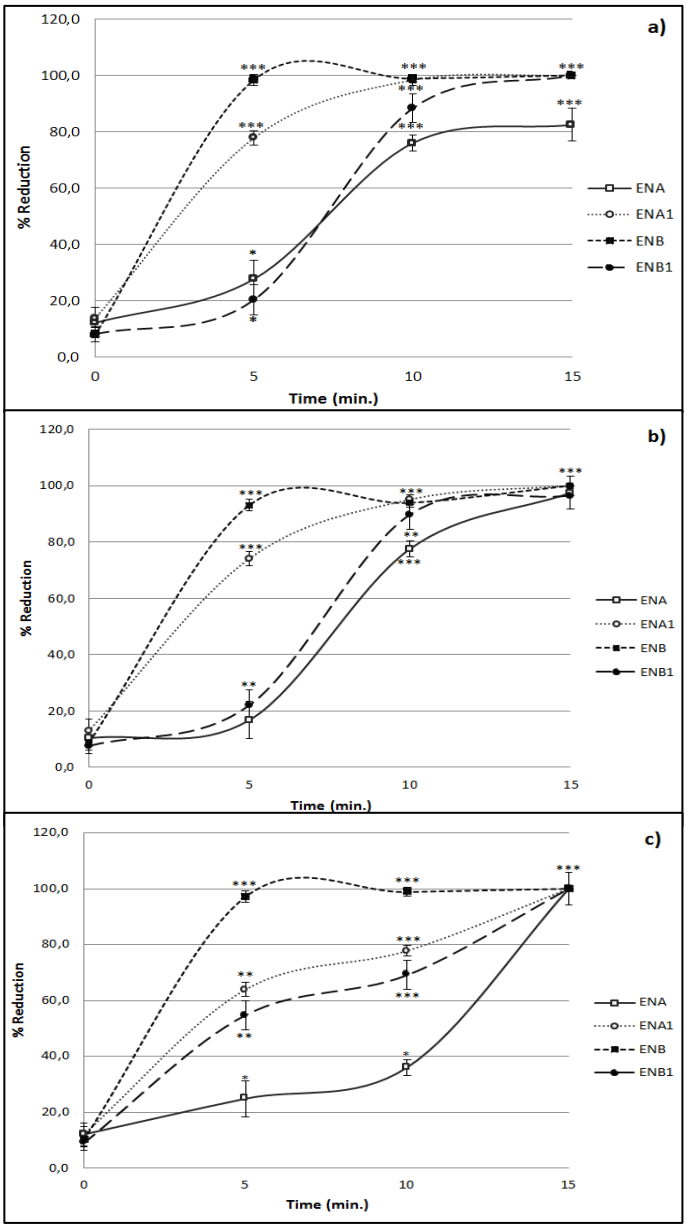


Figure 1. Degradation of the ENs in the aqueous model system during the heat treatment (100 ºC) at different times and pHs: (a) acid solution (pH 4), (b) neutral solution (pH 7) and (c) basic solution (pH 9). $p \leq 0.05$ (*), $p \leq 0.001$ (**) and $p \leq 0.000$ (***) represent significant difference as compared to control values.

Concerning reductions in the basic solution (figure 1c), the highest percentages of reduction after 5 and 10 min of treatment were obtained for ENB (97.1-98.8%). Percentages of reduction of ENA were lower than those obtained for ENA_1 and ENB_1 in the basic solution after 5 and 10 min of thermal treatment. ENs reductions after 15 min of thermal treatment were close to 100% in the basic solution.

In the last years, several studies have been published on the evaluation of the thermal stability of other mycotoxins in aqueous systems, showing different results depending on the studied mycotoxin. Ryu et al. (2003) studied the heat stability of ZEA in aqueous model systems at different temperatures (between 100 and 225ºC) and pHs (4, 7 and 10). Percentages of reduction (23-100%) were positively related to increasing processing temperature. The highest reduction of the ZEA was observed at pH 10 below to 175ºC, whereas above 175ºC, the highest ZEA degradation was observed at pH 7. Pineda-Valdes and Bullerman (2000) evaluated the moniliformin (MON) stability in aqueous buffer solutions at different temperatures (100, 125, 150 and 175ºC), obtaining the highest MON reduction (99%) at pH 10 and 175ºC. In general, the percentages of reduction were dependent with the increasing temperature, time and the pH. On the other hand, it has been demonstrated that FBs stability in aqueous buffered solutions was dependent with decreasing pH and increasing temperature (Humpf & Voss, 2004). Therefore, the results are variable depending on the type of mycotoxin greatly due to the chemical diversity of the mycotoxins.

3.2.2. Pasta resembling system (PRS)

As it is possible to observe in figure 2, the percentages of degradation of ENs A, A_1, B and B_1 in the PRS were similar at the three incubation times and at the tested pHs.

In contrast to the results obtained in the aqueous system, the reduction of all mycotoxins in the PRS system reached percentages higher than 81.0% after 5 and 10 min of treatment. After 15 min of treatment, the percentages of reduction reached 95.2, 98.3, 100.0 and 97.7% for ENA, ENA_1, ENB and ENB_1, respectively. Therefore, similar results were observed between PRS and aqueous system after 15 min of thermal treatment. In general, the values produced in this study evidenced that the pH of the solution is not related with the ENs reduction.

Only few studies on the thermic degradation of the minor *Fusarium* mycotoxins are available in the scientific literature. Meca et al. (2012b) studied BEA reduction in model systems at 160, 180 and 200ºC and different incubation times (from 3 to 20 min). The percentages of reduction in aqueous models (between 58 and 100%) were time and temperature dependent. BEA was totally reduced at 200ºC during 20 min of treatment. In the food system, the percentage of BEA degradation ranged from 20 to 90%. The results obtained by the authors are comparable with the data obtained in the present study considering that the chemical structure of BEA is very similar with the ENs structure. Moreover, Vaclavikova et al. (2013) studied the ENs degradation during beer and bread production, obtaining similar results to those obtained in the present study. During bread making, the impact of the thermal treatments caused the reduction of ENs contamination (reductions of 50-60%). The malting and brewing processes resulted in a reduction of the

mycotoxin concentration due to the high temperatures achieved. ENs levels during the malting step were reduced to 10-30% of their original content in barley, while ENs were not detected in the final beer.

The stability of other *Fusarium* mycotoxins during thermal food processing has been diversely discussed by several researchers. Some studies have supported that the impact of high temperatures is a decisive parameter in mycotoxin reduction. Kottapalli and Wolf-Hall (2008) observed significant DON reductions (79-93%) in malts prepared from barley treated with hot water at 45 and 50ºC during 20 min. Visconti, Haidukourki, Pascale and Silvestri (2004) studied DON degradation during durum wheat processing and spaghetti cooking. DON degradation evidenced after the wheat milling, semolina and spaghetti production was of 23, 63 and 67%, respectively. Cortez-Rocha et al. (2002) investigated the FB_1 levels in corn during alkali-cooking and extrusion processing of alkali-cooked corn, habitually used to produce several products from corn, such as snacks and tortilla products. The levels of FB_1 were reduced to 17% after alkali-cooking (55 min at 95-100ºC), whereas the extrusion processing at 171ºC resulted in a reduction of 99%. Furthermore, other studies have indicated low percentages of mycotoxins reduction after thermal treatments. Scudamore, Hazel, Patel and Scriven (2009) studied the influence of bread, cake and biscuits production on the stability of DON, nivalenol (NIV) and ZEA. The concentrations of NIV and ZEA were constant during food production, whereas the final degradation concentration of DON ranged from 5 to 11%.

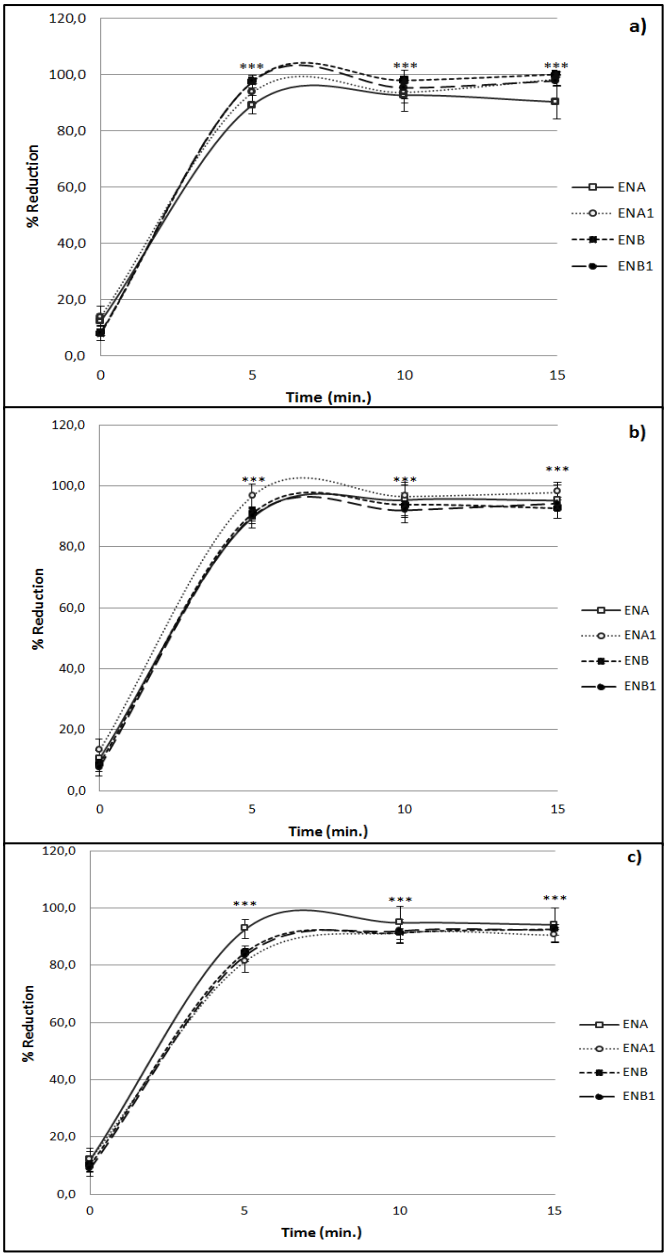


Figure 2. Degradation of the ENs in the PRS during the heat treatment (100 ºC) at different times and pHs: (a) acid solution (pH 4), (b) neutral solution (pH 7) and (c) basic solution (pH 9). $p \leq 0.000$ (***) represent significant difference as compared to control values.

The pH influence on the ENs stability has not been observed in this study, but some authors have reported the importance of the pH on mycotoxin degradation. Abramson et al. (2005) studied the thermal degradation of the mycotoxin DON in feed naturally contaminated with the presence and absence of a water solution of sodium carbonate. Thermal treatments were carried out at 80ºC during 0, 1, 3, 5 and 8 days, obtaining high percentages of DON degradation after 1 day when sodium carbonate solution was added to the feed. However, when samples were heated without sodium carbonate solution, DON reduction was observed after 8 days.

3.3. LC-MS-LIT Characterization of ENs degradation products

The samples positive to ENs degradation, were also injected in the LC-MS-LIT in the ER scan modality (m/z=200-900) to determine ENs degradation products produced through the heat treatments employed. The abundance of ENs degradation products identified in this study increased with time of heating and with decreasing concentrations of ENs.

3.3.1. ENA

In the aqueous system, two degradation products derived from ENA were formed after heat treatments at the three tested pHs (Table 1 and Figure 3a). The abundance of degradation product 1 was positively related to increasing time of thermal treatment and decreasing ENA levels, whereas the abundance of degradation product 2, remained constant after thermal treatments. In figure 3a is evidenced the LC-MS-LIT chromatogram obtained in the modality of ER scan (MS^1) for ENA treated during 10 min at pH 4 in the PRS system. The chromatogram shows the presence of ENA and two degradation products.

Table 1. LC-MS-LIT data (MS^1 and MS^2) of the degradation products of ENs type A obtained in the aqueous system and in the pasta resembling system.

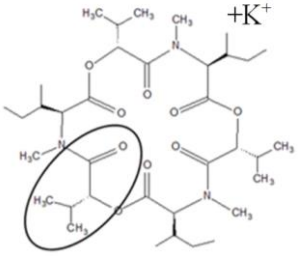
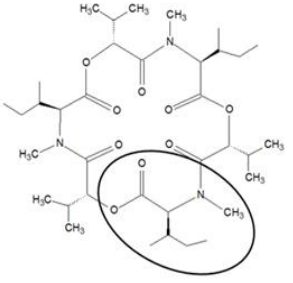
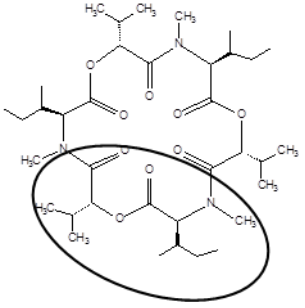
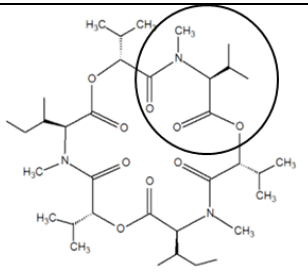
Mycotoxin (type of model system)	Degradation product	$[M+H]^+$ m/z	Fragment	Structure	MS^2 fragments	
					m/z	Fragment
ENA (aqueous system)	[ENA+K-HyLv]+ (ENA degradation product 1)	640.6	HyLv		427.7 314.6	$[ENA+K-2HyLv-Ile+H_2O]^+$ $[ENA-HyLv-2Ile]^+$
ENA (aqueous system)	$[ENA-Ile]^+$ (ENA degradation product 2)	541.9	Ile		- (1)	- (1)

Table 1. cont.

Mycotoxin (type of model system)	Degradation product	$[M+H]^+$ m/z	Fragment	Structure	MS² fragments	
					m/z	Fragment
ENA (pasta resembling system)	$[ENA\text{-}HyLv\text{-}Ile]^+$	455.2	HyLv+Ile		- (1)	- (1)
ENA_1 (aqueous system/ pasta resembling system)	$[ENA_1\text{-}Val]^+$	541.3	Val		441.1	$[ENA_1\text{-}HyLv\text{-}Ile]^+$
					428.1	$[ENA_1+K\text{-}2HyLv\text{-}Val+H_2O]^+$
					414.1	$[ENA_1+Na\text{-}2HyLv\text{-}Val+H_2O]^+$
					214.1	[HyLv+Val]

(1) Data non available

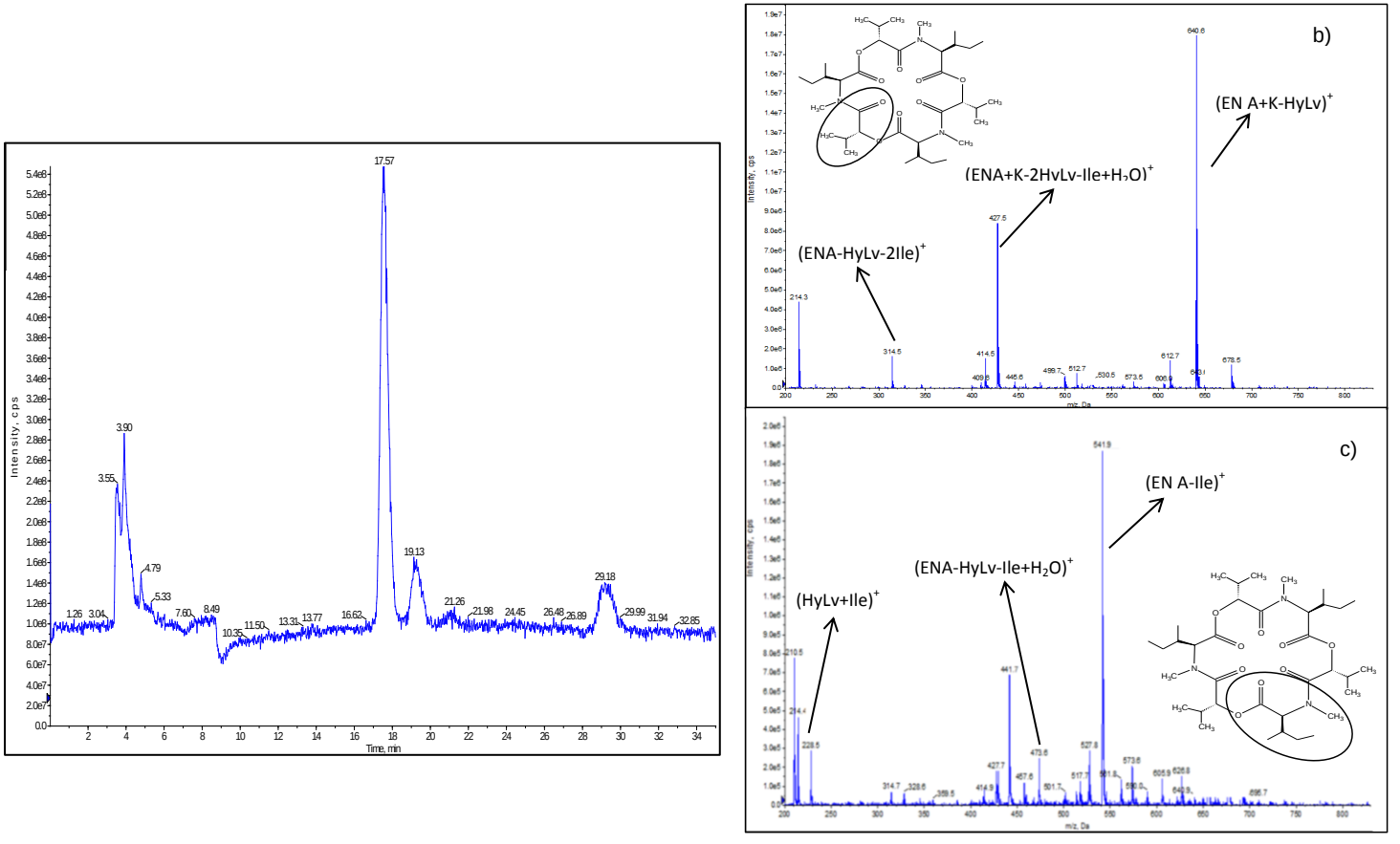


Figure 3. a) LC-MS-LIT chromatogram of ENA treated during 10 min at pH 4 in the PRS system, b) LC-MS-LIT spectra of ENA degradation product 1 and c) LC-MS-LIT spectra of ENA degradation product 2.

The degradation product 1 (figure 3b) was characterized as potassium adduct of ENA with the loss of a structural component of ENs structure as HyLv evidenced in the fragment with a m/z of 640.6. The formation of this new degradation product was also confirmed by the fragments with m/z of 427.5 and 314.5 (figure 3b). The first one corresponding to the potassium adduct of ENA with the loss of 2HyLv and also by the loss of another amino acid that characterized ENs structure as the Ile. The second fragment (m/z 314.5) corresponding to ENA with the loss of HyLv and 2Ile groups. To obtain more information and also to confirm the structure of degradation product 1, the sample was also injected in the modality EPI scan to obtain the MS^2 scan of the neo forming compound using as fragmenting signal the ion with a m/z of 640.6. In figure 4a is possible to observe the LC-MS-LIT chromatogram in MS^2 of the degradation product 1. Figure 4b shows the EPI-LIT spectra that present several important fragments that confirm the structure of degradation product 1 that are the signals with m/z of 427.7 and 314.3. The fragments present the same signal identified in the MS^1 spectra. The fragment with m/z 427.7 corresponds to the potassium adduct of EN A with the loss of 2HyLv and 1Ile, whereas the second one was identified as ENA with the loss of 1HyLv and 2Ile.

The degradation product 2 was identified as ENA with the loss of an Ile. In figure 3c is shown the MS-LIT spectra corresponding to the ENA degradation product 2. The structure of this new compound was also confirmed by the signals with m/z of 473.8, 427.7 and 228.5. The first one was identified as ENA with the loss of HyLv and Ile, whereas the second one (m/z 427.7) was identified as the potassium adduct of ENA with the loss of two groups of HyLv and one Ile group. The loss of HyLv and Ile was confirmed by

the m/z 228.5 corresponding to the fragment HyLv+Ile. Unfortunately no MS^2 EPI spectrum is available to confirm the structure of the second ENA degradation product.

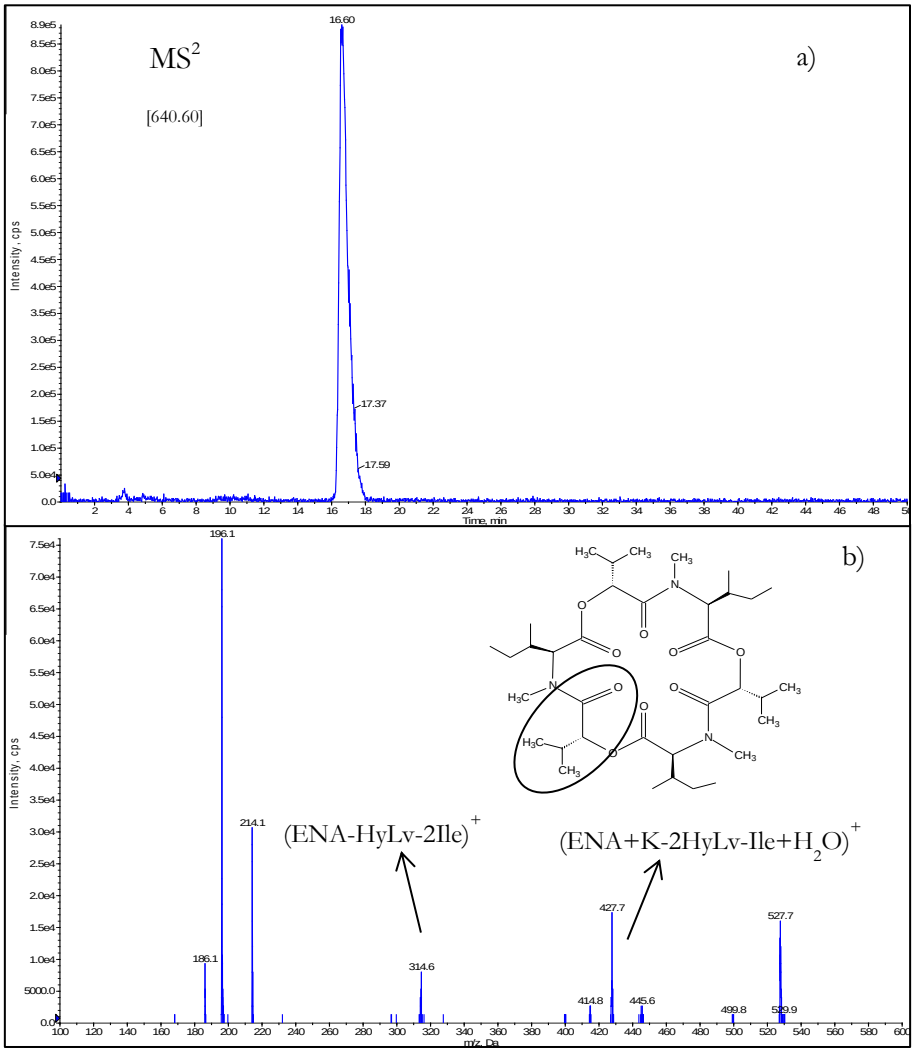


Figure 4. a) LC-MS-LIT chromatogram obtained in EPI mode (MS^2) using as fragmenting ion the signal with a m/z of 640.6 of the ENA degradation product 1 and b) EPI mass spectrum obtained in MS^2 of the degradation products 1 of the ENA.

In contrast to the two degradation products of ENA identified in the aqueous system, only one degradation product of ENA was identified in PRS (Table 1). The degradation products of ENA in the aqueous system were characterized as ENA with the loss of HyLv (m/z 640.6) and as ENA with the loss of Ile (m/z 541.9), whereas the degradation product of ENA in the PRS was characterized as ENA with the simultaneous loss of HyLv and Ile (m/z 455.2). The abundance of this degradation product was positively related to increasing time of thermal treatment and the decreasing ENA levels. This product was identified in the neutral, acid and basic solutions. The structure of this compound from the PRS was confirmed by the signals with m/z of 311.2 and 228.2. The first one corresponding to ENA with loss of one HyLv and two Ile, and the second one corresponding to the group HyLv and Ile. No MS^2 EPI spectrum is available to confirm the structure of the ENA degradation product identified after the treatments in the PRS.

3.3.2. ENA_1

Similar LC-MS-LIT chromatograms and mass spectrums were obtained in the aqueous system and in the PRS at the three tested pH. Two peaks were observed in the chromatograms related to the treated solutions. One peak corresponding to ENA_1 (m/z 668.3), and another peak that increased with the increment of the incubation times corresponding to a new degradation product of the ENA_1 (m/z 541.3), that was characterized as ENA_1 with the loss of Val (Table 1). The structure of this degradation product was confirmed by different fragments in the MS-LIT spectra obtained in ER mode. The fragment with m/z of 441.2 represents the ENA_1 degradation product with a loss of an Ile and of HyLv. The loss of the amino acids Ile, Val and HyLv from the

structure of ENA_1 was confirmed by the presence of the fragments with m/z of 214.1 and 228.3. To confirm the structure of the ENA_1 degradation product formed after the heat treatment, the sample was also injected in the modality EPI scan to obtain the MS^2 scan of the degradation product isolated using as fragmenting signal the ion with a m/z of 541.3 (Table 1). In particular the fragment with a m/z of 441.1 was identified as ENA_1 with the loss of a HyLv and of an Ile, whereas the signals with m/z of 414.1 and 428.1 were identified as the sodium and potassium ENA_1 adduct with the loss of 2HyLv and 1Val. The loss of HyLv and Ile was confirmed in the EPI mass spectra by the presence of the signal with a m/z of 214.1.

3.3.3. ENB

The LC-MS-LIT chromatograms observed in the aqueous system were very similar to those obtained in the PRS. The chromatograms showed one peak corresponding to ENB that decreased with the increment of the incubation times. Other peaks were observed related to two degradation products of ENB that increased with the decrease of ENB. Both peaks appeared in the acid, basic and neutral solutions.

The degradation product 1 was identified as ENB with the loss of the amino acid Val (Table 2). The formation of this new product is confirmed by the presence in the spectra of some diagnostic signals, as the fragment with a m/z of 427.3, identified as ENB with the loss of structural components HyLv and Val, and also by the fragment with m/z of 214.2 identified as ENB with the loss of 2HyLv and 2Val. The formation of this new product was confirmed by the analysis in MS^2 mode of the neo forming product and in particular in the EPI (MS^2) spectra (Table 2). There are some signals that testify the formation

of the reduction product as the ions with m/z of 427.3 and 214.1, present also in the MS^1 spectra, and also by the fragment with m/z of 399.4 that represent ENB with the loss of two units of HyLv and one unit of Val.

The degradation product 2 was identified as ENB with the loss of the HyLv group (Table 2). The structure of this degradation product was confirmed by the presence of different fragments in the MS-LIT spectra obtained in ER mode. The fragment with m/z of 427.3 represents ENB with a loss of Val and HyLv, whereas the fragment with m/z 368.3 is related with the loss of Val and 2HyLv from the structure of ENB. The loss of amino acids Val and HyLv was confirmed by the presence of the fragment with m/z of 214.1. No MS^2 EPI spectrum is available to confirm the structure of the ENB degradation product 2.

3.3.4. ENB_1

Similar results to those obtained for ENB were obtained in ENB_1 during the different treatments applied. Two degradation products of ENB_1 were identified after the treatments (Table 2). The degradation product 1 was identified as ENB_1 with the loss of the aminoacid Val (m/z 527.3). This compound was confirmed by the signals with m/z of 427.3, 414.3 and 214.2. The first one was identified as EN B_1 with the loss of HyLv and Ile, whereas the second one represent potassium adduct of ENB_1 with the loss of 2HyLv and of a Val. The last fragment was identified as the ENB_1 with the loss of the three structural components that characterize the ENB_1 structure: two units of HyLv, one unit of Val, and one unit of Ile. An additional confirmation of the formation of this degradation compound was carried out with the technique of the MS-EPI, where in the MS^2 spectra evidenced some fragments that confirm

the formation of the ENB_1 degradation product. In particular in the MS^2 spectra are evidenced some ions already identified in the MS^1 spectra with m/z of 427.3, 414.1 and 214.1. Also, another fragment was identified in the MS^2 spectra (m/z 399.2) as the ENB_1 sodium adduct with the loss of 2HyLv and 1Val (Table 2).

The degradation product 2 of ENB_1 was characterized as ENB_1 with the loss of the HyLv group (Table 2). The formation of this new product is confirmed by the presence in the spectra of some diagnostic signals, as the fragments with a m/z of 457.5, 427.3, 414.5 and 214.1. The first fragment represents ENB_1 with a loss of Val and HyLv, whereas the second fragment represents ENB_1 with the loss of Ile and HyLv. The fragment with m/z of 414.5 is related to the structure of the potassium adduct of ENB_1 with the loss of two units of HyLv and one unit of Val. The loss of the amino acids Val and HyLv was confirmed by the presence of the fragment with m/z of 214.1. The confirmation of the structure of the degradation product 2 was not possible, since a MS^2 EPI spectrum was not available.

Table 2. LC-MS-LIT data (MS^1 and MS^2) of the degradation products of ENs type B obtained in the aqueous system and in the pasta resembling system.

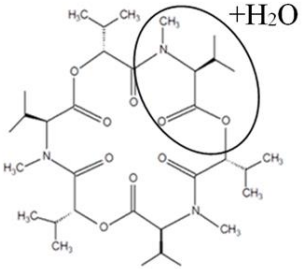
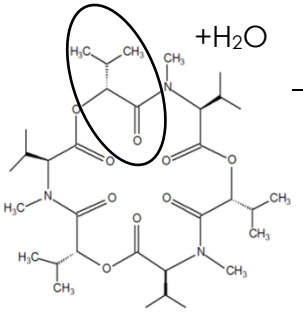
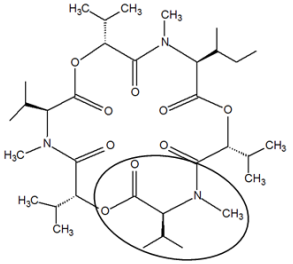
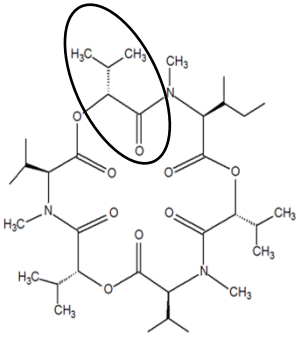
Mycotoxin (Type of model system)	Degradation product	$[M+H]^+$ m/z	Fragment	Structure	MS^2 fragments	
					m/z	Fragment
ENB (aqueous system/ pasta resembling system)	$[ENB\text{-}Val+H_2O]^+$	527.2	Val		427.3	$[ENB\text{-}HyLv\text{-}Val]^+$
					399.4	$[ENB+K\text{-}2HyLv\text{-}Val+H_2O]^+$
					214.1	$[HyLv+Val]^+$
ENB (aqueous system/ pasta resembling system)	$[ENB\text{-}HyLv+H_2O]^+$	573.2	HyLv		- (1)	- (1)

Table 2. cont.

Mycotoxin (Type of model system)	Degradation product	$[M+H]^+$ m/z	Fragment	Structure	MS^2 fragments	
					m/z	Fragment
ENB_1 (aqueous system/ pasta resembling system)	$[ENB_1\text{-Val}]^+$	527.3	Val		427.3	$[ENB_1\text{-HyLv-Ile}]^+$
					414.1	$[ENB_1+K\text{-2HyLv-Val}+H_2O]^+$
					399.2	$[ENB_1+Na\text{-2HyLv-Val}+H_2O]^+$
					214.1	$[HyLv+Val]^+$
ENB_1 (aqueous system/ pasta resembling system)	$[ENB_1\text{-HyLv}]^+$	573.2	HyLv		- (1)	- (1)

(1) Data non available

4. CONCLUSIONS

The time of thermal treatment affected to the ENs reduction, whereas the variation of the pH not produced any effect in the ENs stability. The decrease of the ENs levels during the thermal treatments has been related to the formation of new compounds derived to the ENs. The application of the LC-MS-LIT permitted the elucidation of the new degradation products formed during the treatments. Degradation products of ENs have been identified for the first time in this pilot study. Future studies will be focused on the study of the toxicological activity of the ENs degradation products on several cell lines. This information is necessary to evaluate the potential health risk of the population to the formation of these new compounds in the processed foodstuffs.

ACKNOWLEDGMENTS

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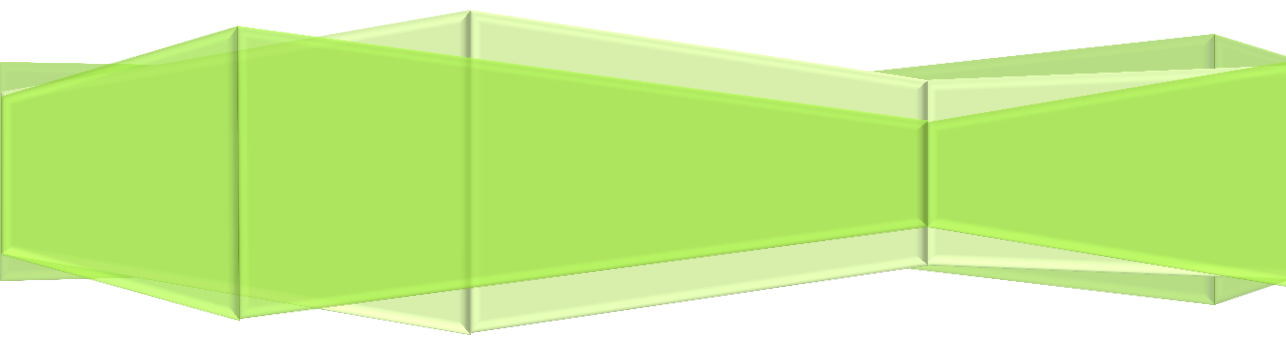
3.9. Effects of technological processes on enniatins levels in pasta



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Effects of technological processes on enniatins levels in pasta

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ABSTRACT

BACKGROUND: Potential human health risks posed by enniatins (ENs) require its control primarily from cereal products, creating a demand for harvesting, food processing and storage techniques capable to prevent, reduce and/or eliminate the contamination. In this study, different methodologies to pasta processing simulating traditional and industrial processes were developed in order to known the fate of the mycotoxins ENs. The levels of ENs were studied at different steps of pasta processing. The effect of the temperature during processing was evaluated in two types of pasta (white and whole-grain pasta). Mycotoxin analysis was performed by LC-MS/MS.

RESULTS: High reductions up to 50% and 80% were achieved during drying pasta at 45-55ºC and 70-90ºC, respectively. The treatments at low temperature (25ºC) did not change ENs levels. The effect of pasta composition did not evidence a significant effect on the stability of ENs. The effect of the temperature allowed a marked mycotoxin reduction during pasta processing. Generally, ENA_1 and ENB showed higher thermal stability than ENA and ENB_1.

CONCLUSIONS: The findings from the present study suggested that pasta processing at medium-high temperatures is a potential tool to remove an important fraction of ENs from the initial durum wheat semolina.

Keywords: Enniatins, mycotoxin reduction, thermal stability, pasta processing, LC-MS/MS.

1. INTRODUCTION

Pasta processing is a practice that involves different mechanical and thermal steps, such as kneading, extrusion, shaping and drying[1]. The production of pasta with high quality is very dependent on the drying conditions. Drying pasta has undergone numerous improvements over the past years, being the time and temperature the most critical parameters of the industrial process[2]. Several time-temperature cycles have been described in the literature[3,4]. However, it has not been proposed any recommendation to standardize a general drying process. Menesatti *et al.*[5] classified pasta processing according to the application of traditional or industrial process. In traditional process, drying means are based on long drying times at low temperatures (29-40ºC). As regards industrial scale, drying systems at high temperatures (70-90ºC) have been widely established in modern plants to shorten drying cycle (big factories), while lower/medium temperatures (40-60ºC) are usually adopted in factories with reduced production (small or medium producers)[5,6].

Enniatins (ENs) involve a group of *Fusarium* mycotoxins with ionophoric activity structurally related to cyclodepsipeptides. Currently at least 23 ENs of natural origin have been identified. These toxins have been grouped into different types based on the functional groups present in their elementary structure. ENs only differ by alternating amino acid residues, including *N*-methylated L-alanine, L-valine, and L-isoleucine units. Enniatins A, A_1, B and B_1 (ENA, ENA_1, ENB and ENB_1, respectively) are the most commonly studied ENs, mainly due to their widespread presence in foodstuffs[7]. At the moment, limited toxicity data is available in the literature. Several *in vitro* toxicological studies have evidenced that ENs produce enzyme inhibition, oxidative stress induction, immunotoxicity and myelotoxicity, among other

systemic disorders[8]. In a preliminary study, Prosperini *et al.*[9] demonstrated that chemical interactions between binary, ternary and quaternary combinations of ENA, ENA_1, ENB and ENB_1 could produce a general additive effect. Usually, human and animal exposure to ENs occur by the consumption of contaminated cereals (rice, maize, wheat, oat, barley, etc.) and derived products, such as biscuits, bread, pasta or feed[10-13]. Therefore, potential human health risks posed by ENs require its control primarily from cereal products, creating a demand for harvesting, food processing and storage techniques capable to prevent, reduce and/or eliminate the contamination. In the last years, physical, biological and chemical decontamination methods have been tested in order to inactivate or reduce mycotoxins from food and feed matrices. Decontamination strategies should not generate toxic compounds as well as induce no modifications to the nutritional and sensory properties of the final product. Physical methods are the most suitable for this purpose, whereas most of the chemical and biological processes are impractical and require extreme conditions compromising the consumer acceptability to the product[14]. In this regard, food industry is in search of effective applications to mycotoxin detoxification, which could be adapted or reproduced in food processing[15,16]. However, certain mycotoxins are heat-stable compounds and can persist to food processing. Aflatoxins (AFs) and ochratoxin A (OTA) are stable during roasting, deoxynivalenol (DON) during cooking and frying, and fumonisins (FBs) during pasteurization[17]. To date, relatively few studies have been focused to evaluate the influence of technological processing on ENs levels. In a previous study, the influence of pH and temperature on ENs levels was evaluated using a model pasta resembling system. The results evidenced a clear decrease of ENs contents after 15 min of thermal treatment at 100ºC[18]. Other

authors[19,20] evaluated the levels of ENs type B during bread-making process, resulting in high percentages of reduction (from 25% to 60%). Furthermore, the influence of beer production has been evaluated obtaining low concentrations of ENs after this process[19,21].

Recently, the EFSA Panel CONTAM (European Food Safety Authority Panel on Contaminants in the Food Chain) provided specific recommendations to improve the risk characterisation associated to emerging *Fusarium* mycotoxins, including that studies should be conducted on the fate of these mycotoxins during the preparation of grain-based products, including bread, pasta and fine bakery wares[8]. To the authors knowledge, until now no studies have been developed concerning the fate of ENs during pasta processing. The aim of the present study was to investigate the impact of different conditions of pasta processing, including the effect of the temperature and the influence of the food composition on ENs levels. Three treatments were assessed at low, medium and high thermal conditions within the processing of white and whole-grain pasta.

2. MATERIALS AND METHODS

2.1. Chemicals

Solvents (acetonitrile and methanol) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Potato Dextrose Broth (PDB) was purchased from Insulab (Valencia, Spain). Deionized water was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain) before use.

Individual standards of ENA, ENA_1, ENB and ENB_1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of ENA, ENA_1, ENB and ENB_1 at a concentration of 1000 μg mL^{-1} were prepared in methanol. These stock solutions were then diluted with pure methanol in order to obtain appropriate working solutions. All solutions were stored in glass-stoppered bottles and darkness in security conditions at -20ºC.

2.2. Starting material

In order to obtain durum wheat semolina contaminated by ENA, ENA_1, ENB and ENB_1 in suitable concentrations and to standardize the pasta processing experiments, the durum wheat semolina used as starting material was contaminated as follows. Inoculation experiments of a blank of commercial durum wheat semolina were perfomed with an ENs producing strain of *Fusarium tricinctum*[22]. *Fusarium tricinctum* strain (CECT 20150) was obtained from the Spanish Type Culture (CECT Valencia, Spain). A suspension of conidia in concentration of 10^6 conidia mL^{-1} of *Fusarium tricinctum* in PDB, was used for inoculation. Conidial concentration was measured by optical density at 600 nm[23]. For fermentation experiments, 250 g of durum wheat semolina were inoculated with macronidia suspensions of *Fusarium tricinctum* and incubated during 42 days at 25°C on an orbital shaker (IKA Ks 260 basic, Stanfen, Germany). Then, the contaminated semolina was autoclaved at 121ºC for 15 min in a Presoclave II-75 autoclave (P Selecta, Barcelona, Spain) to promote fungi inactivation. Finally, the autoclaved semolina was dried at 100ºC to reach 15% of moisture content. Drying was performed in a pilot-scale EC25GE pasta drier (Italgi, Genova, Italy) with a

controlled relative humidity of 75%[24]. ENs concentrations from 10 to 70 mg Kg^{-1} were detected in the contaminated semolina.

In order to obtain concentration levels comparable to those obtained in real samples[25], it was formulated the called Working Semolina as starting material. Working Semolina (500 g) for white and whole-grain pasta, was formulated by mixing the contaminated semolina with a blank commercial white semolina and with commercial whole-grain semolina (1/10 w/w), respectively. The mixtures of contaminated and commercial semolina were systematically combined to obtain a homogenous Working Semolina with final levels of ENs between 1 and 7 mg Kg^{-1}.

2.3. Pasta processing protocol

Two types of pasta were processed: white and whole-grain pasta. Whole-grain pasta was selected due to the increasing demand of the consumer for natural and healthy food, and because of the highest mycotoxin-levels usually found in whole-grain foods[12]. The process to making pasta was divided in two stages: fresh pasta making and fresh pasta drying. The diagram of the experimental plan is shown in Fig. 1. All experiments were performed in triplicate.

2.3.1. Fresh pasta making

Pasta was manufactured according to the standard process, that includes three steps: kneading, extrusion and shaping (Fig. 1a)[4]. Kneading process was carried out by mixing 500 g of Working Semolina with tap water (30%). The mixture was kneaded for 10 min to obtain a stiff and homogeneous dough. The extrusion and shaping steps were carried out with a pilot-scale pasta Micra

extruder (Italgi, Genova, Italy). The dough was pressed several times until obtain the desired thickness (2.5 mm). Finally, the dough was shaped through the die to obtain a spaghetti form (diameter of 2.1 ± 0.2 mm). Fresh pasta was sampled at the exit of the pasta extruder. An aliquot of 30 g was directly subjected to mycotoxin analysis. In addition, 600 g of fresh pasta were immediately subjected to fresh pasta drying.

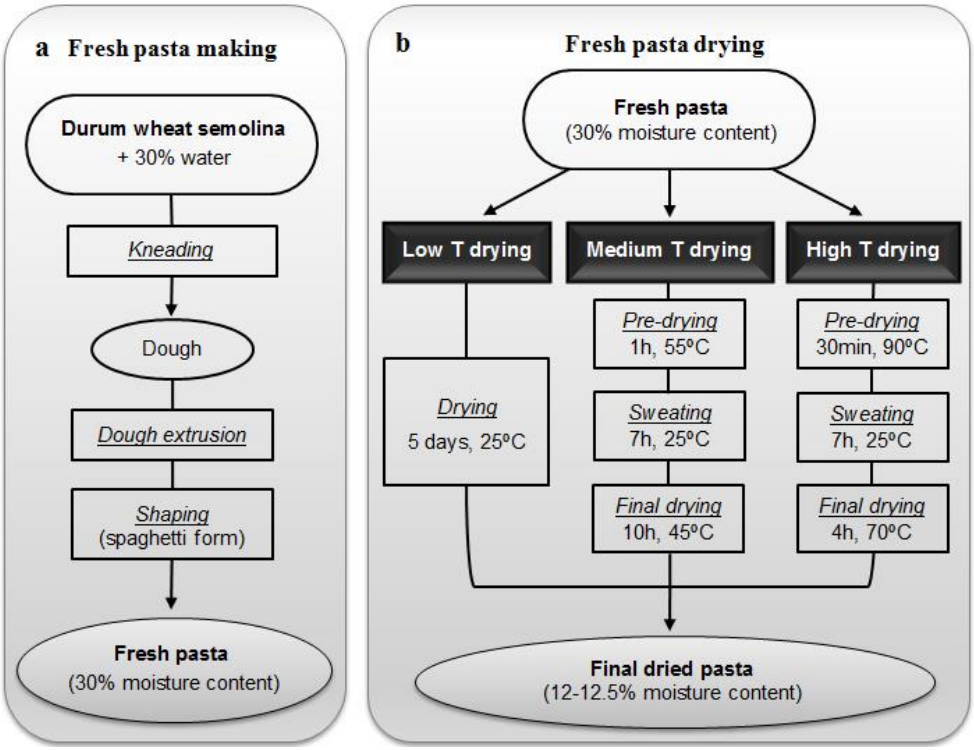

Fig. 1. Experimental plan for pasta processing: **a** fresh pasta making, and **b** fresh pasta drying according to three different drying programs: low, medium and high temperature of drying.

2.3.2 Fresh pasta drying

Fresh pasta contained about 30% moisture content. In order to obtain dried pasta, the following step involved the pasta drying to reduce the moisture content until 12-12.5%. The experimental plan for pasta drying was performed according to the traditional and industrial processes described in the literature[5]. Based on these processes, drying pasta was conducted by three different drying programs: low, medium and high temperature (T) of drying. For this purpose, fresh pasta was divided in three fractions of 200 g. The specific conditions of drying programs are summarized in Fig. 1b.

Low T drying reflected the typical practice of traditional homemade drying. Fresh pasta (30% moisture content) was dried during five days at 25ºC to reach 12-12.5% moisture content. Drying was performed in a pilot-scale EC25GE pasta dryier (Italgi, Genova, Italy) with a controlled relative humidity of 80%. Aliquots of 30 g of pasta were sampled after each day of drying and then, were rapidly stored in a dark and dry place at 4°C until mycotoxin analysis.

Medium T drying simulated the drying process applied in industries with reduced capacity of production and long drying times at 45-55ºC. High T drying simulated the process developed in large-scale industries with short time-temperature cycles at 70-90ºC. The industrial time-temperature cycle involves three steps: pre-drying, sweating and final drying. Pre-drying step allowed a fast drying leading to a dry area on the outside of pasta, while the interior remains with water. Sweating step provided an appropiate stabilization leading to an uniform moisture content in the product. The last period involved final drying of pasta to reach 12-12.5% water content (Fig. 1b)[4]. All drying steps were perfomed in a pilot-scale EC25GE pasta dryier (Italgi,

Genova, Italy). Aliquots of 20 g of intermediate products were sampled after each key step (pre-drying, sweating and final drying) and were rapidly stored in a dark and dry place at 4°C until mycotoxin analysis. All samples were analized in triplicate.

2.4. Mycotoxin analysis

2.4.1. Enniatins extraction from pasta

The extraction method was previously reported by Serrano *et al.*[12]. Samples of pasta (5 g) were extracted with 50 mL of acetonitrile (AcN) using a Ika T18 basic Ultra-Turrax (Staufen, Germany) during 3 min. After, centrifugation for 15 min at 3554 g and 5ºC, the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Flawil, Switzerland). The extract was dissolved with 5 mL of AcN, and was evaporated to dryness by nitrogen gas at 35ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and was filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) prior the injection in the LC-MS/MS system.

2.4.2. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 2 mm I.D., 3 μm particle size)

analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using gradient elution that started at 90% of A (AcN) and 10% of B (10 mM ammonium formate in MeOH), increased linearly to 50% B in 10 min. After, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 mL min^{-1}. The analysis was performed in positive ion mode. The electrospray ionisation source values and the analyser settings are shown in Table 1 Supplementary data. The mass spectrometer was operated in Multiple Reaction Monitoring (MRM) mode. The method was optimized according to the criteria which establishes that a substance can be identified using LC-MS/MS in MRM mode by at least two transitions[26]. The most abundant product ion was selected for quantification and the second one for confirmation. Therefore, the quantification of each mycotoxin was carried out with the primary transition (transition of quantification) and the confirmation with the second transition (transition of confirmation). The MRM ratio was monitored. Table 2 Supplementary data shows the precursor and product ions of each mycotoxin for acquisition in MRM mode, and the related cone voltage and collision energy values.

2.5. Statistical analysis

The experiments were carried out in triplicates. Statistical analysis was conducted by one-way analysis of variance (ANOVA) and by least significance difference (LSD) test (assuming equal variance) to evaluate the significant variations ($p<0.05$), using Statgraphics X64 statistical software.

Table 1 supplementary data. Electrospray ionisation source values and analyser settings.

Electrospray ionisation source values	
Capillary voltage	3.50 kV
Extractor	5 V
RF lens	0.5 V
Source temperature	100ºC
Desolvation temperature	300ºC
Desolvation gas flow	800 L h^{-1} (nitrogen 99.99% purity)
Cone gas flow	50 L h^{-1} (nitrogen 99.99% purity)
Analyser settings	
Resolution	12.0 (First and third quadrupoles)
Ion energy	0.5
Entrance energy	-3
Exit energy	1
Multiplier	650
Collision gas pressure	3.83 x 10^{-3} mbar (argon 99.995% purity)
Interchanel delay	0.02 s
Total scan time	1.0 s
Dwell time	0.1ms

Table 2 supplementary data. Precursor and product ions for selected mycotoxins and MRM optimized parameters.

Mycotoxin	Precursor ion (m/z)	Product ion (m/z) [a]	Cone (V)	Collision energy (eV)
ENA	681.9 $[M+H]^+$	228.2Q	40	35
		210.0q		35
ENA1	667.9 $[M+H]^+$	228.2Q	40	35
		210.0q		35
ENB	639.8 $[M+H]^+$	214.2Q	40	35
		196.2q		35
ENB1	654.9 $[M+H]^+$	214.2Q	40	35
		196.2q		35

[a] Q, Quantification transition q, Confirmation transition

3. RESULTS AND DISCUSSION

3.1. Method performance

The method validation included the evaluation of linearity, limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision). 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. LODs were calculated using a signal-to-noise ratio of 3. LOQs

were calculated using a signal-to-noise ratio of 10. LOQs were of 0.5 μg Kg^{-1} for ENA, ENB and ENB_1, while the LOQ for ENA_1 were of 0.25 μg Kg^{-1}. LODs were of 0.15 μg Kg^{-1} for ENA, ENB and ENB_1, while the LOD for ENA_1 was of 0.08 μg Kg^{-1}. In order to determine the linearity, matrix-assisted calibration curves were constructed for each studied mycotoxin. ENs exhibited good linearity over the working range: from 0.25 μg Kg^{-1} to 20 mg Kg^{-1} for ENA_1, and from 0.5 to 20 mg Kg^{-1} for ENA, ENB and ENB_1.The regression coefficient of calibration curves was higher than 0.992. The accuracy was evaluated through recovery studies using spiked blank samples at two concentration levels (LOQ and 100 x LOQ). Intra-day precision was performed by five determinations at each addition level in the same day. Inter-day precision was assessed by one determination at each addition level during five days. The mean recoveries and the corresponding relative standard deviations (RSDs) are presented in Table 3 Supplementary data. RSDs ranged from 4% to 11% for intra-day precision, and from 6% to 15% for inter-day precision. Recovery values were excellent at the range 85-110% and 86-112% for low spiked level (LOQ) and high spiked level (100xLOQ), respectively. The results obtained in the present study were within the limits set by Commission Decision, 2002/657/EC[26].

Table 3 supplementary data. Recovery values (%) and relative standard deviations (%) for ENA, ENA_1, ENB and ENB_1.

		Recovery ± RSD			
		ENA	ENA_1	ENB	ENB_1
Intra-day precision [a]	Low level (LOQ)	92±5	88±7	109±8	99±10
	High level (100 x LOQ)	91±4	86±9	112±6	97±11
Inter-day precision [b]	Low level (LOQ)	93±8	85±11	110±8	97±13
	High level (100 x LOQ)	90±6	88±8	109±9	95±15

[a] Number of replicates: 5

[b] Different days: 5

3.2. Pasta processing design

As described above, pasta processing was divided in two stages: fresh pasta and fresh pasta drying. Thermal treatment was not involved during fresh pasta making, which presented a short storage period. On the other hand, dried commercial pasta should be strong and have a long storage life. Drying process is the most critical phase of pasta processing, because the quality of the final dried pasta is highly dependent upon drying process. Drying pasta has undergone numerous improvements over the past years. Several programs of temperatures have been described in the literature[5,27]. However, it has not been proposed one drying standard process**.** Whereas traditional-home means are based on long drying times at low temperatures, high and ultra-high temperatures have been widely established for most industries worldwide. Industries with reduced production apply temperatures of 40-60°C, while drying systems above 70°C are commonly used at great industries. Although a general procedure for drying pasta has not been widespread, different authors

have been described a general process that involves three steps: pre-drying, sweating and final drying. The first step (pre-drying) allows a rapid drying during 30-60 min at 55-110°C, that lead to a dry area on the outside of pasta, while the interior remains with water. To provide the appropiate stabilization of the product, a second period of sweating allows to equilibrate the percentage of water inside and outside of the pasta during 4-8 h at 25°C. The last period involves slow drying of pasta at 45-70°C for 6-16 h to reach to a water content between 12% and 12.5%[4]. Taking into account the different described drying processes in literature, it was decided to drying pasta according to three drying programs at different T and times of drying: low T drying, medium T drying and high T drying (Fig. 1b).

3.3. Influence of pasta processing on ENs levels

Levels of ENA, ENA_1, ENB and ENB_1 were determined at different stages of pasta processing. Given the significant differences in the water content of pasta after each drying step, all concentrations were converted to dry weight values to obtain comparable results. The conversion to dry weight values was conducted by the moisture determination of each intermediate product.

3.3.1. Fresh pasta making

ENs levels in white Working Semolina were 1.40±0.25, 3.16±0.40, 7.45±0.39 and 6.60±0.59 mg Kg^{-1} of ENA, ENA_1, ENB and ENB_1, respectively. ENs levels in whole-grain Working Semolina were 1.44±0.40, 3.04±0.51, 7.33±0.94 and 6.05±0.77 mg Kg^{-1} of ENA, ENA_1, ENB and ENB_1, respectively. Making pasta stage (kneading, dough extrusion and

shaping) did not result in a significant modification on ENs levels ($p>0.05$). In fact, all steps for fresh pasta making are mechanical without thermal treatment (Fig. 1a). The contents of ENs in white fresh pasta were 1.29±0.32, 3.05±0.53, 7.02±0.58 and 6.36±0.72 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively, and in whole-grain fresh pasta were 1.30±0.45, 2.71±0.65, 7.10±0.78 and 5.96±0.64 mg Kg^{-1} of ENA, ENA_1, ENB and ENB_1, respectively.

3.3.2. Low temperature of drying

Drying pasta was performed for five days at 25ºC. ENs concentrations were determined after each day of drying. ENs levels are presented in Fig. 2. After five days of drying, ENs concentrations were similar to those presented in fresh pasta. The process did not showed a significant reduction on ENA, ENA_1, ENB and ENB_1 levels ($p>0.05$). Moreover, no significative differences between white and whole-grain pasta were observed. Real concentrations taking into account the water content of final dried pasta (humidity of 12%), were of 1.19, 2.44, 6.44 and 5.42 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively. Therefore, the treatments at low temperatures within a short period of time, did not influenced the ENs contents.

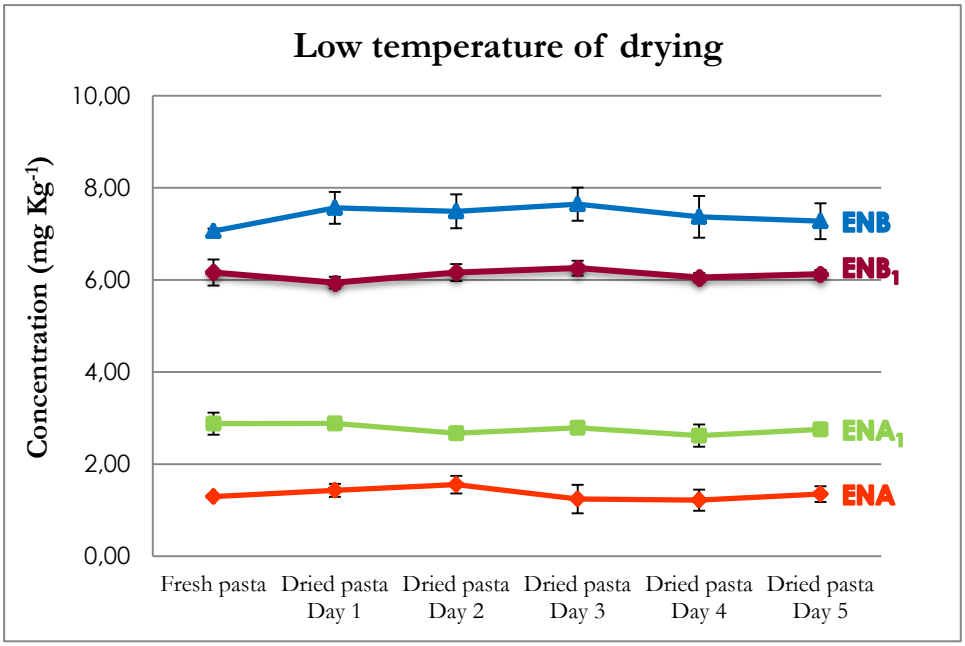

Fig. 2. Levels of concentration (as a dry weight basis) of ENs (mg Kg^{-1}) after each day of drying pasta at low temperature (25°C).

3.3.3. Medium temperature of drying

The drying process applied in the study at medium T drying involves three stages, pre-drying, sweating and final drying (Fig.1b). The concentration and the percentages of reduction after each step are reported in Table 1. Fig. 3 show the total reduction related to the full process. Pre-drying step contributed significantly to decrease ENs levels. The concentrations detected in pre-dried pasta were about 14-29% lower than those detected in fresh pasta. ENB was the mycotoxin most stable under pre-drying conditions. This fact could be due to the high melting point of ENB (172-174°C)[28]. The intermediate stage of

sweating (25ºC) did not reduce significatively the ENs levels (reductions of 0.1-6.4%), while the step of final drying (10h, 45°C) showed the maximum percentages of reduction, from 15 to 37%. According to the results of the LSD test, the reductions only were significant in the last step of drying ($p<0.05$ for ENA and ENA_1, $p<0.001$ for ENB, and $p<0.005$ for ENB_1).

Considering the global process at medium T drying, the total ENs reduction was assessed at 40-45% for white pasta. No significative differences were obtained between the percentages of reduction obtained for ENA, ENA_1, ENB and ENB_1. Concentration reductions ranged from 1.29 to 0.71 mg Kg^{-1} for ENA, from 3.05 to 1.76 mg Kg^{-1} for ENA_1, from 7.02 to 4.02 mg Kg^{-1} for ENB, and from 6.36 to 3.80 mg Kg^{-1} for ENB_1. Real concentrations taking into account the water content of final pasta (humidity of 12%) were of 0.60, 1.51, 3.88 and 3.32 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively.

Data concerning the global process for whole-grain pasta, showed total reductions of 51% for ENA and 56% for ENB_1, while total reductions were of 37% for ENA_1 and 39% for ENB. Concentration levels were reduced from 1.30 to 0.57 mg Kg^{-1} for ENA, from 2.71 to 1.71 mg Kg^{-1} for ENA_1, from 7.10 to 4.36 mg Kg^{-1} for ENB and from 5.96 to 2.90 mg Kg^{-1} for ENB_1. ENs contents in final dry pasta (humidity of 12%), were 0.51, 1.69, 3.84 and 2.50 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively.

The percentages of reduction between white and whole-grain pasta were comparable in all cases.

Table 1. Effect of treatment at medium temperature of drying on white and whole-grain pasta: ENs concentration (mg Kg^{-1}) and percentages of ENs reduction after each step of processing (%). All results are expressed as dry weight basis.

Sample	ENA		ENA_1		ENB		ENB_1	
	Conc ± RSD (mg Kg^{-1})	% reduction	Concentration ± RSD (mg Kg^{-1})	% reduction	Conc ± RSD (mg Kg^{-1})	% reduction	Conc ± RSD (mg Kg^{-1})	% reduction
White pasta								
Fresh pasta	1.29±0.32	-	3.05±0.53	-	7.02±0.58	-	6.36±0.72	-
Pre-drying (1h, 55°C)	1.01±0.15	21.7	2.50±0.45	17.9	5.95±0.69	15.2	5.24±0.56	17.6
Sweating (7h, 25°C)	0.99±0.09	2.0	2.50±0.49	0.1	5.94±0.65	0.2	5.13±0.63	2.1
Final drying (10h, 45°C)	0.71±0.08	28.3	1.76±0.60	29.7	4.02±0.60	32.3	3.80±0.49	25.9
Whole-grain pasta								
Fresh pasta	1.30±0.45	-	2.71±0.65	-	7.10±0.78	-	5.96±0.64	-
Pre-drying (1h, 55°C)	0.93±0.20	28.5	2.05±0.70	24.3	6.10±0.77	14.1	4.73±0.77	20.6
Sweating (7h, 25°C)	0.87±0.22	6.4	2.01±0.55	1.9	5.97±0.61	2.1	4.64±0.56	1.9
Final drying (10h, 45°C)	0.57±0.11	34.5	1.71±0.42	14.9	4.36±0.33	27.0	2.90±0.42	37.5

3.3.4. High temperature of drying

The drying process at high T drying concerns three steps: pre-drying, sweating, and final drying (Fig. 1b). The concentrations and the percentages of reduction are reported in Table 2. Fig. 3 show the total reduction related to the full process. ENs showed significant reductions during the preliminary treatment at 90°C for 30min, reaching to reduction values of 21-54% ($p<0.001$). As is evidenced in the treatment at medium T drying, no significant modifications were obtained on ENs levels during sweating step at 25ºC ($p>0.05$). Results from the last step of drying (4h, 70ºC) also reflected a significant percentage of ENs degradation ($p<0.000$). Levels of ENs declined to 47-71% in white pasta, while in whole-grain pasta declined to 34-52%. Therefore, drying at 90ºC for 30 min resulted in higher percentages of reduction than those obtained by drying at 70ºC for 4h. These results indicated that the effect of the temperature have a marked influence on the ENs levels detected in the final product. Taking into account data concerning complete process of drying, a significant decrease occurred in white pasta after the total process at high T drying (ENs dropped to 60-83% of their original content). Similar total reduction of ENs levels has been reported on whole–grain pasta (51-72%). ENA_1 and ENB were more stable than ENA and ENB_1. The treatment of white pasta at high T showed final concentrations of 0.21, 1.13, 2.81 and 1.18 mg Kg^{-1} of ENA, ENA_1, ENB and ENB_1, respectively. The treatment of whole-grain pasta at high T resulted to a final concentration of 0.36, 1.26, 3.46 and 1.73 mg Kg^{-1} of ENA, ENA_1, ENB and ENB_1, respectively. Considering the water content of final dry pasta (humidity of 12%), real ENs concentrations in white pasta were 0.23, 1.01, 2.50 and 1.04 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively, and ENs concentrations

in whole-grain pasta were 0.33, 1.10, 3.04 and 1.51 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively.

Table 2. Treatment at high temperature of drying: ENs concentration (mg Kg^{-1}) and percentages of ENs reduction after each step of processing (%) on white and whole-grain pasta. All results are expressed as dry weight basis.

Sample	**ENA**		**ENA_1**		**ENB**		**ENB_1**	
	Conc ± RSD (mg Kg^{-1})	% reduction	Conc ± RSD (mg Kg^{-1})	% reduction	Conc ± RSD (mg Kg^{-1})	% reduction	Conc± RSD (mg Kg^{-1})	% reduction
White pasta								
Fresh pasta	1.29±0.32	-	3.05±0.53	-	7.02±0.58	-	6.36±0.72	-
Pre-drying (30min,90°C)	0.72±0.11	44.4	2.38±0.77	21.9	5.40±0.70	23.1	2.95±0.56	53.6
Sweating (7h, 25°C)	0.72±0.16	0.0	2.33±0.56	2.1	5.29±0.55	2.0	2.94±0.63	0.4
Final drying (4h, 70°C)	0.21±0.08	70.7	1.13±0.42	51.5	2.81±0.42	46.9	1.18±0.23	59.9
Whole-grain pasta								
Fresh pasta	1.30±0.45	-	2.71±0.65	-	7.10±0.78	-	5.96±0.64	-
Pre-drying (30min,90°C)	0.75±0.24	42.1	2.02±0.69	25.4	5.59±0.77	21.3	3.13±0.45	47.4
Sweating (7h, 25°C)	0.74±0.18	1.2	1.98±0.65	2.0	5.23±0.61	6.4	3.09±0.49	1.4
Final drying (4h, 70°C)	0.36±0.16	51.9	1.26±0.60	36.4	3.46±0.33	33.7	1.73±0.66	44.1

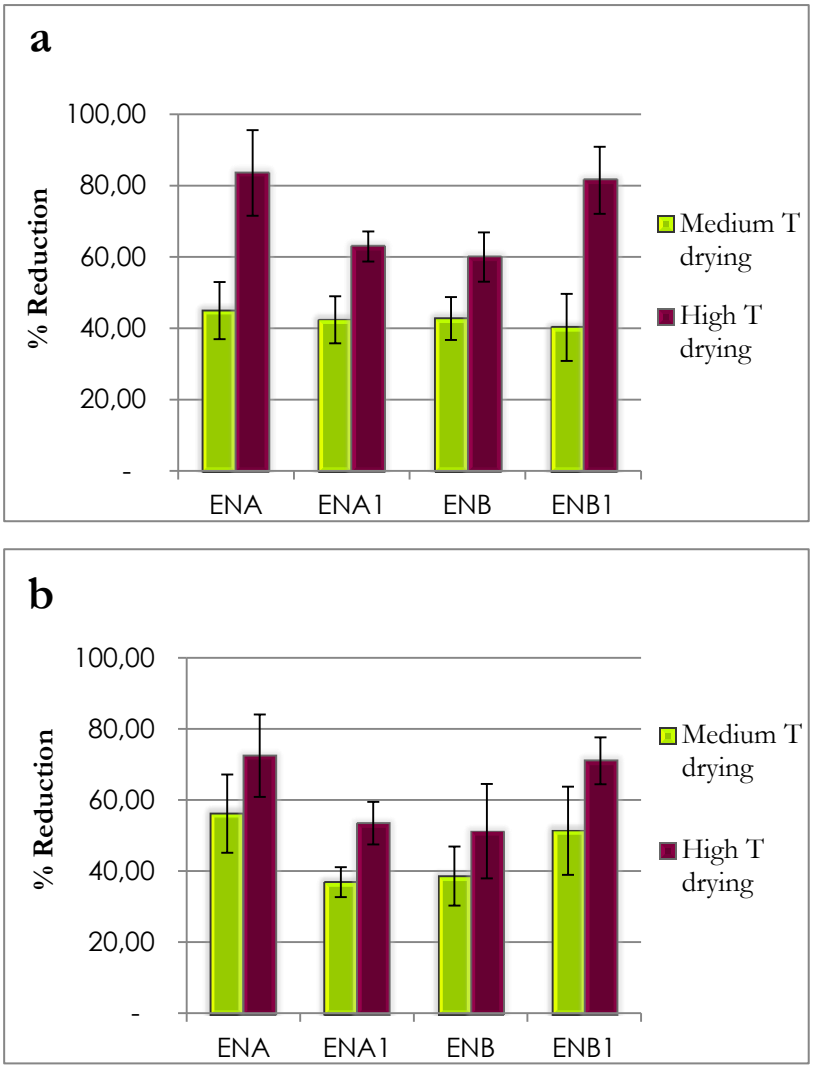

Fig. 3. Percentages of reduction after pasta processing at medium and high temperatures: **a** white pasta, **b** whole-grain pasta. Percentages were calculated on a dry weight basis.

3.4. Medium versus high temperature of drying

Figs. 3a and 3b show the percentages of total reduction considering the global pasta processing at medium and high T drying for white and whole-grain pasta. Regarding white pasta (Fig. 3a), reductions of 40-45% were reported under drying at medium T (45-55ºC), while drying at higher T (70-90ºC) produced the highest percentages of reduction (60-83%). According to the LSD test, significant differences on the ENs reduction were achieved with the high and medium drying ($p<0.000$). With respect to whole-grain pasta, drying at medium and high temperatures resulted in reductions of 31-56% and 51-72%, respectively. Drying treatments on whole-grain pasta resulted in similar reductions to those obtained on white pasta, with no significant differences ($p>0.05$) (Fig. 3). Therefore, it was concluded that the influence of the type of pasta did not affect to the ENs levels upon pasta processing. The same trend was observed in the study conducted by Meca *et al.*[29], who affirmed that no significant differences in the levels of BEA were found in the use of the different flour matrices during the bread-making process.

According to our results, Menesatti *et al.*[5] developed a method to distinguish between different parameters to spaghetti processing, and it was demonstrated that the production parameters such as drying temperature and the time of treatment deeply influenced the variation of the VIS NIR spectra far more than the kind of semolina (organic or conventional).

Although ENs are structurally similar compounds, each type of EN may present a different response depending on their intrinsic stability. On this account, the reduction effect related to the different treatments was studied individually for each mycotoxin. ENA and ENB_1 showed similar response to the different treatments with a comparable reduction levels. Medium T drying

process showed reductions of 40-50% compared with their original levels, while reductions of 70-80% were reported for high T drying. Therefore, the increase of drying temperature from 45-55ºC to 70-90ºC results in a clear reduction of ENA and ENB_1. On the other hand, ENA_1 and ENB were the most persistent toxins after pasta processing. Comparable reductions of ENA_1 and ENB were achieved under the treatments at medium T drying (40%) and high T drying (50-60%). These results demonstrated a major stability of ENA_1 and ENB with respect to ENA and ENB_1.

The decreasing levels of ENs could be due to the low thermal stability of these toxins. Recently, the review paper conducted by Berthiller *et al.*[30] reported that the reduction on mycotoxin levels during technological processes can be related to the transformation of these toxins to degradation products or to masked mycotoxins. These findings support the results obtained on the present study.

Although no studies are available in the literature on the effect of pasta processing on ENs, the results from the current study are comparable to those published for other food processes. Different technological processes such as bread and beer making have been performed to evaluate their influence on ENs stability. In this regard, Hu *et al.*[20] and Vaclavikova *et al.*[19] studied the fate of ENB and ENB_1 during bread-making. Baking step at 200ºC (25 min) and at 240ºC (14 min) showed reductions between 9% and 30% for ENs type B. Considering the complete process of breadmaking, the authors reported levels of 25-60% lower in bread than in the initial wheat flour. In the current study, reductions of 40-80% were obtained for ENB and ENB_1.

Moreover, the same authors investigated the influence of beer processing on ENs stability[19,21]. During malting step, reductions of 28-59% were reported

when the temperatures reached to 80ºC. During brewing process, the step involving thermal treatment (100ºC) showed a marked influence in decreasing ENs contamination. These results also were supported in our previous study, which showed great ENs reductions of 81-100% during boiling (100ºC) for 15 min in a model pasta resembling system[18]. It was explained that the thermal and biological degradation contributed significantly to decreasing ENs levels during these processes. The results are in compliance with those obtained in the present study that suggested reductions of 21-54% on ENs after the treatment at 90ºC.

4. CONCLUSIONS

The application of thermal treatments at different temperatures as well as the specific features of each technological process, gave different reductions on ENs contamination. The drying process at medium temperatures (45-55ºC) resulted in a ENs reduction of 40-45%, whereas the use of higher temperatures (70-90ºC) dropped to 51-83% the ENs levels. Although, the drying process at low temperature (25ºC) for 5 days, did not affect to ENs levels. No significant differences were observed depending the type of pasta. Therefore, the effect of the pasta composition did not evidence an effect on the stability of ENs. Although ENs levels were reduced, it would be important to consider the fate of masked mycotoxins as well as the relating new formed products from the thermal degradation for a reliable assessment of human exposure relating to contaminated pasta. In the present study it was evidenced the importance to select a suitable technological processes to minimize the final enniatins contents.

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3.10. Development a mitigation strategy of enniatins in pasta under home-cooking conditions

LWT- Food Science and Technology (Under review)

Development a mitigation strategy of enniatins in pasta under home-cooking conditions

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ABSTRACT

The effect of pasta cooking on the enniatins (ENs) contents was evaluated in white and whole-grain pasta. The cooking of pasta was performed for 10 min in boiling water. ENs were extracted from raw and cooked pasta by Solid-Liquid extraction with acetonitrile, and from cooking water by Dispersive Liquid-Liquid Micro-Extraction (DLLME) with a mixture of AcN and carbone tetrachloride. Mycotoxin analysis was performed by Liquid Chromatography coupled to Tandem Mass Spectrometry. The results indicated that the effect of pasta cooking have a marked influence on the reduction of ENs levels. Reductions of 98-100% for ENA, 94-95% for ENA_1, 14-49% for ENB and 53-65% for ENB_1 were achieved after the process. ENs were detected at low concentration levels in water samples. Moreover, it was developed a mitigation strategy to reduce the ENs contents by the combining effect of the thermal treatment and the pH during cooking pasta. The modification of the pH in the cooking water to pH 4 resulted in percentages of reduction close to 100%, while the modification to pH 9 provided percentages from 44 to 100%. Two degradation products were elucidated after the thermal treatment by Liquid Chromatography-Quadrupole-Linear Ion Trap-Mass Spectrometry.

Keywords: enniatins, cooking pasta, mitigation strategy, LC-MS/MS.

Chemical compounds studied in this article:

Enniatin A (PubChem CID: 16727691); Enniatin A_1 (PubChem CID: 16727692); Enniatin B (PubChem CID: 164754); Enniatin B_1 (PubChem CID: 11262300).

1. INTRODUCTION

Fusarium species produce a wide variety of mycotoxins, including trichothecenes, zearalenone, fumonisins and other mycotoxins called emerging *Fusarium* mycotoxins, that comprise the group of enniatins (ENs), beauvericin (BEA), fusaproliferin (FUS) and moniliformin (MON). It has been reported that emerging *Fusarium* mycotoxins occur in the field, mainly in cereals from different regions in the world and their products (Santini, Meca, Uhlig, & Ritieni, 2012). In particular, ENs are a group of structurally related metabolites receiving increasing interest in the last years. *In vitro* studies have evidenced that ENA, ENA_1, ENB and ENB_1 are enzyme inhibitors, oxidative stress inductors, immunotoxic and myelotoxic compounds (Ficheux, Sibiril, & Parent-Massin, 2012; Prosperini, Juan-García, Font, & Ruiz, 2013). Recently, the EFSA (European Food Safety Authority) Panel on Contaminants in the Food Chain (CONTAM) emitted a Scientific Opinion related to the risks to human and animal health associated to ENs and BEA, concluding that there is insufficient information for the risk characterisation. Moreover, the CONTAM Panel provided specific recommendations to improve the risk assessment to this group of mycotoxins. The recommendations included that further studies should be conducted on the fate of emerging mycotoxins during the preparation of grain-based products, specially focused in bread, pasta and fine bakery wares (EFSA CONTAM Panel, 2014). Furthermore, it could be interesting to develop effective methods and preventive measures to reduce the presence of ENs.

In the last years, several studies have been focused in the development of strategies to reduce mycotoxin levels during food and feed production. Different industrial processes such as cleaning, milling, extrusion,

pasteurization, baking, roasting, brewing, cooking and frying, have been proposed as the most effective practices to reduce mycotoxin contents. Legislated mycotoxins were the most studied mycotoxins, including trichothecenes, fumonisins, zearalenone, aflatoxins, ochratoxin A and patulin (Kabak, 2008; Milani & Maleki, 2014). However, limited data are available on the effects of food processing on ENs contents. Recent studies have evidenced that ENs levels are reduced through common industrial processes, such as bread-making, beer and pasta production (Serrano, Font, Mañes, & Ferrer, 2015a; Vaclavikova et al., 2013; Hu, Koehler, & Rychlik, 2014b). Furthermore, it must be considered that mycotoxin reduction can result in a chemical modification on the mycotoxin structure and in the formation of new degradation or transformation products. Moreover, mycotoxins are capable of binding to several components of the food matrix leading to conjugated new compounds or modified mycotoxins. In a previous research, different degradation products derived from ENA, ENA_1, ENB and ENB_1 were elucidated by Liquid Chromatography-Quadrupole-Linear Ion Trap-Mass Spectrometry (LC-Q-LIT-MS) in a model system simulating cooking pasta. The abundance of these degradation products seemed to be quite dependent on the ENs reduction levels (Serrano, Meca, Font, & Ferrer, 2013b).

According to the European Commission, efforts should be made to reduce the presence of *Fusarium* toxins from cereals and cereal products (Commission Recommendation, 2006). On this account, raw pasta is considered as a potential cereal product subject to ENs contamination and one of the main contributors to the chronic dietary exposure (EFSA CONTAM Panel, 2014). In this context, the aims of the present study were: a) to provide information on the fate of ENA, ENA_1, ENB and ENB_1 during the cooking

process of different types of pasta (white and whole-grain) and, b) to develop a mitigation strategy to reduce ENs levels during pasta cooking. To achieve these purposes, the influence of the pH on boiling water was investigated to evaluate the combined impact of the thermal treatment and the pH on the concentration and the chemical structure of ENs. LC-Q-LIT-MS was employed in order to obtain structural information of the potential new products derived from ENs degradation.

2. MATERIALS AND METHODS

2.1. Chemicals

Acetonitrile (AcN), methanol (MeOH), carbone tetrachloride (CCl_4) and sodium chloride (NaCl) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) and formic acid were supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Lemon juice and table salt were purchased from valencian supermarkets. Sodium carbonate was adquired from valencian pharmacy. Deionized water was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain) before use.

Individual standards of ENA, ENA_1, ENB and ENB_1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of ENA, ENA_1, ENB and ENB_1 at a concentration of 1000 μg mL^{-1} were prepared in methanol. These stock solutions were then diluted with pure methanol in order to obtain appropriate working solutions. All solutions were stored in glass-stoppered bottles and darkness in security conditions at -20ºC.

2.2. Preparation of pasta contaminated with enniatins

Two types of raw pasta were selected to develop the study: white and whole-grain pasta. Whole-grain pasta was selected due to the increasing demand of the consumer for natural and healthy food, and because of the highest mycotoxin-levels usually found in whole-grain foods (Serrano, Font, Mañes, & Ferrer, 2013a).

To ensure the homogeneity and reliability of the study, the samples of raw pasta were processed in the laboratory starting from durum wheat semolina previously contaminated by ENA, ENA_1, ENB and ENB_1. Semolina contamination was performed by inoculation experiments of a blank of commercial durum wheat semolina with a ENs producing strain of *Fusarium tricinctum* (CECT 20150, Spanish Type Culture, Valencia, Spain) (Meca et al., 2010). For fermentation experiments, 250 g of durum wheat semolina were inoculated with conidia suspensions of *Fusarium tricinctum* (concentration of 10^6 conidia mL^{-1} in PDB) and incubated during 42 days at 25°C on an orbital shaker (IKA Ks 260 basic, Stanfen, Germany). Then, the contaminated semolina was dried at 100ºC to reach 15% of moisture content (Batey, 2010) to promote fungi inactivation. Drying was performed in a pilot-scale EC25GE pasta dryier (Italgi, Genova, Italy) with a controlled relative humidity of 75%. In order to obtain semolina in concentration levels comparable to those achieved in real samples (Zinedine, Meca, Mañes, & Font, 2011), the contaminated semolina was mixed with commercial semolina to obtain the called "Working Semolina". "Working Semolina" to process white pasta was formulated by mixing one batch of contaminated semolina with a blank of commercial white semolina (1/10 w/w). "Working Semolina" to process whole-grain pasta was formulated by mixing other batch of contaminated

semolina with a blank of commercial whole-grain semolina (1/10 w/w). The two types of "Working Semolina" contained concentrations of ENs between 1 and 7 mg Kg^{-1}.

Pasta processing was conducted by mixing 500 g of "Working Semolina" with tap water (30%). The mixture was kneaded for 10 min to obtain a stiff and homogeneous dough. The dough was pressed several times until obtain the desired thickness (2.5 mm), and shaped through the die to obtain a spaghetti form (diameter of 2.1 ± 0.2 mm) in a pilot-scale pasta Micra extruder (Italgi, Genova, Italy). Finally, drying pasta was conducted as follows: the first step of drying allowed a rapid drying during 30 min at 90°C. To provide the appropiate stabilization of the product, a second period of sweating was performed during 7 h at 25ºC. Finally, pasta was dried at 70°C for 4 h to obtain raw pasta with a water content of 12.5% (Delcour & Hoseney, 2010). Drying was performed in a pilot-scale EC25GE pasta dryer (Italgi, Genova, Italy) with a controlled relative humidity of 80%. The homogeneity of the ENs levels in processed raw pasta was tested by analyzing three replicates of each type of pasta.

2.3. Pasta cooking experiments

The experiments to evaluate the ENs stability were conducted according to the common homemade process to pasta cooking (Riva, Mariotti, & Saccone, 2006). A schematic diagram of the cooking protocol is shown in figure 1a. Raw pasta (50 g) was cooked for 10 min in 500 mL of boiling tap water containing 5 g of table salt. This process was conducted in two types of pasta: white and whole-grain pasta. Samples of raw pasta, cooked pasta and the cooking water were collected separately and stored in a dark and dry place at -

20°C until mycotoxin analysis. The experiments were repeated in duplicate. ENs analysis was performed by three replicate trials for each sample.

2.4. Mitigation strategy

The mitigation strategy consisted in the study of the combining effect of the thermal treatment and the pH. In order to reduce the ENs levels from cooked pasta ready for consumption, it was developed a new protocol to pasta cooking based on the pH modification in the cooking water. Lemon juice was selected to obtain water in acid medium (pH 4). Sodium carbonate was employed to achieve the basic medium (pH 9). These two substances are non-toxic, stable, water-soluble and easily obtained by the population. Therefore, the experiments could be adapted to the homemade cooking processes to establish future recommendations.

The experiments were performed according to the diagram represented in figure 1b. Water at pH 4 was prepared by addition of 15 mL of lemon juice to 500 mL of tap water under continuous stirring. Water at pH 9 was prepared by addition of 20 g of sodium carbonate to 500 mL of deionized water under continuous stirring. Measurements of pH were performed employing a GLP21 Crison pH-meter (Crison Instruments, S.A., Barcelona, Spain) with a Hamilton pH electrode (Fisher Scientific, Madrid, Spain). Then, cooking of pasta was conducted according to the procedure described in the section 2.3.

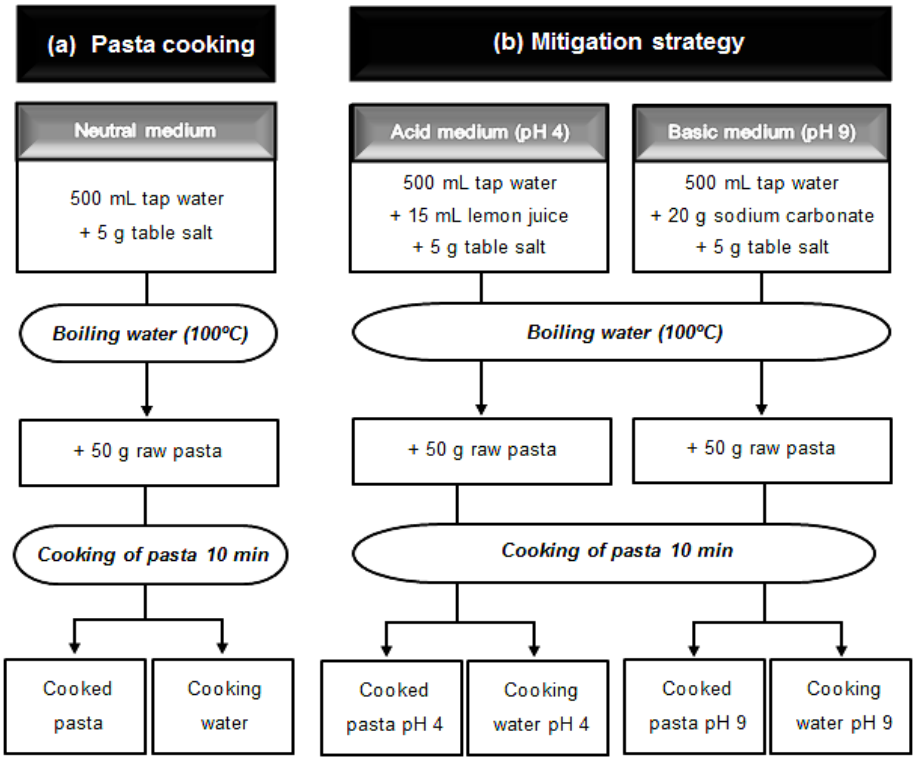

Figure 1. (a) Pasta cooking protocol, (b) mitigation strategy to reduce ENs levels during pasta cooking at acid and basic pH.

2.5. Method of extraction

2.5.1. Enniatins extraction from pasta

Samples of pasta (5 g) were extracted with 50 mL of AcN using a Ika T18 basic Ultra-Turrax (Staufen, Germany) during 3 min. After, centrifugation for 15 min at 3500 g and 5ºC, the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Postfach, Switzerland). The extract was dissolved with 5 mL of AcN, and was evaporated to dryness by nitrogen gas at 35ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After

solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and was filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) prior the injection in the LC-MS/MS system.

2.5.2. Enniatins extraction from cooking water

The extraction from cooking water was performed by the use of the dispersive liquid-liquid microextraction (DLLME). A mixture of 900 μL of AcN (as disperser solvent) and 100 μL of CCl_4 (as extraction solvent), was rapidly injected into 5 mL of cooking water containing 1 g of table salt. The mixure was shaken on a Vortex for 1 min. Then, the mixture was centrifugated for 15 min at 3500 g (10ºC), and the droplet formed was collected by a 100 μL syringe and it was evaporated to dryness by nitrogen gas at 35ºC using a multi-sample Turbovap LV Evaporator. Then, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and filtered through 13 mm/0.20 μm nylon filter prior the injection in the LC-MS/MS system.

2.6. LC-MS/MS

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 2 mm I.D., 3 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using gradient elution that

started at 90% of A (AcN) and 10% of B (10 mM ammonium formate in MeOH), increased linearly to 50% B in 10 min. After, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 mL min^{-1}. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0,5 V; source temperature, 100ºC; desolvation temperature, 300ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L h^{-1}; cone gas 50 L h^{-1} (nitrogen 99.99% purity).

The cone voltage selected was 40 V and the collision energy selected was 35 Ev for all ENs. 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 99.995% purity) pressure, 3.83 x 10^{-3} mbar; interchanel 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. According with the 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 follow precursor ion and product ions were selected for each mycotoxin: the precursor ion m/z 681.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA, the precursor ion m/z 667.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA_1, the precursor ion m/z 639.8 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB, the precursor ion m/z 654.9 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB_1.

2.7. LC-Q-LIT-MS

An Applied Biosystems/MDS SCIEX Q TRAP TM Linear Ion Trap mass spectrometer (Q-LIT-MS) (Concord, Ontario, Canada), coupled with a Turbo Ion Spray source, was used to the elucidation of the ENs degradation products. This instrument is based on a triple-quadrupole path (QqQ) in which the third quadrupole can also be operated as a LIT with improved performance. In the Q-LIT-MS configuration, the Q TRAP TM can also operate in Enhanced Resolution (ER) and in enhanced product ion (EPI) scan modes. Applied Biosystem/MDS SCIEX Analyst software version 1.3.2 was used for data acquisition and processing. A Gemini (150 x 2.0 mm, 5 μm) Phenomenex column was used for separation. The mobile phase (AcN/water 70/30 v/v with 0.1 % of formic acid) was delivered in an isocratic manner at a constant flow rate of 0.3 mL min^{-1}. The MS was operated in the positive ion electrospray mode using the following parameters: cone voltage 40 V, capillary voltage 3.80 kV, source temperature 350ºC, desolvation temperature 270ºC and collision gas energy 5 eV. The analyses were carried out using: 1) the ER mode for newly formed compounds in the mass range from 200 to 800 Da; 2) the EPI mode to obtain a MS^2 scan of a fragment of the adducts.

2.8. Statistical Analysis

The experiments were carried out in triplicates. Statistical analysis was conducted by one-way analysis of variance (ANOVA) and by least significance difference (LSD) test (assuming equal variance) to evaluate the significant variations ($p<0.05$), using Statgraphics X64 statistical software.

3. RESULTS AND DISCUSSION

3.1. Method performance

Method validation was performed according to the guidelines established by the European Union (Commission Decision, 2002; Commission Regulation, 2006). The validation included the determination of linearity, the evaluation of the matrix effect (ME), limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision).

The method validation to determine ENs in the samples of raw pasta was reported previously by Serrano et al. (2013c). The validation assays were implemented in this study to cooked pasta and the method validation was updated with aceptable results (Table 1). A modified DLLME procedure optimized previously in our laboratoy was selected to determine ENs in the samples of water (Serrano, Font, Mañes, & Ferrer, 2015b). The validation parameters are reported in Table 1.

Table 1. Analytical parameters for determination of ENs in cooked pasta and cooking water: correlation coefficient, matrix effect (%), limits of detection and quantification (μg Kg^{-1}), recoveries (%) and relative standard deviations (%).

Type of sample	Mycotoxin	Matrix effect (%)[a]	LOD (μg Kg^{-1})[b]	LOQ (μg Kg^{-1})[b]	Intra-day precision[c] Recovery ± RSD (%)		Inter-day precision[d] Recovery ± RSD (%)	
					Low level (10xLOQ)	High level (100xLOQ)	Low level (10xLOQ)	High level (100xLOQ)
Cooked pasta	ENA	80	0.21	0.70	89.2 ± 9.3	92.3 ± 3.9	90.2 ± 4.2	88.1 ± 10.8
	ENA_1	76	0.29	0.96	85.5 ± 4.0	89.4 ± 8.7	92.2 ± 9.8	97.3 ± 5.5
	ENB	65	0.52	1.73	94.7 ± 5.3	96.3 ± 3.0	87.1 ± 6.7	93.9 ± 10.0
	ENB_1	69	0.51	1.70	88.2 ± 3.7	90.0± 8.4	84.6 ± 7.4	87.8 ± 8.2
Cooking water	ENA	104	0.06	0.20	79.4 ± 7.2	75.4 ± 7.3	87.6 ± 5.1	79.9 ± 14.1
	ENA_1	101	0.13	0.44	79.6 ± 10.6	81.0 ± 7.8	86.8 ± 9.6	82.5 ± 8.9
	ENB	96	0.17	0.58	68.5 ± 13.0	81.9 ± 5.7	86.8 ± 5.9	83.5 ± 6.3
	ENB_1	98	0.09	0.30	71.6 ± 10.6	75.2 ± 6.8	84.4 ± 6.7	85.3 ± 9.3

[a] Matrix effect (%): 100 x (slope of matrix matched water/slope of standard in solvent)
[b] LOD and LOQ in μg L^{-1} for samples of cooking water
[c] Number of replicates: 5
[d] Different days: 5

3.2. Fate of enniatins during cooking pasta

The ENs concentrations in raw and cooked pasta (white and whole-grain) are reported in Table 2. Levels in raw pasta were considered to be the initial concentrations. Results evidenced that the concentration levels of ENs in cooked pasta were lower than those detected in raw pasta. The final

concentrations of ENs in cooked pasta were below 0.6 mg Kg^{-1}, with the exception of the ENB, that was of 1.23-1.69 mg Kg^{-1}.

Figure 2 shows the percentages of reduction for each mycotoxin after the cooking process. In order to improve the discussion of the results, the percentages of ENs reduction were calculated over a dry weight basis for raw and cooked pasta. The conversion to dry values was performed through the moisture determination of each kind of pasta. In general, different percentages of reduction were obtained after the cooking process depending on the type of EN. The most significant decrease was evidenced in the levels of ENs type A (reduction rate close to 100%). Reductions of 53-65% were achieved for the ENB_1. ENB was the most stable mycotoxin with percentages of reduction of 14-49%.

Concerning the results depending on the type of pasta, significant differences were obtained for ENB and ENB_1 with higher reduction rates in whole-grain pasta than those obtained in white pasta ($p=0.001$). However, no significant differences were obtained for ENA and ENA_1 in both types of pasta ($p>0.05$).

The results obtained in the current study are in agreement with previous studies suggesting that food processing may reduce the risk of ENs contamination in the final product ready for consumption. In a recent study, the levels of ENA, ENA_1, ENB and ENB_1 were studied during the different steps of pasta manufacturing: the steps involving temperatures of 45-90ºC resulted in percentages of reduction from 40% to 90% (Serrano, Font, Mañes, & Ferrer, 2015a). In other studies relating the stability of ENB and ENB_1 during beer production (from barley to beer), high reductions close to 100% were also evidenced (Vaclavikova et al., 2013; Hu et al., 2014a). Recently, two

studies discussed the effect of bread-making on the levels of ENs type B. The baking step at 240ºC for 14min resulted in reductions of 50-60% according to Vaclavikova et al. (2013). Hu et al. (2014b) reported reductions of 25-41% in similar conditions of baking. In contrast to the results obtained in the present study, the study conducted by Nijs et al. (2013) evidenced that cooking of pasta contaminated at low ENs concentrations did not affect to ENs stability. Therefore, it is important to consider that the behaviour of ENs is dependent on the type of sample, the initial concentration of ENs and the specific conditions achieved in the developed studies.

With the purpose to determine if the ENs reduction is related to the leaching of ENs contents from pasta into the cooking water, the samples of water were analyzed after the cooking process. Results relating the levels of ENs in the cooking water are reported in Table 2. Low concentration levels were detected in the samples of water, ranging from no detectable to 0.05 mg L^{-1}. These results evidenced a very low transfer of ENs from pasta into cooking water. Therefore, the high percentages of reduction reached after pasta cooking cannot be attributed to the leaching of ENs into the cooking water.

Data on the effects of cooking pasta on the distribution of other *Fusarium* toxins has been widely described in the last years. In several studies involving the fate of deoxynivalenol (DON) during pasta cooking, the authors concluded that an important reduction up to 75% on DON levels occurred during cooking with a significant transfer of DON from raw pasta into the cooking water (Visconti, Haidukowski, Pascale, & Silvestri, 2004; Cano-Sancho, Sanchis, Ramos, & Marín, 2013). Other studies also demonstated that the cooking process with larger amounts of water is a suitable tool to reduce DON

contents from different cereal products (pasta, noodles or rice) and consequently, to reduce the risk for the consumer (Kushiro, 2008). However, it was not possible to perform the same reasoning for ENs, which behaved differently to DON during cooking pasta. A possible explanation to these findings is that DON is a mycotoxin with higher water-solubility than ENs. Other explanation could be that ENs were degraded during the treatments, resulting in the formation of new degradation products. On this account, analysis of ENs degradation products was performed in the samples of cooking water (see section 3.4.).

Table 2. Concentration levels of ENA, ENA_1, ENB and ENB_1 in raw pasta, cooked pasta (over a wet weight) and cooking water, after the cooking treatment and after the mitigation strategy of pasta cooking at pH 4 and pH 9.

Type of pasta	Mycotoxin	Concentration ± RSD (mg Kg^{-1})						
		Raw pasta	Pasta cooking		Mitigation strategy pH 4		Mitigation strategy pH 9	
			Cooked pasta	Cooking water	Cooked pasta	Cooking water	Cooked pasta	Cooking water
White pasta	**ENA**	**0.21±0.10**	0.06±0.11	nd	nd	nd	0.03±0.05	nd
	ENA_1	**1.13±0.34**	0.04±0.02	0.05±0.03	nd	0.08±0.03	0.31±0.11	0.05±0.04
	ENB	**2.81±0.66**	1.69±0.29	<LOQ	nd	0.05±0.04	1.10±0.30	< LOQ
	ENB_1	**1.18±0.26**	0.29±0.18	0.01±0.02	nd	0.12±0.05	0.27±0.13	0.03±0.01
Whole-grain pasta	**ENA**	**0.36±0.15**	nd	nd	0.02±0.03	nd	nd	nd
	ENA_1	**1.26±0.32**	0.05±0.06	0.01±0.02	0.12±0.14	0.06±0.04	0.03±0.10	0.02±0.01
	ENB	**3.46±0.53**	1.23±0.36	< LOQ	0.18±0.25	0.02±0.01	0.01±0.01	< LOQ
	ENB_1	**1.73±0.33**	0.57±0.13	0.03±0.02	0.02±0.05	0.08±0.03	0.43±0.14	< LOQ

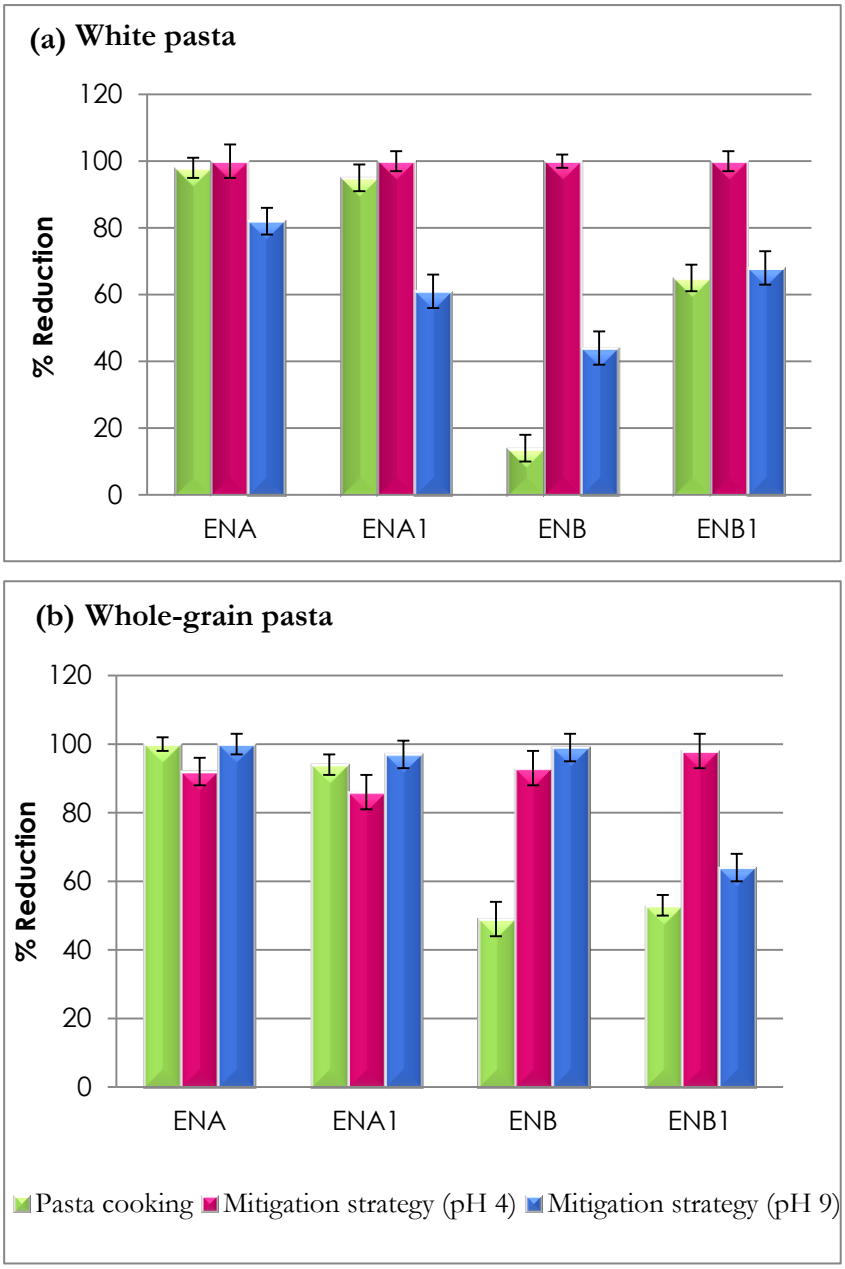

Figure 2. Percentages of ENs reduction after cooking and after the mitigation strategy at pH 4 and pH 9, in: (a) white pasta, (b) whole-grain pasta.

3.3. Mitigation strategy

The mitigation strategy to reduce ENs levels during cooking of pasta was based on the pH modification (pH 4 or pH 9) in the cooking water prior to the treatment. Results are showed in the Table 2 and Figure 2. In all cases, high reductions on ENs levels were achieved after the cooking process ($p<0.05$).

The mitigation strategy at pH 4 resulted in the highest reductions (86-100%). All mycotoxins were behaved in a similar way. Relating the results depending on the type of pasta, no detectable levels of ENs were achieved in white pasta after cooking, while final levels in whole-grain pasta were of 0.02-0.18 mg Kg^{-1}. Low concentration levels were detected in cooking water (nd-0.12 mg L^{-1}).

With respect to the mitigation strategy at pH 9, it should be noted that reductions of 44-100% were achieved after the process. ENB_1 behaved similarly ($p>0.05$) in both types of pasta (percentages of reduction of 64-68%). However, significant differences ($p<0.05$) were obtained in the results of white and whole-grain pasta for ENA, ENA_1 and ENB. Levels dropped to 44-82% of its original levels in white pasta, while the levels in whole-grain pasta resulted in reductions of 97-100%. Final levels in cooked pasta ranged from 0.03 to 1.10 mg Kg^{-1} for white pasta, and from no detectable to 0.43 mg Kg^{-1} for whole-grain pasta. Levels in cooking water ranged between no detectable and 0.05 mg L^{-1}.

In summary, the levels of all ENs were reduced significantly with the treatment at pH 4. The effect of the pH 9 also resulted in high reductions, but only in some cases depending on the mycotoxin and the type of pasta.

In a previous study conducted by Serrano et al. (2013b), the thermal stability of ENs was evaluated in a model system simulating the cooking of pasta under similar conditions to those applied in the present study (cooking

water at pH 4, 7 and 9). Percentages of ENs reduction were close to 100% after 10 min of cooking, without differences in the results depending on the type of pH. According to the results obtained in the present study, the percentages of reduction evidenced in the samples of pasta were lower than those obtained in the study employing the model system. The same trend was observed in a preceding study conducted by Meca et al. (2012), which found degradation levels of BEA in crispy breads lower than those obtained in the model solution after the bread-making process. These authors indicated that a possible explanation to this fact is probably that food matrices present a protective effect toward the mycotoxins during the thermal treatment.

3.4. Degradation products

To obtain structural information on the ENs during the thermal treatment, samples of raw pasta, cooked pasta and cooking water were extracted according to the methodology described in the section 2.5, and analysed by LC-Q-LIT-MS in the ER scan modality (m/z 200-900). A total of two new compounds were elucidated after pasta cooking (Figures 3 and 4). The same compounds were elucidated after the mitigation strategy at basic and acid pH. These compounds were detected in the samples of cooked pasta and cooking water, but not in the samples of raw pasta. Therefore, the two elucidated compounds were probably formed as a result of the thermal treatment.

Figure 3 shows the LC-MS-LIT chromatograms of a sample of uncooked white pasta, the related cooked pasta and the cooking water after the mitigation strategy at basic pH. As it can be seen, the sample of uncooked pasta was initially contaminated by the four ENs (figure 3a). However, the

chromatogram related to the sample of cooked pasta shows four peaks corresponding to ENB, ENB_1, ENA_1 and ENA, and two new peaks corresponding to two different compounds (figure 3b). The chromatogram of the sample of cooking water (figure 3c) shows two peaks at the same retention time (t_R) to those detected in cooked pasta (t_R=17.7 min and t_R=19.6 min). In accordance to the results explained above, the four ENs were not detected in the cooking water chromatogram.

The two new peaks detected in cooked pasta and cooking water were elucidated as two ENA-related chemicals derived from the ENA degradation. Figure 4a shows the proposed structure and the LC-Q-MS-LIT spectra of the first compound (t_R = 17.7 min) called "degradation product 1". The degradation product 1 (m/z 577.7) was elucidated as the potassium adduct of ENA with the loss of the structural amino acid isoleucine (Ile). In figure 4b is reported the LC-Q-MS-LIT spectra of the second compound (t_R = 19.6 min) called "degradation product 2" and their potential structure. The degradation product 2 (m/z 640.2) was elucidated as the potassium adduct of ENA with the loss of the structural fragment of 2-hydroxi-3-methyl butanoic acid (HyLv) and the protonation of the molecul. The presence of the protonated molecule was also identified in the spectrum by the signal with m/z 601.5.

The profile of the elucidated compounds was in accordance with the degradation products characterised by Serrano et al. (2013b), which developed a preceding study involving the behaviour of each isolated EN in a model system simulating cooking of pasta.

Given the results obtained in the present study, the high decreasing levels of ENA after the cooking treatment could be attributed to the transformation of ENA to degradation products.

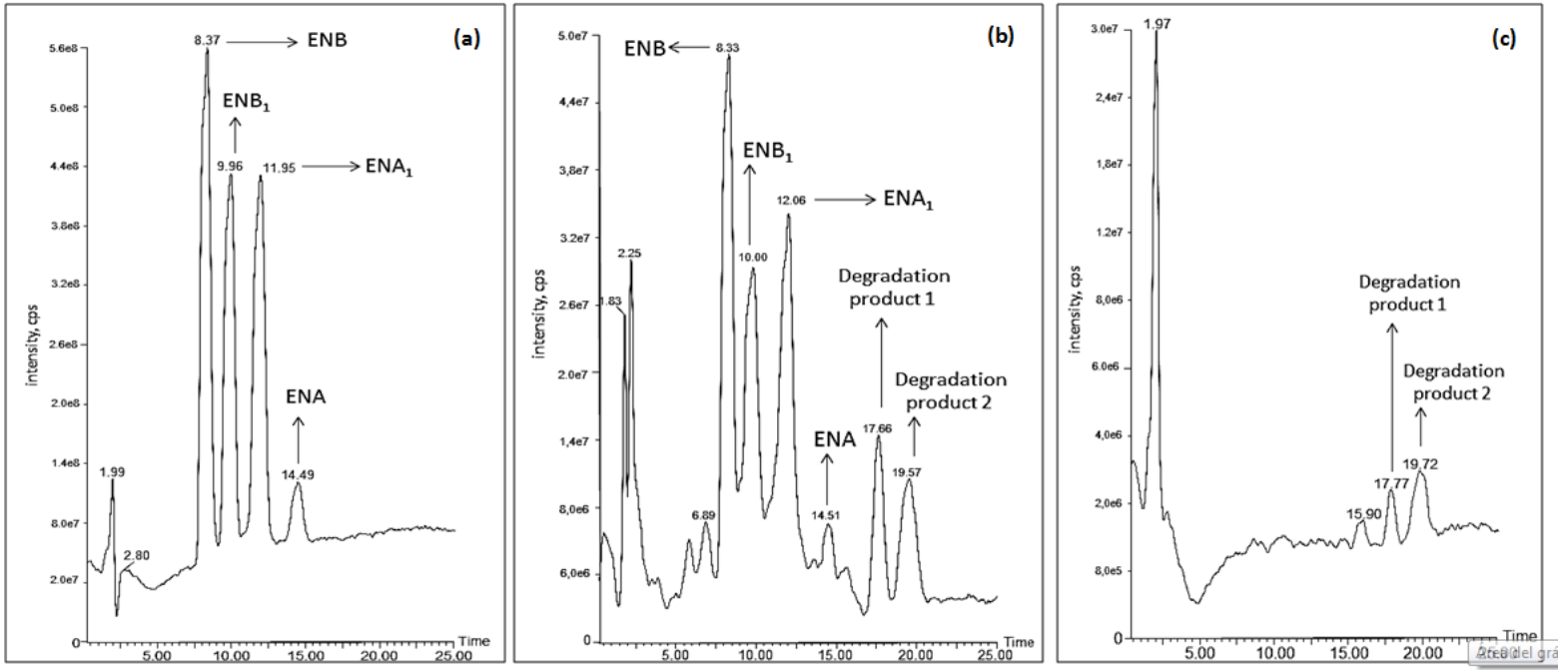

Figure 3. LC-Q-MS-LIT chromatogram in ER scan modality (m/z 200-900) of: (a) uncooked white pasta, (b) cooked white pasta after the mitigation strategy at basic pH, and (c) cooking water after the mitigation strategy at basic pH.

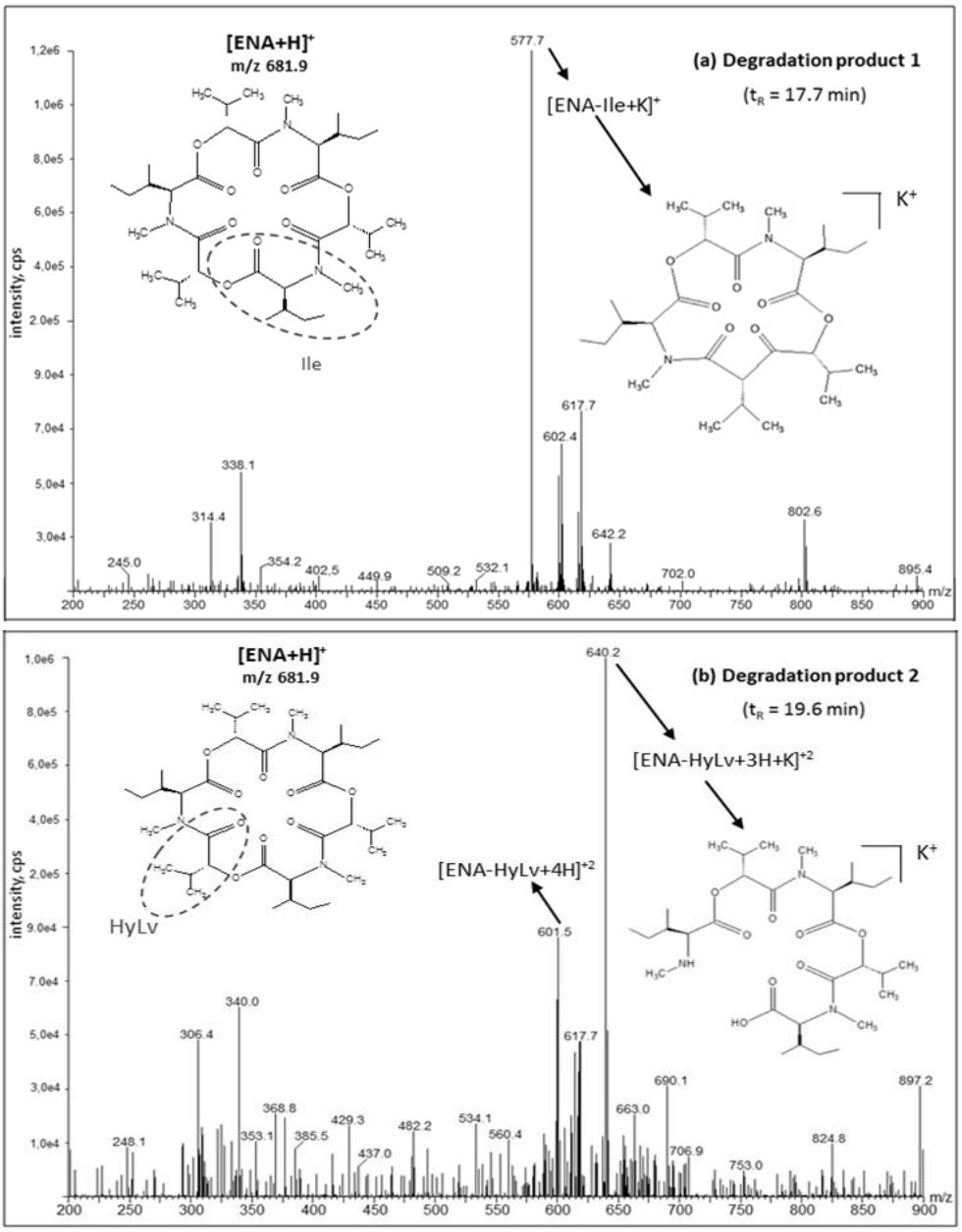

Figure 4. Proposed structure and LC-Q-MS-LIT spectra of the degradation products elucidated in pasta and water after cooking of pasta: (a) degradation product 1 [ENA-Ile+K]$^{+}$, (b) degradation product 2 [ENA-HyLv+3H+K]$^{+2}$.

4. CONCLUDING REMARKS

ENs levels were reduced after the cooking of pasta from 14% to 100%. Percentages of reduction were dependent on the type of EN. Levels of ENs in cooked pasta were from not detectable to 1.69 mg Kg^{-1}. A mitigation strategy has been developed to reduce the ENs contents during pasta cooking by the modification of the pH. The results showed that the combining effect of the thermal treatment and the acid pH offered reductions close to 100% on ENs levels. Therefore, the mitigation strategy at acid pH was proposed as an easy and effective protocol to reduce ENs levels in pasta during homemade pasta cooking. Moreover, two degradation products derived from ENA were tentatively elucidated in pasta and water after the cooking process. The transformation of ENs to degradation products could be a good mechanism of mycotoxin depletion. Future toxicological studies should be developed to determine the toxicity of these new compounds.

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3.11. Evolution of enniatins levels and Maillard Reaction in pasta during one year of storage

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Evolution of enniatins levels and Maillard Reaction in pasta during one year of storage

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ABSTRACT

The influence of the temperature and storage time on the enniatins (ENs) contents was evaluated in different kinds of pasta. White, whole-grain and egg pasta contaminated by ENs were stored over one year at two storage temperatures (4ºC and 25ºC). The concentration levels of ENs A, A_1, B and B_1 were analyzed once a month by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS QqQ). Moreover, water activity and the color parameters a*, b* and L*, were also measured once a month. The ENs contents decreased after one-year storage, without significant differences in the type of pasta. Concentrations of ENs type A showed a strong trend of decline with the increasing time of storage, whereas this trend was less evident for ENs type B. Considering the sum of four ENs, the achieved reductions were statistically significant after the fifth month. According to the multiple regression analysis, the reductions were dependent of the storage time following a linear model. The storage temperature did not show any effect on the ENs stability. It was observed a browning in the samples dependent on the time of storage. There was obtained a significant negative correlation between ENs concentration and browning, indicating a potential contribution of ENs in the Maillard Reaction during storage, which could be explain the decreasing levels of ENs.

Keywords: Enniatins, storage, browning, Maillard Reaction, pasta, LC-MS/MS.

1. INTRODUCTION

Mycotoxins are compounds that can cause a wide range of diseases in humans and animals. Enniatins (ENs) are a group of mycotoxins produced by several species of *Fusarium* genera on agricultural commodities worldwide. The major sources of dietary intake of *Fusarium* toxins are products made from cereals, in particular wheat and maize. The growing of *Fusarium* fungi and mycotoxin production often appears to be dependent of different factors, such as water activity (a_w), temperature or pH (Magan and Aldred, 2007).

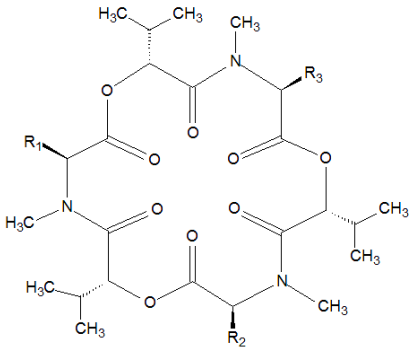
Different studies have described the toxicological effects posed by ENs, including myelotoxicity, immunotoxicity and enzyme inhibition in several cell lines (Escrivá et al., 2015). Governments have general concerns on the harmful effects of mycotoxins on the health of humans and animals. In the last years, different guidelines and legislative limits have been established for the most studied mycotoxins in different commodities, feeds and foodstuffs, but any regulation has been set for ENs (Commission Regulation, 2006). According to the recent Scientific Opinion of the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain, there is still limited information to perform a reliable human risk assessment related to ENs. The Scientific Opinion comprised different recommendations to improve the risk characterization, including that further studies should be performed on their toxicity, mode of action, presence in food and feed, and on the fate of these mycotoxins during the preparation of grain-based products (EFSA, 2014). To achieve the last purpose, it would be of great interest to study the occurrence of ENs during the agricultural practices, harvest logistics, transport, storage and food processing (Milani and Maleki, 2014; Oliveira et al., 2014). To the moment, limited number of studies has been focused on how ENs behaved

from the field to the consumer. These studies have demonstrated that ENs are capable to persist to several food industrial processes such as bread-making or wheat pasta production (Vaclavikova et al.,2013; Serrano et al., 2015). Only the study conducted by Sørensen et al. (2008) has been focused on the fate of ENs during the storage. In this study, the presence of ENB in whole maize silages was monitored over a period of 11 months. A significant reduction on the ENB levels was observed during the first 3 months of storage and then, the levels remained constant.

It is known that long times of cereal storage can be lead to an increment in moisture condensation and then, to local increases in a_w content (Kolmanic et al., 2010). The recommended moisture content for safe storage of cereals is lower than 13%, which corresponds to a a_w lower than 0.7. According to several authors, a_w values higher than 0.7 could activate microbiological growth of *Fusarium* species and subsequent production of mycotoxins (Gnonlonfin et al., 2008; Mohale et al., 2013).

On the other hand, it is known that different reactions can take place in foodstuffs during long storage periods. Maillard Reaction (MR) has an important role in the nutritional quality and safety of cereal products. The progress of the reaction is directly influenced by many factors such as temperature, a_w and the components of the food system (Acquistucci, 2000). The chemical reactions involved in the MR and the related products (MR products), are responsible of undesired effects on foodstuffs, such as color alterations from non-enzymatic browning, and a reduction in the nutritive value (Capuano et al., 2009; De Pilli et al., 2014). The MR products are a result of non-enzymatic reactions between free amino groups and the carbonyl groups of reducing sugars. On this account, ENs are compounds with a

potential structure to participate in the MR, with an structure formed by a cyclic hexadepsipeptide with three alternating amino acid residues (Figure 1).



	R_1	R_2	R_3
ENA	*sec*-butyl	*sec*-butyl	*sec*-butyl
ENA$_1$	*sec*-butyl	*sec*-butyl	*iso*-propyl
ENB	*iso*-propyl	*iso*-propyl	*iso*-propyl
ENB$_1$	*iso*-propyl	*iso*-propyl	*sec*-butyl

Fig. 1. Chemical structures of enniatins A, A_1, B and B_1 (ENA, ENA_1, ENB and ENB_1, respectively).

In the last years, different studies have been developed in order to advise suitable indicators between the MR and the quality of pasta. The most common determination to achieve this purpose is the evaluation of the colorimetric indices, particularly of red index (a*) and luminosity index (L*), that are strictly linked to the development of the MR (Cavazza et al., 2013).

Wheat pasta constitutes one of the most important cereal products consumed in the world and it is considered one of the main sources of ENs in the human diet (EFSA, 2014). To the authors' knowledge, there is a lack of

information on the fate of ENs during the shelf-life period of foodstuffs after food processing. Moreover, the relationship between the presence of ENs in pasta and their interaction in the MR is a relevant feature that has not been addressed in literature so far.

In this context, the present study was aimed to report the evolution of the ENs levels, water activity and color development in pasta during one year of storage at two temperatures (4ºC and 25ºC). The study has been focused on different types of pasta that represent a significant part of the daily human diet, including white, whole-grain and egg pasta.

2. MATERIALS AND METHODS

2.1. Chemicals

Acetonitrile (AcN) and methanol (MeOH) were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Deionized water was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were filtered through a 0.45 μm HV filter provided by Scharlau (Barcelona, Spain) before use.

Individual standards of ENA, ENA_1, ENB and ENB_1 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of ENA, ENA_1, ENB and ENB_1 at a concentration of 1000 μg mL^{-1} were prepared in methanol. These stock solutions were then diluted with pure methanol in order to obtain appropriate working solutions. All solutions were stored in glass-stoppered bottles and darkness in security conditions at -20ºC.

2.2. Preparation of pasta contaminated by enniatins

Three types of pasta were selected to develop the experiments: white pasta, whole-grain pasta and egg-pasta (containing 20% of egg).

To ensure the homogeneity and reliability of the study, the samples of raw pasta were processed in the laboratory starting from durum wheat semolina previously contaminated by ENA, ENA_1, ENB and ENB_1. Semolina contamination was performed by inoculation experiments of a blank of commercial durum wheat semolina with a ENs producing strain of *Fusarium tricinctum* (CECT 20150, Spanish Type Culture, Valencia, Spain) (Meca et al., 2010). For fermentation experiments, 750 g of durum wheat semolina were inoculated with conidia suspensions of *Fusarium tricinctum* (concentration of 10^6 conidia mL^{-1} in PDB) and incubated during 42 days at 25°C on an orbital shaker (IKA Ks 260 basic, Stanfen, Germany). Then, the contaminated semolina was dried at 100ºC to reach 15% of moisture content (Batey, 2010) to promote fungi inactivation. Drying was performed in a pilot-scale EC25GE pasta dryier (Italgi, Genova, Italy) with a controlled relative humidity of 75%. In order to achieve concentration levels in semolina comparable to those obtained in real samples, it was formulated the called "Working Semolina", by mixing contaminated semolina with a blank of commercial semolina (1/10 w/w). Commercial white semolina was used for processing white pasta and egg-pasta, and commercial whole-grain semolina was used for processing whole-grain pasta. Final levels of ENs in Working Semolina were of 1-7 mg Kg^{-1}. Pasta processing was conducted according to the protocol described by Serrano et al. (2015).

2.3. Packaging and storage conditions

The storage period started just after pasta production (time 0). Storage was conducted for 12 months at two temperatures: 4°C and 25ºC. To storage purposes, 50 g of pasta were packaged into transparent Polypropylene-PP pouches. Each pouch was hermetically sealed in order to simulate the conditions of commercial pasta in supermarkets and households. A total of 72 pouches of pasta were packaged: 24 pouches of white pasta, 24 pouches of whole-grain pasta and 24 pouches of egg-pasta. For each kind of pasta, 12 pouches were palce in a storage chamber at a constant temperature of 25 ± 2ºC, and 12 pasta pouches were placed in a storage chamber at 4 ± 2ºC.

Water activity, color parameters (L*, a*, b* and ΔE*) and ENs contents were determined at time 0 (just after pasta manufacture) and then, at regular time intervals of one month over a one year storage period. Samples were analysed in triplicate at each storage time.

2.4. Water Activity Determination

A Portable Water Activity Meter Pawkit (AquaLab Decagon Devices Inc., WA, USA) with accuracy of ± 0.02 a_w and a wide operating range (0- 1.0 a_w), was used to determine a_w in the samples of pasta. Measurements were done at 25 ± 2°C. According to the manufacturer´s instructions, the device was calibrated with standards of saturated saline solutions of LiCl (a_w = 0.26 ±0.02) and NaCl (a_w = 0.77 ± 0.02), within the range of a_w expected for pasta samples.

2.5. Color Determination

Color was measured using a Konica Minolta CM-3500 spectrocolorimeter (Valencia, Spain). The results were expressed according to the CIELAB system

with reference to illuminant D65 and a visual angle of 10º. The measurements were made with an 8 mm diameter diaphragm inset with optical glass. The parameters determined were L* (luminosity or brightness: L*=0 black, and L*=100 white), a* (red-green component: a*<0 greenness, and a*>0 redness), and b* (yelow-blue component: b*<0 blueness, and b*>0 yellowness). To evaluate color changes between the samples, it was calculated the total color difference (ΔE*) as follows: $\Delta E^* = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$ (Francis & Clydesdale, 1975).

2.6. Determination of mycotoxins

2.6.1. Extraction method

The extraction method was previously reported by Serrano et al. (2013). Samples of pasta (5 g) were extracted with 50 mL of AcN using a Ika T18 basic Ultra-Turrax (Staufen, Germany) during 3 min. After, centrifugation for 15 min at 3554 g and 5ºC, the supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Flawil, Switzerland). The extract was dissolved with 5 mL of AcN, and was evaporated to dryness by nitrogen gas at 35ºC using a multi-sample Turbovap LV Evaporator (Zymark, Hoptikinton, USA). After solvent evaporation, the extract was reconstituted with 1000 μL of AcN/MeOH (50/50 v/v), and was filtered through 13 mm/0.20 μm nylon filter (Membrane Solutions, Texas, USA) prior the injection in the LC-MS/MS system.

2.6.2. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, a

pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software version 4.1 were used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 2 mm I.D., 3 μm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a security guard cartridge C_{18} (4 mm x 2 mm I.D.), using gradient elution that started at 90% of A (AcN) and 10% of B (10 mM ammonium formate in MeOH), increased linearly to 50% B in 10 min. After, it was decreased linearly to 10% of B in 3 min. Afterwards, the initial conditions were maintained for 2 min. Flow rate was maintained at 0.2 mL min^{-1}. The analysis was performed in positive ion mode. The electrospray ionisation source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens 0,5 V; source temperature, 100ºC; desolvation temperature, 300ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L h^{-1}; cone gas 50 L h^{-1} (nitrogen 99.99% purity).

The cone voltage selected was 40 V and the collision energy selected was 35 Ev for all ENs. 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 99.995% purity) pressure, 3.83 x 10^{-3} mbar; interchanel 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. The method was optimized according to the criteria established by the European Commission, which establishes that a substance can be identified using LC-MS/MS in MRM mode by at least two transitions (Commission Decision, 2002). The most abundant product ion was selected for quantification and the second one for confirmation. Therefore, the quantification of each mycotoxin was carried out with the primary transition (transition of quantification) and the confirmation with the second transition

(transition of confirmation). The MRM ratio was monitored. The following precursor and product ions for acquisition in MRM mode were selected for each mycotoxin: the precursor ion m/z 681.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA, the precursor ion m/z 667.9 $[M+H]^+$ and the product ions m/z 228.2 and 210.0 for ENA_1, the precursor ion m/z 639.8 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB, the precursor ion m/z 654.9 $[M+H]^+$ and the product ions m/z 214.2 and 196.2 for ENB_1.

2.7. Statistical analysis

The experiments were carried out in triplicates. Statistical analysis was conducted by one-way analysis of variance (ANOVA) and by least significance difference (LSD) test (assuming equal variance) to evaluate the significant variations ($p<0.05$). A multiple regression analysis was performed to study the influence of storage time on ENs contents. A multivariable analysis was conducted with the aim of determining whether there were correlations between color, aw and ENs contents (Pearson's test). All statistical analysis were performed using Statgraphics Centurion XVI statistical software (Statpoint Technologies Inc., USA).

3. RESULTS AND DISCUSSION

3.1. Method performance

The method validation included the evaluation of linearity, limits of detection (LODs), limits of quantification (LOQs), recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision). 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. LODs were calculated using a signal-to-noise ratio of 3. LOQs were calculated using a signal-to-noise ratio of 10. LOQs were of 0.5 μg Kg^{-1} for ENA, ENB and ENB_1, while the LOQ for ENA_1 were of 0.25 μg Kg^{-1}. LODs were of 0.15 μg Kg^{-1} for ENA, ENB and ENB_1, while the LOD for ENA_1 was of 0.08 μg Kg^{-1}. In order to determine the linearity, matrix-assisted calibration curves were constructed for each studied mycotoxin. ENs exhibited good linearity over the working range: from 0.5 μg Kg^{-1} to 700 μg Kg^{-1} for ENA, from 0.25 μg Kg^{-1} to 2.5 mg Kg^{-1} for ENA_1, from 0.5 μg Kg^{-1} to 3 mg Kg^{-1} for ENB and ENB_1. The regression coefficient of calibration curves was higher than 0.992. The accuracy was evaluated through recovery studies using spiked blank samples at two concentration levels (LOQ and 100 x LOQ). Intra-day precision was performed by five determinations at each addition level in the same day. Inter-day precision was assessed by one determination at each addition level during five days. The mean recoveries and the corresponding relative standard deviations (RSDs) are presented in Table 1 Supplementary data. RSDs ranged from 4% to 11% for intra-day precision, and from 6% to 15% for inter-day precision. Recovery values were excellent at the range 85-110% and 86-112% for low spiked level (LOQ) and high spiked level (100xLOQ), respectively. The results obtained in the present study were within the limits set by Commission Decision, 2002/657/EC.

Table 1 supplementary data. ENs Recovery values (%) and relative standard deviations (%).

		Recovery ± RSD			
		ENA	ENA_1	ENB	ENB_1
Intra-day precision[a]	Low level (LOQ)	92±5	88±7	109±8	99±10
	High level (100 x LOQ)	91±4	86±9	112±6	97±11
Inter-day precision[b]	Low level (LOQ)	93±8	85±11	110±8	97±13
	High level (100 x LOQ)	90±6	88±8	109±9	95±15

[a] Number of replicates: 5
[b] Different days: 5

3.2. ENs levels

Starting concentrations in white pasta (t=0) were of 0.42±0.08, 1.92±0.40, 2.46±0.32 and 1.74±0.44 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively. Concentrations (t=0) in whole-grain pasta were of 0.60±0.06, 2.29±0.37, 2.41±0.28 and 1.91±0.19 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively. Initial concentrations in egg-pasta (t=0) were of 0.51±0.03, 2.15±0.24, 2.61±0.47 and 1.96±0.44 mg Kg^{-1} for ENA, ENA_1, ENB and ENB_1, respectively.

Figure 2 shows the results obtained for the ENs concentrations at time 0 and after each month of storage. A general decrease on the ENs levels was achieved in all samples of pasta after one-year storage period.

According to the results of the statistical analysis, comparable results were obtained in white, whole-grain and egg pasta over the period examined ($p>0.05$). Therefore, there were not significant differences on ENs levels depending on the type of pasta.

With respect to the results depending on the temperature of storage, the statistical analysis showed that no differences were observed on the final levels of ENs between the storage at 25ºC or 4ºC. Therefore, the storage temperature did not show any effect on the ENs stability.

The percentages of decreasing levels of ENs type A achieved during the storage were greater than those obtained for ENs type B. The concentrations of ENA decreased to 35-57%, being the levels detected at the end of the storage of 0.20-0.39 μg Kg^{-1}. It was observed a decrease rate of 33-44% on the ENA_1 levels, with final concentrations of 1.07-1.50 μg Kg^{-1}. Percentages of ENB reduction were of 11-33%, and concentrations at the end of the storage ranged from 1.66 to 2.14 μg μg Kg^{-1}. ENB_1 showed a low rate of mycotoxin reduction between 7% and 19%, and final concentrations of 1.54-1.73 μg Kg^{-1}. The concentrations of ENA and ENB were statistically significant from the fifth month ($p<0.05$), while the concentrations of ENA1 and ENB1 showed a significant reduction from the third ($p<0.000$) and ninth month ($p<0.05$), respectively. Considering the sum of four ENs (Figure 3), the reductions were statistically significant from the sixth month ($p<0.000$).

Comparable findings to those achieved in the present study were showed by other authors. Kolmanic et al. (2010) developed a study to evaluate the fate of deoxynivalenol (DON) and nivalenol (NIV) in wheat flour. A decrease rate of 1-29% was reported on the mycotoxin levels after 120 days of storage, without significant differences depending on the temperature of flour storage (10ºC or 25ºC). Sørensen et al. (2008) evaluated the presence of ENB in whole maize stored in silage stacks over a period of 11 months. The obtained results indicated that ENB contents were reduced during the first 3 months of maize ensiling and then, the ENB concentration remained constant. The authors

justify that ENs could be degraded or transformed by different microbes, such as lactic acid bacteria in the first months of ensiling, leading to a reduction on the ENs levels. However, it is quite unlikely that strains of lactic acid bacteria are present in raw pasta during the storage, given the low moisture content of raw pasta.

Other explanation to the decreasing levels of ENs during the storage could be the possible structural transformation of ENs over this period. Different reactions such as biochemical processes, transformation reactions or Maillard Reaction could be responsible of the mycotoxin changes in the stored pasta. Therefore, special attention should be focused in the structural transformations of the targeted mycotoxins during the storage (see section 3.5.).

On the other hand, a study was conducted to describe the evolution of ENs levels throughout the time of storage by a multiple regression analysis. This can contribute to understand of the chemistry and physics taking place in the food matrix during different processes (van Boekel, 2008). According to the multiple regression analysis, the concentration reductions of total ENs were dependent of the storage time following a linear model. It was obtained high correlation coefficients between the concentrations of total ENs and the time of storage (r=-0.8356 for white pasta, r=-0.9181 for whole-grain pasta, r=-0.8254 for egg-pasta). According to the linear model, the R^2 of the equations explained the 80-95% of the experimental results (Figure 3).

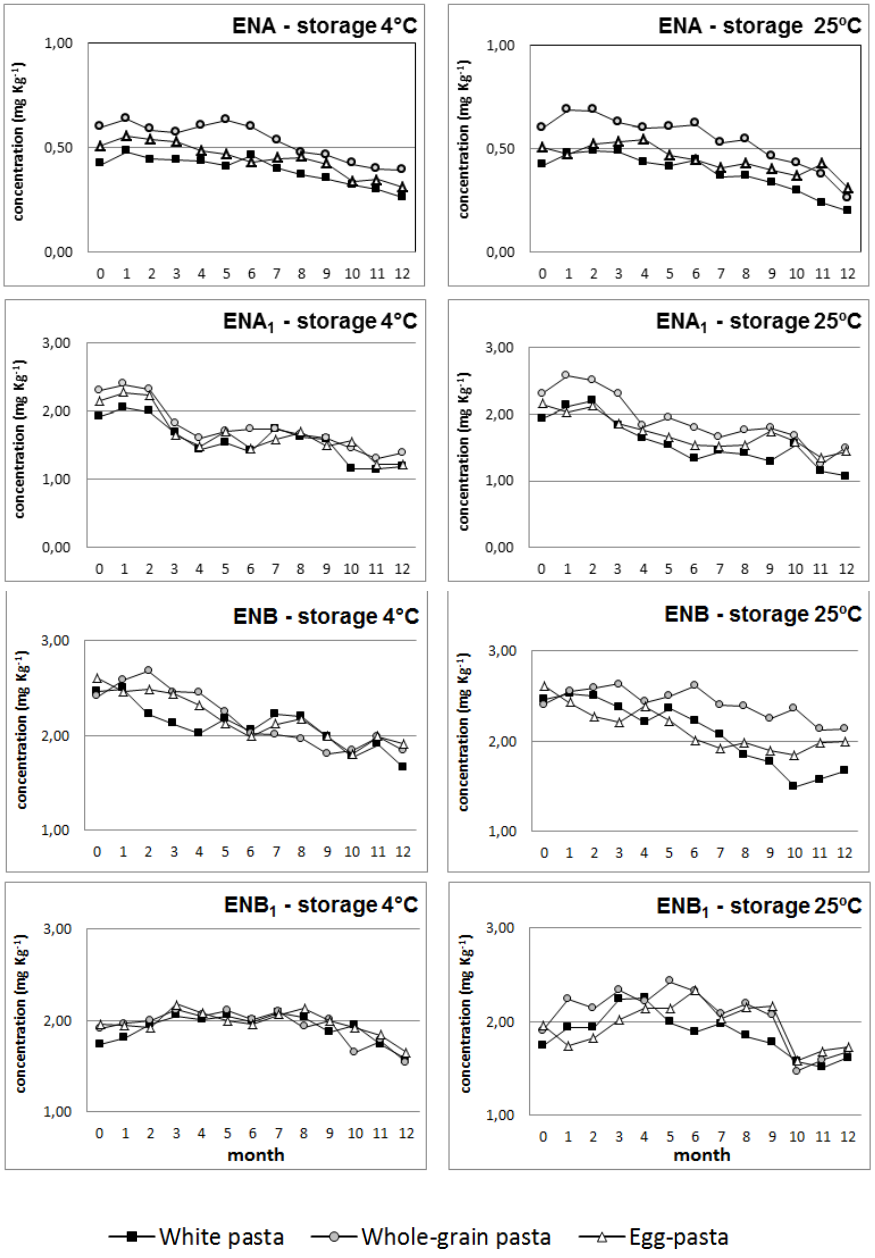


Fig. 2. ENs concentration after each month of pasta storage at 4ºC and 25ºC.

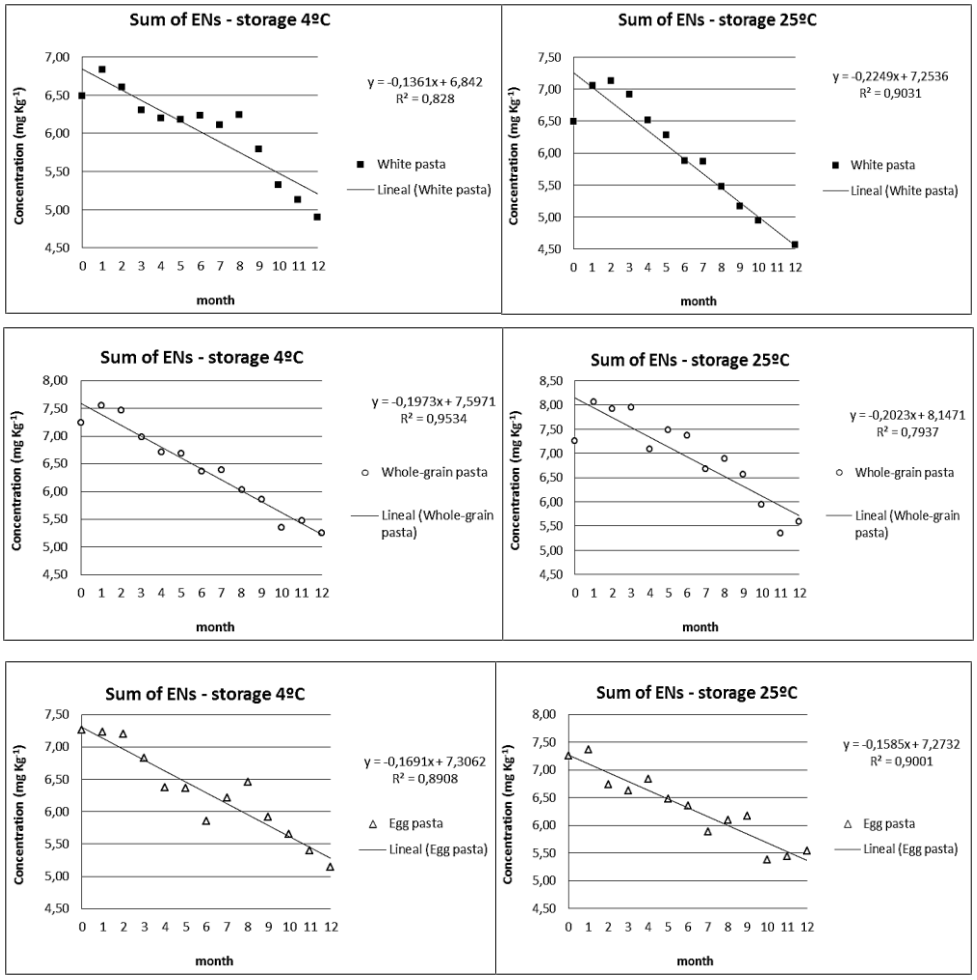


Fig. 3. Concentrations of total ENs during one-year storage at 4ºC and 25ºC for each type of sample.

3.3. Water activity

Table 1 shows the values of a_w for each type of pasta at time 0 and after one-year of storage at 4ºC and 25ºC. The a_w showed a slightly increasing during the storage at both temperatures (from 0.40-0.42 to 0.46-0.51). These results were supported by those obtained by Pacin et al. (2009), which reported that a_w increased slightly during the storage of maize silo bags for 226 days.

Microbiological growth of *Fusarium* species usually occurs at a_w values higher than 0.7 (Gnonlonfin et al., 2008). Taking to account this consideration, there is not any possibility of *Fusarium* fungal growing, in spite of a_w increased to 0.46-0.51 after one-year storage. Therefore, microbial spoilage and mycotoxin production in stored pasta were not expected in the present study. These results are consistent to those achieved relating the decreasing levels of ENs.

Table 1. Average color values (L*, a*, b* and ΔE), average values of water activity (aw) and standard deviations given in brackets, at time 0 and after one year of storage at 4ºC and 25ºC.

Type of sample	Storage	a_w	L*	a*	b*	ΔE
White pasta	*Time 0*	*0.42 (0.01)*	*80.3 (0.1)*	*3.51 (0.01)*	*25.3 (0.2)*	*0*
	One year of storage at 4ºC	0.48 (0.01)	78.8 (0.1)	3.74 (0.05)	25.8 (0.1)	1.60
	One year of storage at 25ºC	0.49 (0.01)	76.9 (0.1)	3.97 (0.06)	25.9 (0.1)	3.41
Whole-grain pasta	*Time 0*	*0.40 (0.01)*	*70.4 (0.2)*	*5.50 (0.01)*	*20.8 (0.1)*	*0*
	One year of storage at 4ºC	0.47 (0.01)	64.7 (0.6)	5.66 (0.06)	20.8 (0.3)	5.71
	One year of storage at 25ºC	0.47 (0.01)	61.1 (0.8)	5.96 (0.11)	20.2 (0.1)	9.29
Egg-pasta	*Time 0*	*0.41 (0.01)*	*80.2 (0.1)*	*4.06 (0.01)*	*30.0 (0.1)*	*0*
	One year of storage at 4ºC	0.46 (0.01)	73.9 (0.4)	4.53 (0.08)	30.1 (0.2)	6.38
	One year of storage at 25ºC	0.51 (0.01)	74.4 (0.2)	4.87 (0.01)	32.5 (0.1)	6.41

With respect to the results relating the type of pasta, the samples of white and whole-grain pasta showed an increment of 0.07 in the a_w over one-year storage, without differences between the storage at 4ºC and 25ºC. Regarding the samples of egg-pasta, it was achieved an increment from 0.41 to 0.46 after storage at 4ºC. However, the increase was more significant after the storage at 25ºC, with a variation of a_w from 0.41 to 0.51. Similar trend was reported by Sharma et al. (2014) in different processed cereal brans stored for six months. The results from these study indicated that a_w after storage at 5-8ºC was lower than the reported a_w after storage at room temperature.

3.4. Color

Detailed data of L*, a* and b* after each month of storage is presented in figure 4. Table 1 shows the chromatic parameters L*, a*, b* and ΔE* at the beginning and the end of storage. The parameter ΔE* was calculated with respect to the values obtained at time 0 and time 12 (see equation in section 2.4.). The determination of this parameter allowed the evaluation of the human eye´s ability to discriminate the color changes in the samples. The values used to determine obvious total color differences were the following: ΔE*<1 color differences are not obvious for the human eye, 1<ΔE*<3 minor color differences could be appreciated by the human eye depending of the hue, and ΔE*>3 color differences are obvious for the human eye (Bodart et al., 2008). As can be observed in table 1, visible color differences by the human eye were obtained after one year of storage for all types of pasta at 4ºC and 25ºC (ΔE* higher than 3.0), with the exception of white pasta after the storage at 4ºC (ΔE*=1.60). These results evidenced the darkening in the samples over the storage period.

Moreover, it should be noted that values of ΔE* were higher in the samples stored at 25ºC than those reported in samples stored at 4ºC. These results are in agreement with the results obtained by Ferrer et al. (2005), which evidenced highest ΔE* in the samples stored at the highest tested temperatures.

Relating the results on the luminosity parameter (L*), all samples of pasta showed a significant decrease in the luminosity during the storage period ($p<0.05$). The decreasing levels of L* indicated the increasing darkness in the samples, that seemed to be quite dependent with the time of storage. Data regarding the parameter a* indicated a significant increment of this value after one-year storage ($p=0.03$), which is associated to a more red chroma in the sample. The color parameter b* did not present significant differences before and after the storage ($p>0.05$). Therefore, the differences in the color observed in the ΔE* parameter are attributable to the changes in the components a* and L*.

According to Bahloul et al. (2009), the increasing levels on the parameter a* are related to the formation of brown pigments in the samples. The browning development during the storage of processed foods can be influenced by diverse factors, such as enzymatic browning or polymerization of anthocyanins. Moreover, the increasing browning is usually associated to the formation of novel compounds such as advanced products formed during the MR. On this account, MR involves different chemical reactions such as non-enzymatic browning, which is favored during prolonged periods of storage (Klimeczak et al., 2007).

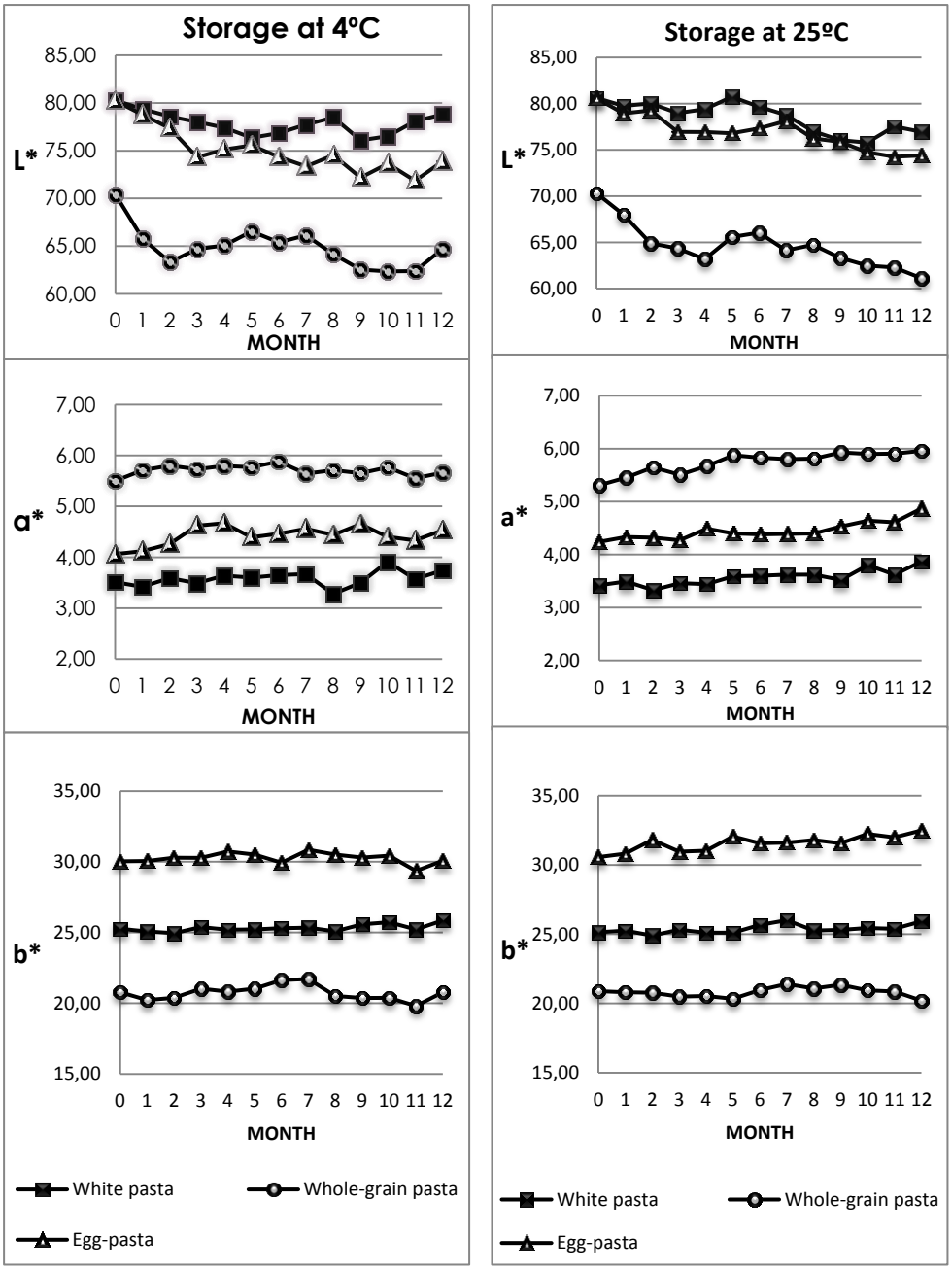


Fig. 4. Color values (L*, a*, b*) after each month of pasta storage at 4ºC and 25ºC.

The relationship between the color changes and the presence of MR products in cereals has been reported by different studies. It is well known that changes in the yellow index (parameter b*) are correlated to enzymatic reactions, whereas the variations in the red index (parameter a*) are related to the development of the MR (Acquistucci, 2000). De Pilli et al. (2014) showed that a decrease of L* and an increasing of red chroma were expected when the MR takes place in the samples of pasta. Recently, Pasqualone et al. (2014) evidenced that b* parameter is also related to the earlier phase of MR, while a* and L* parameters are related to intermediate and advanced phases, respectively. In the present study, variations on the parameters L* and a* have been reported in the samples of pasta. According to the above-mentioned studies, it could be concluded that long times of storage promote the development of the MR, resulting in a formation of intermediate and final products of MR.

3.5. Relation between variables

In this study, mycotoxin concentration and sample browning seemed to be dependent on the time of storage. The fate of a_w was less significant due to the few changes achieved after one-year storage. The decreasing levels of ENs achieved during the storage, and the possible structural transformation of ENs, could be correlated with the increasing browness of the pasta samples. According to Feillet et al. (2000), the brownness and the MR are usually promoted in pasta by drying cycles at temperatures higher than 60ºC and low moisture content (lower than 15%) in dried pasta. In the present study, the pasta used to develop the experiments of storage, was previously dried at temperatures of 70-90ºC to obtain a moisture content of 12%. Therefore, it is

very probable that MR takes place during the thermal treatments, and during the following period of storage. On this account, the development of the MR could be correlated with a loss of the ENs contents during one-year storage. It is well know that MR comprises different reactions, such as protein-carbohydrate reactions, which involve the free amino acids, small peptides, proteins and reducing sugars, leading to a degradation of amino acids and to changes in the structure of the protein (Acquistucci, 2000). Given the potential structure of ENs to participate in the MR (cyclic hexadepsipeptides containing three alternating amino acid residues), the losses of ENs levels probably are attributed to the formation of advanced MR compounds as a result of the degradation of amino acid residues from the ENs.

It was applied a multivariable analysis to establish the correlation between the ENs contents and the color changes. There was obtained a significant negative correlation between ENs concentrations and browning (Pearson correlation coefficient=-0.7162, $p<0.000$). In light of the results obtained in this analysis, it could be concluded that the achieved decreasing levels of ENs are due to the potential contribution of ENs in the Maillard Reaction, and the formation of products structurally related to these mycotoxins.

4. CONCLUSIONS

The results obtained in the current study demonstrated that storage of pasta over one-year affect the ENs stability. The reductions achieved on the levels of ENs type A were higher than those achieved for ENs type B. The concentrations were not significantly affected by the storage temperature and by the type of pasta. Considering the sum of four ENs, they behaved according to a linear model during the time of storage with a negative

correlation. The browning of the samples was observed, mainly due to the increasing red color and the decrease of the luminosity. The browning of the samples and the decreasing levels of ENs were correlated for first time in the present study, being very probable the participation of ENs in the advanced stages of the MR. Although an important decreasing of ENs levels was reported after 12 months of storage, further studies are necessary in order to fully understand the fate of ENs in the advanced stages of the MR throughput long storage periods of different cereal products. This information is necessary to evaluate the potential health risk related to the formation of these novel compounds during the storage.

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4. General discussion

4. DISCUSIÓN GENERAL

Para realizar la evaluación del riesgo se han validado nuevos métodos de análisis de micotoxinas en alimentos, agua y fluidos biológicos de acuerdo a la normativa europea (Commission Decision, 2002; Reglamento (CE) Nº 401/2006), y se han determinado los niveles de concentración en alimentos de elevado consumo para estimar la ingesta diaria y evaluar la exposición de la población a diferentes grupos de micotoxinas. Se ha evaluado la bioaccesibilidad de las micotoxinas emergentes de *Fusarium* y se ha estudiado el comportamiento que presentan durante los procesos de fabricación, cocción y almacenamiento de la pasta.

4.1. Validación de la metodología analítica para la determinación de las micotoxinas en alimentos

4.1.1. Optimización del método por LC-MS/MS

Se ha optimizado un procedimiento analítico por LC-MS/MS para la determinación de las ENA, ENA_1, ENB, ENB_1, BEA y FUS. La optimización de las condiciones del espectrómetro de masas se realiza por infusión de cada patrón de micotoxina en modo "full SCAN", en modo de ionización positivo (ESI+) y negativo (ESI-), con el fin de seleccionar el ión precursor con la relación m/z más abundante. Todas las micotoxinas han presentado una buena eficiencia en el modo ESI+, hallándose moléculas protonadas $[M + H]^+$, y aductos de sodio $[M + Na]^+$ y potasio $[M + K]^+$. La adición de modificadores como el formiato de amonio y el ácido fórmico, y la selección del adecuado voltaje de cono han permitido suprimir la formación de los aductos de sodio y potasio, dando lugar a una mayor sensibilidad del método y a una mayor fiabilidad en la cuantificación.

El método se ha optimizado conforme a las directrices establecidas por la Comisión Europea (Commission Decision, 2002), donde se establece que una sustancia puede ser identificada usando LC-MS/MS en el modo de monitorización de reacciones múltiples (MRM) por al menos dos transiciones, de cuantificación y de confirmación. 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 (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, probando tanto la elución isocrática como en gradiente, con diferentes combinaciones de disolventes de fase móvil. Se selecciona el modo de elución en gradiente con diferente composición de AcN y MeOH en presencia de formiato de amonio, ya que da lugar a una eficiente separación de las micotoxinas en un menor tiempo de análisis (Serrano et al., 2013a)

El método optimizado permite la separación de las ENs y la BEA en los primeros 4 minutos de análisis, y de la FUS a los 13.5 minutos. El tiempo total de análisis es de 15min, mientras que los métodos propuestos por otros autores requieren tiempos superiores a 20 min (Blesa et al., 2012; Juan et al., 2013; Hu y Rychlik, 2014).

4.1.2. Optimización de los métodos de extracción

Se han empleado diferentes técnicas de extracción de acuerdo a las características de la matriz alimentaria y a las micotoxinas a determinar.

❖ *Extracción de micotoxinas en cereales y productos derivados*

La determinación de las micotoxinas tradicionales AFs, TCs, FBs, OTA y ZEA, y de la micotoxina emergente BEA en cereales y productos a base de cereales, se realiza por dispersión de matriz en fase sólida (MSPD) seguido de cuantificación por LC-MS/MS. El método empleado proporciona recuperaciones del 68.7-89.6% con límites de cuantificación de 0.25-85.24 μg Kg^{-1} en función del tipo de micotoxina. Este método se ha aplicado al análisis de 265 muestras de cereales procesados y sin procesar (ver sección 4.3.) (Serrano et al., 2012a).

❖ *Extracción de micotoxinas emergentes de Fusarium en pasta y papillas infantiles*

Se ha optimizado un método para realizar la extracción de las ENs, la BEA y la FUS en pasta y papillas infantiles. Estos alimentos han sido seleccionados por ser susceptibles de contaminación por micotoxinas de *Fusarium* y por ser productos de elevado consumo por la población adulta e infantil, respectivamente. La optimización del método se realiza mediante un estudio comparativo de la extracción sólido-líquido (S-L) usando Ultra-Turrax, microondas y baño de ultrasonidos (Serrano et al., 2013a).

Se han ensayado distintos volúmenes y disolventes de diferente polaridad. El empleo de 50 mL de AcN da lugar a los porcentajes de recuperación más elevados. El tiempo de extracción usando el Ultra-Turrax se optimiza en 3 min

a temperatura ambiente. En la extracción empleando el microondas se estudian diferentes combinaciones de tiempo-potencia del instrumento, obteniendo los mejores resultados con un tiempo de extracción de 2 min a una potencia de 250W, lo que corresponde a una temperatura de 45ºC. En la extracción con ultrasonidos se evalúan diferentes tiempos y temperaturas, seleccionándose un tiempo óptimo de 30min a 40ºC.

El calentamiento de la muestra durante la extracción por microondas y por ultrasonidos produce un aumento de la solubilidad de ciertas sustancias presentes en la matriz alimentaria, como lípidos y otros compuestos, dando lugar a un incremento importante en el efecto matriz con respecto al obtenido en la extracción por Ultra-Turrax. Debido a ello, en el caso de la extracción por microondas y ultrasonidos es necesario aplicar una etapa de purificación para reducir la presencia de sustancias interferentes en el extracto final, así como para evitar el deterioro de la columna cromatográfica y del equipo LC-MS/MS. La etapa de purificación se realiza por extracción en fase sólida (SPE) con columnas de C18 y se estudian diferentes disolventes de elución. Se selecciona la mezcla de MeOH/AcN (50/50 v/v) con formiato de amonio (20 mM) como disolvente de elución, puesto que da lugar a extractos considerablemente más limpios con una reducción del efecto matriz.

Los tres procedimientos han dado lugar a resultados aceptables para las ENs y la BEA, siendo la extracción S-L con Ultra-Turrax la que ofrece un mayor porcentaje de recuperación (86-109%) en comparación con el baño de ultrasonidos (69-83%) y el microondas (75-90%). Por lo que respecta a la FUS, no se obtienen buenos resultados con la extracción por microondas y por ultrasonidos (recuperación <45%) (Serrano et al., 2013a). Estos resultados son debidos a que se produce la deacetilación de la FUS en las condiciones de

temperatura seleccionadas (Wu et al., 2003). No obstante, el empleo de estas técnicas requiere la aplicación de una etapa adicional de purificación, dando lugar a un aumento en el coste y tiempo de análisis.

Por lo tanto, se selecciona la extracción S-L con Ultra-Turrax, puesto que presenta una elevada eficacia de extracción para todas las micotoxinas de interés, sin necesidad de una etapa adicional de purificación. Este método ha sido validado obteniéndose resultados satisfactorios en cuanto a la linealidad (r^2> 0.992), exactitud (recuperaciones del 85-112%), y precisión inter-día e intra-día, con una desviación estándar relativa (RSD) del 4-11% y 5-15%, respectivamente. Los límites de cuantificación (LOQs) alcanzados oscilan entre 0.05 y 0.5 μg Kg^{-1}, siendo del mismo orden o inferiores a los obtenidos en otros estudios (Jestoi, 2007; Lindblad et al., 2013; Hu y Rychlik, 2014). El estudio del efecto matriz revela una supresión de la señal del 20-48%, sin diferencias en cuanto al tipo de pasta. En ausencia de un patrón interno fiable para estas micotoxinas, la cuantificación de las muestras se realiza mediante el empleo de rectas de calibrado adicionadas de matriz (Serrano et al., 2013a).

Considerando los resultados obtenidos en el estudio anterior, se desarrolla un método de extracción para tratar las muestras de papillas infantiles. Partiendo de la extracción empleando el Ultra-Turrax, se optimizan la cantidad de muestra inicial, el volumen y tipo de disolvente, y el tiempo de extracción. Los resultados obtenidos muestran una mayor eficacia del método empleando 3g de muestra y 30mL de acetato de etilo con un tiempo de extracción de 5 minutos. Una vez optimizados los parámetros críticos de la extracción, se realiza la validación del método: se obtienen porcentajes de recuperación del 70-89% y con RSDs inferiores al 11%, indicando una óptima repetibilidad y

reproducibilidad del método. Los LOQs obtenidos son del mismo orden a los hallados para la pasta (0.30-0.64 μg Kg^{-1}) (Serrano et al., 2012b).

El método propuesto mediante extracción S-L con Ultra-Turrax y determinación por LC-MS/MS permite la determinación de las micotoxinas emergentes de *Fusarium* con una elevada especificidad y sensibilidad.

❖ *Extracción de micotoxinas emergentes de Fusarium en agua*

La optimización de un método para la determinación de micotoxinas emergentes en agua se realiza por micro-extracción líquido-líquido dispersiva (DLLME). Para optimizar el método se realizan pruebas con diferentes volúmenes de muestra y disolvente, y con diferentes tipos de disolventes. Los disolventes seleccionados son el tetracloruro de carbono (CCl_4) como disolvente de extracción, y el AcN como disolvente de dispersión. El método consiste en la adición de 1mL de AcN/CCl_4 (9/1 v/v) a 5 mL de agua que contiene 1 g de NaCl. La dispersión del disolvente de extracción en el seno de la muestra favorece la extracción de los analitos al disolvente de extracción. El método desarrollado proporciona excelentes porcentajes de recuperación del 75-100% con una RSD<14%, y con LOQs inferiores a 0.6 μg L^{-1}(Serrano et al., 2015c).

4.2. Validación de la metodología analítica para la determinación de las micotoxinas emergentes de *Fusarium* en fluidos biológicos

La evaluación de la exposición mediante la determinación de micotoxinas en muestras de origen biológico, es una interesante medida alternativa a los estudios de estimación de ingestas. Con el fin de proporcionar un nuevo procedimiento para evaluar la reciente exposición a las micotoxinas emergentes, se ha optimizado un método para la determinación de ENA, ENA_1, ENB, ENB_1 y BEA en orina y plasma humanos. Este estudio se lleva a cabo durante la estancia realizada en el grupo de Seguridad y Calidad Alimentaria y Ambiental en la Universidad La Sapienza de Roma.

Para la optimización del método se realiza la evaluación de una etapa inicial de pre-tratamiento de acuerdo al tipo de muestra, y una etapa posterior de purificación y preconcentración de la muestra (Serrano et al., 2015e). La separación y cuantificación de las micotoxinas se realiza por LC-MS/MS QqQ según el método desarrollado por Capriotti et al., (2014).

Las pruebas para optimizar el pre-tratamiento de las muestras de orina se basan en la dilución de la muestra (5mL) con diferentes volúmenes de agua (100-1000 mL), y en el ajuste de diferentes pH (pH=2.0-7.0), necesario para asegurar la reproducibilidad del método. La dilución 1:100 seguida de ajuste de pH a 4.00, da lugar a una importante mejora en la eficacia y la reproducibilidad del método.

El pre-tratamiento del plasma humano se aplica principalmente para la desproteinización de la muestra. Se estudian diferentes volúmenes de muestra (100-500 μL) y de disolvente (1mL-10mL). Asimismo, se evalúa la eficacia de diferentes tipos disolventes y mezclas de ellos. La combinación de 250 μL de

plasma con 10 mL de H_2O/MeOH (60/40 v/v) da lugar a los mejores resultados de recuperación.

Tras la optimización del pre-tratamiento inicial acorde a cada matriz biológica, se optimiza la etapa de purificación de la muestra por SPE con columnas de carbón activo. Para ello, se estudian diferentes volúmenes y tipos de disolventes de las etapas de lavado y elución. La etapa de lavado empleando 2mL de MeOH en presencia de ácido fórmico (0.2%) da lugar a los mejores resultados, logrando una importante reducción en el efecto matriz sin comprometer la elución de los analitos de interés. Por lo que respecta a la etapa de elución, se obtiene una efectiva elución de los analitos con 10mL de una mezcla de diclorometano/MeOH (80/20 v/v) y ácido fórmico al 0.2%.

Se obtienen óptimos resultados en cuanto a la linealidad, exactitud y precisión, con $r^2 > 0.991$, recuperaciones del 85-109% para la orina, y del 90-120% para el plasma, y valores de RSD inferiores al 20%. El estudio del efecto matriz demuestra una supresión de la señal de hasta el 18% y un aumento de hasta el 9%, dependiendo del tipo de micotoxina. Aunque el efecto matriz observado no es muy acusado, para una adecuada cuantificación de micotoxinas se decide emplear rectas de calibrado adicionadas de blanco de matriz, dada la ausencia de un estándar interno admisible.

El método desarrollado da lugar a extractos exentos de interferencias con un efecto matriz despreciable, lo que permite aumentar en gran medida la sensibilidad. Los LOQs alcanzados con este método (15-35 ng L^{-1} y 30-65 ng L^{-1}, para la orina y el plasma) son del orden de 5 veces inferior a los obtenidos en plasma por Devreese et al. (2013), y del orden de 500 veces inferior a los hallados para la ENA en orina y plasma de ratas (Juan et al., 2014a).

La metodología analítica desarrollada constituye una potente herramienta para la evaluación del grado de exposición a las micotoxinas emergentes de *Fusarium*, existiendo posibilidad de incluir en un futuro la determinación de los metabolitos derivados de las biotransformaciones que puedan sufrir estas micotoxinas en el organismo.

Tras la validación del método, se analizan 10 muestras de orina y 10 muestras de plasma procedentes de diferentes individuos, habiéndose detectado niveles traza (<LOD) en una muestra de orina y en una muestra de plasma de un mismo individuo. Los estudios disponibles hasta el momento han analizado muestras de plasma y orina procedentes de animales expuestos a las micotoxinas emergentes (Devreese at al., 2013; Ivanova et al., 2014; Manyes et al., 2014). Por lo general, los autores han descrito una rápida absorción, distribución y biotransformación de las ENs y la BEA, lo que en parte explicaría los resultados obtenidos en el presente estudio en el caso de que los individuos implicados hubieran estado expuestos a estas micotoxinas.

4.3. Presencia de micotoxinas en alimentos

4.3.1. Micotoxinas en cereales y productos derivados

Los niveles de concentración de las AFs, OTA, ZEA, FBs, DON, NIV, DAS y BEA se han determinado en un total de 265 muestras de cereales en grano (n=135) y cereales procesados (n=130) procedentes de países del área Mediterránea (España, Italia, Túnez y Marruecos). En el 53% de las muestras analizadas se ha detectado alguna micotoxina. Destaca la elevada prevalencia obtenida en las muestras procedentes de Túnez (96%). Por lo general, las máximas concentraciones se han detectado en muestras de arroz de Marruecos y en muestras de cereales procesados de Túnez (Serrano et al., 2012a).

El NIV es la micotoxina con una mayor prevalencia en las muestras analizadas (51%), con niveles de concentración de 100-961 μg Kg^{-1}. Esta micotoxina se encuentra en todos los grupos de cereales analizados, procedentes de los 4 países. Los resultados obtenidos son del mismo orden a los hallados en otros estudios (Milanez et al., 2006). La BEA es la segunda micotoxina más frecuente, con una incidencia en el 14% de las muestras en concentraciones de 2-844 μg Kg^{-1}. Estos rangos de concentración son inferiores a los detectados en otros estudios para cereales (Meca et al., 2010).

La ZEA es la única micotoxinas que no se ha detectado en ninguna de las muestras objeto de estudio.

El DAS, DON y toxinas T-2 y HT-2 se han encontrado en un bajo porcentaje de las muestras (<10%), principalmente arroz, maíz y productos a base de trigo. El DAS se detecta en concentraciones de 6-97 μg Kg^{-1}. El DON se detecta en concentraciones (63-296 μg Kg^{-1}) inferiores a los contenidos máximos establecidos por el Reglamento (CE) Nº1881/2006 de la Comisión. Las concentraciones detectadas de las toxinas T2 y HT2 (13-87 μg Kg^{-1}) en algunas muestras de Túnez y Marruecos son superiores a los valores fijados en la Recomendación 2013/165/UE de la Comisión. Por lo general los niveles de concentración detectados para las micotoxinas de *Fusarium* fueron del mismo orden o inferiores a los hallados en otros estudios (Milanez et al., 2006; González-Osnaya et al., 2011).

Las FB1 y FB2 se han detectado en un rango de concentración de 121-184 μg Kg^{-1}, inferior a los contenidos máximos establecidos en la Unión Europea (Reglamento (CE) Nº1881/2006). Otros estudios han indicado un mayor porcentaje de muestras positivas en concentraciones superiores a las detectadas en este estudio (Cavaliere et al., 2007; Silva et al., 2009).

Las concentraciones de las AFs oscilan entre 4 y 67 μg Kg^{-1}, siendo la AFB1 la AF detectada con una mayor frecuencia. Los niveles de concentración de la OTA son de 75-112 μg Kg^{-1}. Los contenidos de AFs y OTA en las muestras de Túnez y Marruecos han superado los límites establecidos en la Unión Europea. (Reglamento (CE) Nº1881/2006). No obstante, existen varios estudios que muestran resultados similares o superiores a los obtenidos en el presente estudio en cereales y productos derivados procedentes de países caracterizados por un clima templado (Blesa et al., 2004; Ghali et al., 2008; Zaied et al., 2009).

La mayoría de muestras han presentado simultáneamente una o dos micotoxinas, destacando las combinaciones de NIV y BEA, y de NIV con alguna de las cuatro AFs en una misma muestra. En las muestras procedentes de países europeos (España e Italia) únicamente se han identificado NIV y BEA, para las cuales no existe ninguna reglamentación para limitar los contenidos en los alimentos. Por tanto, especial atención se debe prestar a los niveles de contaminación de estas dos micotoxinas en materias primas y productos alimenticios, siendo interesante la realización de estudios de toxicidad combinada para evaluar el posible impacto negativo de estas dos micotoxinas cuando se encuentran en combinación en un mismo alimento.

4.3.2. Micotoxinas emergentes de Fusarium en pasta

El método optimizado para determinar ENA, ENA_1, ENB, ENB_1, BEA y FUS en pasta (Serrano et al., 2013a), se ha aplicado al análisis de 114 muestras de pasta comercial. La mayoría de las muestras proviene de España (n=87), seguido de Italia (n=17), Alemania (n=6) y Portugal (n=4). Las muestras objeto de estudio proceden de agricultura ecológica (n=40) y convencional (n=74) (Serrano et al., 2013b).

Un total de 105 muestras (92%) contienen alguna de las micotoxinas objeto de estudio. Los niveles de concentración oscilan entre 0.05 y 979.56 μg Kg^{-1}, siendo del mismo orden a los hallados en otros estudios que analizaron cereales en grano y productos derivados (Malachova et al., 2011; Blesa et el., 2012; Juan et al., 2013). No obstante, ciertos estudios han indicado niveles de concentración considerablemente superiores a los obtenidos en este trabajo. En estos estudios se detectan concentraciones máximas del orden de 1000 mg Kg^{-1} en diversos productos a base de cereales, principalmente procedentes de países con condiciones climáticas características del área mediterránea (Meca et al., 2010; Oueslati et al., 2011; Sifou et al., 2011).

Tanto la incidencia como los niveles de concentración obtenidos en el presente estudio son mayores para las ENs (71-80%, 0.10-979.56 μg Kg^{-1}) que para la FUS y la BEA (11-18%, 0.05-20.96 μg Kg^{-1}). En el grupo de las ENs, las de tipo B se detectan en una mayor concentración e incidencia que las de tipo A. Una tendencia similar se ha observado en diferentes estudios que han evaluado la presencia de estas micotoxinas en otros productos a base de cereales (Uhlig et al., 2006; Yli-Mattila et al., 2006; Mahnine et al., 2011).

Los resultados obtenidos indican la presencia simultánea de varias micotoxinas en un elevado porcentaje de las muestras. El 65% de las muestras contiene dos o más ENs, mientras que el 22% presenta la presencia simultánea de alguna de las cuatro ENs y la BEA o la FUS.

Evaluando los resultados obtenidos en función del tipo de pasta (ecológica o convencional) se observa que el 100% de las muestras de agricultura ecológica están contaminadas por alguna de las seis micotoxinas objeto de estudio, mientras que la incidencia es del 88% en las muestras convencionales. Con respecto a los niveles de concentración, no se obtienen resultados

concluyentes: mientras la concentración media de la ENA y de la FUS es mayor en la pasta orgánica, la concentración media de las ENs tipo B es mayor en la pasta convencional. Hasta el momento únicamente existe un estudio que ha comparado la presencia de las micotoxinas emergentes de *Fusarium* en productos de agricultura ecológica y convencional, en el cual tampoco se obtuvieron diferencias de concentración en cuanto al tipo de pasta (Jestoi et al., 2004b).

4.3.3. Micotoxinas emergentes de Fusarium en papillas infantiles

Las micotoxinas emergentes de *Fusarium* se han determinado en 45 muestras de papillas infantiles destinadas a lactantes entre el 6º y 12º mes de vida. Las muestras se dividen en función de su composición en papillas de 8 cereales (n=8), de 8 cereales enriquecidas con zumo de fruta (n=7), de 8 cereales enriquecidas con miel (n=7), papillas de 3 cereales (n=10) y de 2 cereales (n=7) (Serrano et al., 2012b).

Alrededor del 47% de las muestras estan contaminadas con al menos una micotoxina (n=21). La ENB_1 presentó una mayor incidencia (40%) que el resto de micotoxinas (<20%). El 9% de las muestras positivas presentaron co-presencia de dos ENs, mientras que el resto de muestras únicamente contenían una micotoxina.

De acuerdo con los resultados obtenidos en las muestras de pasta, los niveles de concentración obtenidos en el presente estudio son mayores para las ENs (6.3-149.6 mg Kg^{-1}) que para la FUS y la BEA (nd-1.7 mg Kg^{-1}). Los niveles de concentración de las ENs fueron del mismo orden a los hallados por otros autores en cereales procesados, incluyendo cereales para el desayuno, pasta y arroz, entre otros (Mahine et al., 2011; Meca et al., 2010; Sifou et al., 2011). No obstante, los contenidos determinados en el presente estudio son

superiores a los obtenidos en estudios que han evaluado la presencia de micotoxinas tradicionales o legisladas en diferentes productos infantiles (Cano-Sancho et al., 2012; Rubert et al., 2012; Tavares et al., 2013).

Estos resultados ponen de manifiesto la necesidad de establecer medidas para regular los niveles de las micotoxinas emergentes de *Fusarium*, especialmente en alimentos para consumo infantil, siendo necesarios estudios adicionales dirigidos a este grupo de población.

4.3.4. Micotoxinas emergentes de Fusarium en agua

Para evaluar si existe riesgo para la salud humana y animal por exposición a las micotoxinas emergentes a través del consumo de agua, se han analizado 40 muestras de agua de diferentes tipologías: 20 muestras de agua embotellada, 10 muestras de agua de la red de consumo público, y 10 muestras de agua subterránea y de superficie. Las micotoxinas de interés no han sido detectadas en ninguna de las muestras objeto de estudio (Serrano et al., 2015c). No obstante, existen varios estudios que han detectado AFs y OTA en agua embotellada (Mata et al., 2015), y BEA en aguas de superficie y subterráneas (Schenzel et al., 2010).

4.4. Evaluación de la Exposición

Se ha llevado a cabo la evaluación de la exposición a las micotoxinas basada en un enfoque determinista. Para ello, se calcula la IDE a partir de las concentraciones obtenidas en alimentos, y de los datos disponibles de consumo de alimentos (FAO, 2007; MAGRAMA, 2011).

Se realizan estimaciones para dos grupos de población dependiendo del tipo de alimento. Los datos obtenidos de presencia de micotoxinas en productos a base de cereales han sido empleados para evaluar la exposición de la población adulta, mientras que los datos obtenidos en papillas se emplean para la población infantil (lactantes 6-12 meses de vida).

Los datos relativos al consumo de cereales por la población adulta de España, Túnez, Marruecos e Italia, se obtienen de los balances alimentarios de la FAO (FAO, 2007). Los datos de consumo para evaluar la exposición relativa a la ingesta de micotoxinas a través de la pasta en España, se toman de las bases de datos del Ministerio de Agricultura, Alimentación y Medio Ambiente (MAGRAMA, 2011). Para el cálculo del consumo de alimento expresado en Kg de peso corporal (Kg pc), se considera un peso medio de la población adulta de 70 Kg,

Para la evaluación de la exposición en la población infantil comprendida entre los 6 y 12 meses de vida, se toman datos de consumo de papillas de acuerdo a la recomendación indicada en los envases de las papillas analizadas, y a un peso corporal medio de 8.5 Kg para la población infantil (WHO, 2011).

4.4.1. Población adulta

❖ *Micotoxinas legisladas*

La mayoría de IDEs de las micotoxinas legisladas o tradicionales debidas al consumo de productos a base de cereales, son inferiores a la ingestas diaria tolerable (TDI) y a la ingesta diaria máxima tolerable provisional (PMTDI) establecidas por la JECFA y la SCF (FAO/WHO 2001; SCF 2002).

No obstante, la IDE de NIV correspondiente a la ingesta de productos de Italia y Túnez, es superior a la TDI temporal (t-TDI) representando el 487% y 113% (respectivamente) del valor estipulado de t-TDI (0.7 μg/kg pc/ día). La ingesta estimada de OTA excede a la ingesta tolerable estipulada en las muestras de Túnez (0.1 μg/Kg pc/semana).

Por lo que respecta a las AFs, se estiman valores de IDE de 1.00 ng/Kg pc/día (España), 1-50 ng/Kg pc/día (Túnez) y de 0.004-0.05 ng/Kg pc/día (Marruecos). Los bajos valores obtenidos de IDE relativos a las muestras de Marruecos, son debidos a que únicamente se consideró el consumo de arroz para el cálculo de la ingesta (de acuerdo con los datos disponibles de contenidos de micotoxinas), mientras que para el resto de países se consideró el consumo de más grupos de alimentos, aumentando así, el valor de ingesta estimada (Serrano et al., 2012a).

La TDI de las AFs no ha sido establecida, debido a que son sustancias cancerígenas clasificadas según la IARC en el grupo 1 de compuestos cancerígenos en humanos con evidencia suficiente (IARC, 1993). De acuerdo con el principio ALARA (As Low As Reasonable Achievable), para estos compuestos se recomienda reducir tanto como sea posible la exposición. De acuerdo con ello y considerando que todas las muestras en las que se han detectado AFs presentan niveles de concentración superiores a los contenidos

máximos legislados (Reglamento (CE) Nº1881/2006), se puede concluir que la presencia de AFs en las muestras analizadas representa riesgo para la salud de la población.

❖ *Micotoxinas emergentes de Fusarium*

En la evaluación de la exposición de la población española a partir del consumo de pasta, se obtienen valores de IDE correspondientes a la FUS (0.010 ng/Kg pc/día) y a la BEA (0.50 ng/Kg pc/día) del orden de diez veces inferior a los obtenidos para las ENs (2.12-4.72 ng/Kg pc/día). La evaluación de la exposición se realiza de forma aproximada, ya que no se han establecido ingestas tolerables para las micotoxinas emergentes. La estimación de la exposición se realiza comparando el valor de IDE correspondiente a la suma de las 6 micotoxinas emergentes (0.013 μg/kg pc/día), con las TDIs establecidas para otras micotoxinas de *Fusarium*: 1.0, 0.7, 0.1 y 2.0 μg/Kg pc/día para el DON, el NIV, la suma de las toxinas T-2 y HT-2 y la suma de las fumonisina B_1 y B_2, respectivamente (FAO/WHO, 2001; SCF, 2002). Comparando estos valores, se observa que la IDE total es del orden de 10 o 100 veces inferior que las TDIs establecidas para otras micotoxinas de *Fusarium*. Considerando los resultados obtenidos, se ha estimado que la ingesta de micotoxinas emergentes de *Fusarium* asociada al consumo de pasta, no representa riesgo apreciable para la población. No obstante, se debe de considerar que la IDE estimada únicamente se debe al consumo de pasta, el cual representa un pequeño porcentaje de la dieta total (Serrano et al., 2013b).

En cuanto a la IDE de la BEA presente en las muestras de cereales, se obtienen valores inferiores a las TDIs de las otras micotoxinas de *Fusarium* en el caso de la IDE asociada a las muestras de España (3.0 ng/Kg pc/día) y Marruecos (0.003 ng/Kg pc/día), mientras que la IDE debida a la ingesta de

alimentos procedentes de Túnez (0.37 μg de BEA/Kg pc/día) supera la TDI establecida para las toxinas T-2 y HT-2 (0.1 μg /Kg pc/día) (Serrano et al., 2012a).

Dada la diferencia entre los valores de TDI dependiendo del tipo de micotoxina, y teniendo en cuenta que se están estudiando los posibles efectos tóxicos de las micotoxinas emergentes de *Fusarium*, el riesgo que representan estas micotoxinas en la dieta total para la población adulta podría ser mayor al estimado en estos estudios.

4.4.2. Población infantil

Los valores de IDE obtenidos en este estudio varían dependiendo del tipo de micotoxina emergente (9.3-123.4 μg/Kg pc/día). La IDE total relativa a la suma de todas las micotoxinas emergentes fue de 234.6 μg/Kg pc/día. Este valor es muy superior al obtenido cuando se estima la exposición de la población adulta (0.013 μg/kg pc/día). Esto se debe al bajo peso corporal que presenta el grupo de población infantil junto con una dieta elevada en consumo de cereales.

La IDE relativa a la población infantil no es comparable a los valores de TDIs propuestos para grupos de población en edad adulta. Según el dictamen científico de la SCF (2001) para otros contaminantes alimentarios, se puede considerar que la IDE infantil es del orden de 100 veces inferior a la IDE de adultos. Considerando esta correlación, el valor total de IDE transformado sobre la base de un adulto es de 2.35 μg/Kg pc/día. Este valor es del mismo orden de magnitud que la TDI establecida para las FBs, y muy superior a la TDI para el resto de micotoxinas de *Fusarium*. Por tanto, existe riesgo de exposición potencial para la población infantil a las micotoxinas emergentes de *Fusarium* (Serrano et al., 2012b).

4.5. Evaluación de la bioaccesibilidad de las micotoxinas emergentes de *Fusarium*

El estudio de bioaccesibilidad se realiza mediante la aplicación de un modelo de digestión *in vitro* estático previamente optimizado, empleando muestras de papillas contaminadas por micotoxinas emergentes. El modelo consiste en la simulación de las condiciones fisiológicas del tracto gastrointestinal en un lactante de 6-12 meses (Serrano et al., 2014).

Los resultados obtenidos varían dependiendo del tipo de micotoxina. La ENA_1 es la micotoxina que presenta una mayor bioaccesibilidad, llegando a estar accesible el 32-60% del contenido inicial ingerido en el tracto gastrointestinal. El resto de micotoxinas presentan una menor bioaccesibilidad, con porcentajes del 1.5-8%. Estos resultados demuestran que la concentración de ENs accesible para la absorción intestinal es muy inferior a la concentración determinada en la papilla inicialmente (Serrano et al., 2014). Estos resultados son similares a los obtenidos en otros estudios que han evaluado la bioaccesibilidad de estas micotoxinas simulando condiciones de digestión de un adulto (Meca et al., 2012a).

Los resultados obtenidos en este estudio indican que la cantidad de micotoxinas emergentes accesible para la absorción en el intestino es muy inferior a la ingerida, lo que se traduce en un mayor nivel de protección y un menor riesgo para la salud de la población. No obstante, estos resultados no son concluyentes, ya que la evaluación de la bioaccesibilidad a través de métodos de digestión *in vitro* puede llegar a variar considerablemente dependiendo del tipo de simulación (estática o dinámica), de la composición del alimento, y del nivel de contaminación inicial (Manzini et al., 2015).

4.6. Comportamiento de las micotoxinas emergentes durante los tratamientos tecnológicos

En la actualidad existen limitados 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. De acuerdo a las recomendaciones del reciente dictamen científico de las EFSA para realizar la evaluación del riesgo de las micotoxinas emergentes (EFSA Contam Panel, 2014), se ha evaluado el comportamiento de estas micotoxinas y de los posibles productos de degradación y/o conjugación formados durante los procesos de elaboración y cocción de la pasta, así como durante su almacenamiento.

4.6.1. Evaluación del proceso de fabricación de la pasta

Para llevar a cabo el estudio, se ha simulado el proceso de fabricación de la pasta según indican varios autores (Delcour y Hoseney, 2010; Menesatti et al., 2014), partiendo de harina de trigo contaminada por micotoxinas emergentes (Serrano et al., 2015a). Dada la ausencia de material de referencia contaminado por ENs y BEA, se decide emplear como material de partida, harina de trigo previamente contaminada en el laboratorio por inoculación de *Fusarium Tricinctum* (CECT 1036) productora de estas micotoxinas. Las concentraciones de ENs en la harina de partida son de 1-7 mg Kg^{-1}. La BEA y la FUS no fueron producidas por el hongo, por lo que no se incluyeron en los ensayos.

El estudio de fabricación de la pasta se llevó a cabo en dos etapas: elaboración de la pasta fresca y secado. La elaboración de la pasta fresca comprende las etapas de amasado de la harina con el agua (30%), extrusión de la masa y conformación de acuerdo a la forma deseada. Estas etapas no

presentan ningún efecto significativo en los contenidos de micotoxinas, ya que se trata de etapas mecánicas en las cuales no se aplica tratamiento térmico.

La etapa de secado permite reducir el contenido en humedad de la pasta del 30% al 12%. Para ello, se aplican diferentes ciclos de secado modificando las temperaturas a lo largo del tiempo. Se aplican tres ciclos de secado a baja (25ºC, 5 días), media (45-55ºC, 18 h) y alta temperatura (70-90ºC, 13 h), que simulan las condiciones de secado tradicional en los hogares, en la industria con capacidad de producción media, y en las industrias con producción a alta escala, respectivamente (Menesatti et al., 2014). Los contenidos de ENs se determinan según Serrano et al. (2013a).

Los resultados obtenidos muestran que el secado tradicional (25ºC) no causa ningún efecto significativo en el contenido de ENs. Sin embargo, se obtienen reducciones del 31-56% con el ciclo de secado a media temperatura y del 51-83% con el ciclo a alta temperatura. Las concentraciones finales detectadas en la pasta seca fueron de 0.51-4.36 mg Kg^{-1} y de 0.23-3.04 mg Kg^{-1} tras el secado a media y alta temperatura, respectivamente. Los porcentajes de reducción fueron del mismo orden para la pasta blanca y la pasta integral, sin diferencias estadísticamente significativas.

Actualmente existen tres estudios que han evaluado los contenidos de las micotoxinas emergentes durante la fabricación del pan y de la cerveza. En estos estudios se han obtenido elevados porcentajes de reducción en parte debidos a las elevadas temperaturas alcanzadas (Vaclavikova et al., 2013; Hu et al., 2014a; Hu et al., 2014b).

Por tanto, la aplicación de los tratamientos térmicos adecuados se podría establecer como una estrategia para reducir los contenidos de ENs y minimizar la exposición de la población. No obstante, deben ser estudiados otros

aspectos, como la formación de compuestos derivados de la degradación y/o conjugación de las ENs en el alimento (ver sección 4.6.5.), y el grado de toxicidad de los mismos. Asimismo, se deben considerar los diferentes aspectos que afectan a la calidad y a la aceptabilidad del producto final, como son el valor nutricional y las características organolépticas del mismo.

4.6.2. Evaluación del proceso de cocción de la pasta

Las muestras de pasta procesadas en el estudio anterior aplicando el tratamiento a elevadas temperaturas, han sido sometidas al proceso de cocción según la recomendación del fabricante (Serrano et al. 2015b). Los contenidos de ENs se analizan tanto en las muestras de pasta cruda, como las muestras de pasta cocida y del agua de cocción, según los métodos optimizados previamente en el laboratorio (Serrano et al. 2013a; Serrano et al. 2015c).

El proceso de cocción de la pasta da lugar a la reducción prácticamente total de las ENs tipo A, sin diferencias en el tipo de pasta. La ENB es la micotoxina más estable, con reducciones del 14%-49%. La reducción de la ENB_1 es del 53-65%.

Los contenidos detectados en el agua de cocción son inferiores a 0.05 mg Kg^{-1}, siendo en la mayoría de casos no detectables. Por tanto, la tasa de transferencia de las ENs desde la pasta al agua de cocción es prácticamente despreciable, por lo que no se puede atribuir la reducción de las ENs a este proceso, como ocurre con otras micotoxinas como el DON (Visconti et al., 2004; Cano-Sancho et al., 2013). Las reducciones obtenidas en este estudio pueden ser debidas a la modificación estructural de las micotoxinas de interés, como consecuencia de las elevadas temperaturas aplicadas durante los tratamientos (ver sección 4.6.4.).

4.6.3. Estrategia de descontaminación

Las estrategias de descontaminación propuestas se basan en la modificación del pH del agua de cocción a un pH ácido o básico. Habitualmente, se han desarrollado estudios de este tipo para otras micotoxinas empleando sustancias químicas para tamponar el agua de cocción (Karaca y Nas, 2009; Mishra et al., 2014). Sin embargo, estos estudios carecen de aplicación en sistemas reales de alimentos, puesto que emplean sustancias no aptas para el consumo humano. Es por ello que en el presente estudio se seleccionan compuestos con las características de ser estables al tratamiento aplicado, no tóxicos y solubles en agua. El proceso de cocción a pH ácido (pH 4) se realiza con zumo de limón, y el proceso en medio básico (pH 9) se realiza con carbonato sódico habitualmente empleado como aditivo alimentario (Serrano et al., 2015b).

El proceso a pH ácido da lugar a elevados porcentajes de reducción para todas las ENs (86-100%), con concentraciones finales en la pasta cocida de nd-0.18 mg Kg^{-1}. Las reducciones con la estrategia a pH básico son considerablemente inferiores, obteniéndose concentraciones finales de nd-1.10 mg Kg^{-1}, y por tanto se descarta como una estrategia efectiva para reducir los niveles de las micotoxinas objeto de estudio.

La estrategia de descontaminación aplicando pH ácido es un excelente protocolo para reducir los niveles de ENs, pudiéndose realizar en un futuro la adaptación de esta estrategia tanto en la industria como en los hogares.

4.6.4. Productos de degradación

Un aspecto importante a considerar cuando se reducen los niveles de micotoxinas a través de los tratamientos tecnológicos, es que 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 de degradación y/o conjugación, con una toxicidad diferente a la del compuesto inicial. En este sentido, la detección e identificación de los compuestos generados así como la realización de estudios para evaluar su toxicidad, es un importante aspecto a considerar durante el proceso de evaluación del riesgo (Broekaert et al., 2015).

La identificación de productos de degradación y/o conjugación de las micotoxinas tras el procesado de los alimentos, puede llegar a ser muy compleja debido a la gran cantidad de compuestos que pueden interactuar con la micotoxina en la matriz alimentaria. Debido a ello se realiza un estudio preliminar para evaluar el comportamiento de las micotoxinas emergentes en sistemas modelo: se plantea un sistema modelo acuoso y un sistema modelo compuesto por los principales componentes de la pasta (almidón, gluten y albúmina). Estos sistemas se someten a una temperatura de 100ºC durante 5, 10 y 15 min, simulando las condiciones de cocción de la pasta (Serrano et al., 2013c).

Los resultados en el sistema modelo acuoso evidencian elevadas reducciones tras 15 min de cocción (82-100%), siendo de menor orden de magnitud las reducciones obtenidas tras 5 min y 10 min. Sin embargo, las reducciones obtenidas en el sistema modelo alimentario son similares a los tres tiempos ensayados (81-100%), sin diferencias estadísticamente significativas.

Estos resultados ponen en evidencia la gran diferencia de comportamiento que pueden presentar las ENs dependiendo del sustrato en el que estén presentes.

La elucidación de los productos de degradación derivados de las ENs se lleva a cabo mediante inyección de las muestras en un LC-MS con una trampa lineal de iones (LC-LIT-MS) en la modalidad de "barrido completo" (SCAN). El empleo de esta técnica ha permitido elucidar estructuralmente compuestos de degradación de las ENs, formados tras la aplicación de los tratamientos térmicos. En los cromatogramas obtenidos por LC-LIT-MS se observa un aumento del tamaño del pico correspondiente a los productos de degradación proporcional al aumento del tiempo de calentamiento y a la disminución de las ENs (Serrano et al., 2013c).

Los productos de degradación derivados de la ENA presentan la estructura de la ENA con la pérdida de alguno de sus componentes estructurales, principalmente isoleucina (Ile) o un fragmento de ácido 2-hidroxi-3-methilbutanoico (HyLv). En el caso de la ENA_1, se identifica un único producto de degradación caracterizado por la estructura de la ENA_1 con la pérdida de una unidad de valina (Val). Con respecto a las ENs tipo B, se identificaron dos productos de degradación caracterizados por la estructura de la ENB y la ENB_1 con la pérdida de una unidad de Val y otros dos productos caracterizados por la pérdida de una unidad de HyLv (Serrano et al., 2013c).

Una vez elucidados los productos de degradación en los sistemas modelo, se analizan las muestras de pasta y de agua del estudio de cocción por LC-LIT-MS (Serrano et al., 2015b). De los productos de degradación formados en los sistemas modelo únicamente se detectan dos compuestos derivados de la degradación de la ENA, caracterizados por la pérdida de una unidad de Ile y de una unidad de HyLv. No obstante, dada la complejidad de la matriz y las

posibles reacciones que pueden tener lugar durante el tratamiento térmico, el número de productos de degradación formados durante este proceso podría ser superiror.

Hasta el momento ningún estudio ha evaluado la formación de los productos derivados de la degradación de las ENs tras los tratamientos tecnológicos. No obstante, existen diversos estudios para otras micotoxinas de *Fusarium* que han identificado productos de degradación (Humpf y Voss, 2004; Bretz et al., 2006; Meca et al., 2012b). Algunos de ellos han comparado la toxicidad de estos compuestos con la toxicidad de la micotoxina inicial, concluyendo 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 la hipótesis de que la estructura de las ENs sufre modificaciones durante los tratamientos a los que son sometidos los alimentos. Se deberían realizar futuros estudios para la identificación de estos productos en otros grupos de alimentos, así como para evaluar su toxicidad.

4.6.5. Evaluación del almacenamiento de la pasta

Durante el almacenamiento del alimento el contenido de las micotoxinas puede ser modificado, bien por un aumento de la concentración, si se produce desarrollo fúngico, o bien por reducción respecto a los niveles iniciales (Sørensen et al., 2008; Mohale et al., 2013). En este sentido, en el presente estudio se han evaluado los contenidos de las ENs durante un año de almacenamiento a 25ºC y 4ºC de tres tipos de pasta (blanca, integral y con huevo). La determinación de los contenidos de ENs se realiza una vez al mes durante un año, tomando como punto 0 el análisis de la pasta en el momento del envasado. Asimismo, también se realiza la evaluación de dos parámetros

que pueden estar relacionados con el contenido en micotoxinas: la a_w y el color de las muestras (Serrano et al., 2015d).

Tras un año de almacenamiento, se observa una reducción progresiva en los niveles de ENs. Se han estimado reducciones del 33-57% para las ENs tipo A, mientras que las ENs tipo B presentan reducciones del 11-33%. No se han observado diferencias estadísticamente significativas en los resultados en cuanto a la temperatura de almacenamiento ni en cuanto al tipo de pasta.

Considerando la suma de las cuatro ENs, se ha determinado que las reducciones en los contenidos son significativas a partir del 6º mes de almacenamiento, con un nivel de confianza del 95%. Se ha realizado un estudio aplicando el análisis de regresión múltiple para evaluar la tendencia de reducción de las cuatro ENs durante el almacenamiento. De este estudio se concluye que las 4 ENs se comportan de acuerdo a un modelo lineal, con una correlación negativa entre los niveles de concentración y el tiempo de almacenamiento. El modelo lineal explica el 80-95% de los resultados obtenidos experimentalmente.

En cuanto a los resultados de la a_w, es de destacar que los valores inicialmente determinados en las muestras aumentan en 0.1 tras 12 meses de almacenamiento, obteniéndose valores de 0.46-0.51. Dado que el valor de a_w para el crecimiento del hongo debe ser superior a 0.7, y de acuerdo a los resultados obtenidos en este estudio, se descarta producción de micotoxinas por una posible contaminación fúngica (Gnonlonfin et al., 2008).

Por otra parte, es de destacar que un aumento en la a_w puede llegar a condicionar diversos procesos como el pardeamiento no enzimático, la degradación de vitaminas y la destrucción de pigmentos influyendo por tanto, en el color final de la muestra. El oscurecimiento no enzimático producido

como consecuencia de la Reacción de Maillard (RM), puede estar influenciado por otros factores como la temperatura y la composición del alimento (Acquistucci, 2000). Los productos de la RM son el resultado de reacciones no enzimáticas entre los grupos amino libres de los péptidos y los grupos carbonilo de los azúcares reductores. En este sentido, las ENs presentan una estructura con potencial para participar en la RM, principalmente debido a los grupos amino que se encuentran alternados en el ciclo del hexadepsipéptido. Es por ello, que se consideró de gran interés el estudio de la evolución del color en una tentativa de correlacionar los valores obtenidos con los contenidos de ENs.

Los resultados obtenidos de la evolución del color muestran una disminución significativa del parámetro L*, lo que explica el oscurecimiento de la muestra, y cierto aumento del parámetro a*, demostrando un incremento en las tonalidades rojizas. El parámetro b* (color amarillo) se mantuvo constante con el tiempo. En este sentido, algunos estudios han indicado que el aumento del parámetro a* y la disminución del L* en las muestras de pasta están asociados a la formación de productos de las etapas intermedias o avanzadas de la RM, mientras que las variaciones en el componente b* se deben a las etapas iniciales de la RM (De Pilli et al., 2014; Pasqualone et al., 2014).

El análisis multivariado de los datos ha permitido establecer que existe una fuerte correlación entre la reducción en las concentraciones de las ENs y el aumento de color de las muestras, con un coeficiente de correlación de Pearson de -0.7162. Estos resultados demuestran la posible participación de las ENs en la RM.

Los resultados obtenidos como consecuencia de los tratamientos térmicos y el almacenamiento de la pasta han dado lugar reducciones considerables en los contenidos de ENs, disminuyendo la exposición de la población. No obstante, se plantea como principal hipótesis la posibilidad de que la reducción observada en los contenidos de ENs durante el almacenamiento y durante los tratamientos térmicos de elaboración y cocción de la pasta, pueda ser atribuida a la participación de estas micotoxinas en las etapas de la RM. Es necesario estudiar el mecanismo de reducción de estas micotoxinas durante los diferentes procesos a los que son sometidos los alimentos. Todo ello, permitirá establecer si el comportamiento de las micotoxinas emergentes de *Fusarium* en alimentos procesados representa riesgo para la salud de la población.

5. Conclusions

CONCLUSIONES

1. La revisión bibliográfica realizada pone de manifiesto que se deben realizar un mayor número de estudios para evaluar el riesgo de las micotoxinas.

2. La metodología analítica validada ha permitido la determinación de las aflatoxinas, ocratoxina A y micotoxinas de *Fusarium* en cereales y productos derivados, agua y fluidos biológicos. El nivalenol y las micotoxinas emergentes de *Fusarium* son las micotoxinas con una mayor prevalencia en las muestras de cereales y productos derivados.

3. Un elevado porcentaje de muestras ha presentado varias micotoxinas simultáneamente, destacando las combinaciones de micotoxinas emergentes, de nivalenol y beauvericina, y de nivalenol con alguna aflatoxina.

4. El estudio de bioaccesibilidad pone de manifiesto que las concentraciones de micotoxinas emergentes accesibles son inferiores a las presentes en papillas infantiles.

5. La estimación de la ingesta de aflatoxinas, ocratoxina A y nivalenol por consumo de cereales y productos derivados representa un riesgo potencial para la salud.

6. La estimación de la ingesta de micotoxinas emergentes de *Fusarium* por consumo de pasta no representa riesgo apreciable para la población, mientras que por consumo de papillas infantiles puede representar riesgo potencial.

7. La aplicación de elevadas temperaturas y modificaciones de pH durante los tratamientos tecnológicos, así como largos tiempos de almacenamiento causan reducciones en los contenidos de las micotoxinas emergentes, dando lugar a la formación de nuevos compuestos de degradación de toxicidad desconocida.

8. Se deberían realizar más estudios sobre la presencia de micotoxinas en alimentos, de consumo de la población, y de la influencia de los tratamientos tecnológicos para realizar una evaluación del riesgo con menor incertidumbre como objetivo prioritario en seguridad alimentaria.

CONCLUSIONS

1. The bibliographic review highlighted that more studies should be performed to assess the risk of mycotoxins.

2. The validated analysis methodology allowed the determination of aflatoxins, ochratoxin A and *Fusarium* mycotoxins in cereals and products derived from them, water and biological fluids. Nivalenol and emerging *Fusarium* mycotoxins were the mycotoxins with the highest prevalence in the samples of cereals and cereal-based products.

3. Various mycotoxins co-occurred in a high percentage of samples. Among the most common combinations were of the emerging mycotoxins, nivalenol and beauvericin, and nivalenol with one of the aflatoxins.

4. The bioaccessibility study indicated that the accessible concentrations of emerging mycotoxins were lower than those found in infant formula.

5. The estimated intake of aflatoxins, ochratoxin A and nivalenol from consumption of cereals and cereal-based products represents a potential health risk.

6. The estimated intake of emerging *Fusarium* mycotoxins through pasta consumption did not represent an appreciable risk to the population, whereas the consumption of infant formula may represent a potential risk.

7. The application of high temperatures and pH modifications during industrial treatments, as well as long storage times, result in reductions on

emerging mycotoxins contents, and give rise to the formation of new degradation compounds with unknown toxicity.

8. More studies should be performed on the occurrence of mycotoxins in foodstuffs, consumption in population, and the influence of industrial treatments, in order to reduce the uncertainty in risk assessment which is an imperative objective in food safety.

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Anexo I

Difusión de resultados

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Comparative assessment of three extraction procedures for determination of emerging *Fusarium* mycotoxins in pasta by LC–MS/MS

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ABSTRACT

A new rapid, sensitive, reproducible and reliable method was developed for the quantitative determination of enniatins A, A_1, B and B_1, beauvericin and fusaproliferin in dry and fresh pasta by liquid chromatography-triple quadrupole-tandem mass spectrometry. A comparative study of different rapid and economical extraction procedures was performed for the extraction of these mycotoxins in pasta. For this purpose, three different approaches were studied during the extraction step (Ultra-Turrax, ultrasonic bath and microwave). Optimal extraction conditions were reached using Ultra-Turrax with acetonitrile for 3 min without purification step. The chromatographic separation of the six mycotoxins was accomplished in 15 min. The method was extensively validated with satisfactory results: recovery rates ranged from 86 to 112% and the relative standard deviations were lower than 15%. Limits of detection ranged from 0.02 to 0.15 μg kg^{-1}. The applicability of the method was assessed with the analysis of 30 samples of dry and fresh pasta.

1. Introduction

Mycotoxins are a group of natural substances produced as a result of the secondary metabolism of different species of filamentous fungi, which have been found occurring in foods and feeds. A wide range of undesirable effects on consumer health and economical losses in the world caused by the mycotoxins have been reported during years. The genus *Aspergillus, Fusarium, Penicillium, Claviceps* and *Alternaria* are the major species capable of producing mycotoxins in agricultural commodities during their growth period, and during harvest and post-harvest period under certain environmental conditions (Hussein & Brasel, 2001). According to the Scientific Committee on Food (SCF), the *Fusarium* fungi are probably the most prevalent toxin producing fungi in the temperate regions of America, Europe and Asia (SCF, 2002). The genus *Fusarium* includes more than 100 different phytopathogenic fungi species. Some of these species are known to produce mycotoxins such as deoxynivalenol, zearalenone or fumonisins, for which the European Union has set maximum levels (MLs) in certain foodstuffs (Commission Regulation, 2006b). Also, several *Fusarium* species can produce other mycotoxins without established MLs, such as enniatins (ENs), beauvericin (BEA) and fusaproliferin (FUS), usually called emerging *Fusarium* mycotoxins. Over the last few years, emerging *Fusarium* mycotoxins have acquired importance because of their toxic effects in human and animal health such as cardiotoxicity, cytotoxicity or teratogenicity (Jestoi, 2008). Enniatins A, A_1, B and B_1 (ENA, ENA_1, ENB and ENB_1, respectively) and BEA are ionophoric compounds with similar toxicodynamic actions, which possess similar chemical structures with a cyclic hexadepsipeptide distinguished only by alternating amino acid residues (Fig. 2). FUS is a bicyclic sesterterpene derived from five isoprenic units (Fig. 1). These compounds present similar polarity, both with a hydrophilic and hydrophobic environment (Jestoi, 2008).

Limited studies exist about their incidence in food, which have indicated their presence mainly in grain cereals and in cereal products from different countries in the world (Argentina, South Africa, Morocco, Spain, Italy, Finland, etc.). The highest concentration levels have been found in samples from countries characterized by a Mediterranean climate such as Tunisia, Spain or Morocco (Meca, Zinedine, Blesa, Font, & Mañes, 2010; Oueslati, Meca, Mliki, Ghobel, & Mañes, 2011; Sifou et al., 2011). To carry out the analysis of mycotoxins, specific procedures are required for the extraction and removal of impurities using clean-up steps. Ultra-Turrax and rotatory shaker are the most widely used approaches for the extraction of emerging *Fusarium* mycotoxins. Ultra-Turrax provides extracts with few matrix interferences, so it does not need further

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Co-occurrence and risk assessment of mycotoxins in food and diet from Mediterranean area

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ABSTRACT

The contents of 14 mycotoxins were studied in samples of different cereals and cereal products from four countries of the Mediterranean region. Two hundred and sixty-five samples from Spain, Italy, Morocco and Tunisia were analysed. Samples were extracted with matrix solid-phase dispersion (MSPD) and determined by liquid chromatography–tandem mass spectrometry with a triple quadrupole mass analyser. The percentage of total samples contaminated was 53%. The frequency of contaminated samples from Spain, Italy, Tunisia and Morocco was 33%, 52%, 96% and 50%, respectively. Nivalenol and beauvericin were the most predominant mycotoxins. This is the first international report to study the presence of several mycotoxins in different types of cereal (rice, wheat, maize, rye, barley, oat, spelt and sorghum) and cereal products (snacks, pasta, soup, biscuits and flour) from the Mediterranean area, estimate the intake of mycotoxins and evaluate the risk assessment.

1. Introduction

Mycotoxins are common contaminants of many grains, such as wheat, barley, maize and rice, and they can possess a broad range of toxic properties, including carcinogenicity, neurotoxicity, as well as reproductive and developmental toxicity. The total number of mycotoxins is not known, but the number of potential toxic metabolites of fungi has been estimated to be in the thousands, although to date only about 300 different mycotoxins have been identified (Krska et al., 2008).

To ensure food safety, the European Union has established maximum levels (MLs) for some mycotoxins (zearalenone, ochratoxin A, deoxynivalenol, aflatoxins and fumonisins) in human foods (EC 401/2006), whereas for others (e.g., nivalenol and beauvericin) maximum tolerable levels have not yet been proposed. For the performance of the current legislation, it is fundamental to have reliable and accurate mycotoxin analytical methods. An important and critical step is sample preparation and sample clean-up. Different strategies have been performed according to the sample and analyte properties, including solid phase extraction (SPE), matrix solid-phase dispersion (MSPD), liquid–liquid and solid–liquid partitioning, accelerated solvent extraction (ASE), multifunctional columns (MFC) and immunoaffinity columns (IAC) (Krska et al., 2008; Rubert, Soler, & Mañes, 2011). Several analytical methods were used to measure mycotoxins, including enzyme-linked immunosorbent assay (ELISA) and gas chromatography (GC) with mass spectrometric detection (MS). Nowadays, liquid chromatography (LC) based methods are the most frequently applied, with several analysers such as fluorescence detector (FLD), single-quadrupole mass spectrometer (MS), time-of-flight mass spectrometer (TOFMS), triple-quadrupole tandem mass spectrometer (MS/MS QqQ) and ion trap tandem mass spectrometer (MS/MS QTrap) (Krska et al., 2008). In the last few years, increased efforts have been made to develop analytical methods for the detection of very low concentrations of mycotoxins in cereal samples and for the simultaneous determination of different classes of mycotoxins using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Recently, in our laboratory, a new rapid, sensitive and reproducible analytical strategy has been developed to determine 14 mycotoxins in cereal products using MSPD and LC–MS/MS with a triple quadrupole (QqQ) mass analyser (Rubert et al., 2011).

Several studies have reported high contamination in a wide variety of important agricultural products worldwide; usually fumonisins (FBs), trichothecenes (TCs), zearalenone (ZEN), aflatoxins (AFs), ochratoxin A (OTA) and beauvericin (BEA) have been found to contaminate grains and cereal-based products (corn, rice, sorghum, wheat, barley and oats) (Adejumo, Hettwer, & Karlovsky, 2007; Araguás, González-Peñas, & López De Cerain, 2005; Gaumy, Bailly, Burgat, & Guerre, 2001; González-Osnaya, Cortés, Soriano, Moltó, & Mañes, 2011; Soriano & Dragacci, 2004). Specifically, the Mediterranean area has a climate characterised by high humidity and high temperature which favour the natural occurrence of mycotoxins pre-harvest and/or during transport, processing or storage (SCF, 2002).

In 2001 the Joint FAO/WHO Expert Committee on Food Additives (JECFA), established provisional maximum tolerable daily

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Emerging *Fusarium* mycotoxins in organic and conventional pasta collected in Spain

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ABSTRACT

One of the main sources of emerging *Fusarium* mycotoxins in human nutrition is the cereals and cereal products. In this study, an analytical method to determine enniatins A, A1, B and B1 (ENs), beauvericin (BEA) and fusaproliferin (FUS) based on Ultra-Turrax extraction followed by liquid chromatography coupled to triple quadrupole mass spectrometer detector (MS/MS QqQ), was applied for the analysis of pasta. For this purpose, 114 commercial samples of pasta were acquired from supermarkets located in Valencia. The results showed higher frequencies of contamination in organic pasta than in conventional pasta, while the concentration levels were variable for both types of pasta. In positive samples, BEA levels varied from 0.10 to 20.96 μg/kg and FUS levels varied from 0.05 to 8.02 μg/kg. ENs levels ranged from 0.25 to 979.56 μg/kg, though the majority of the values were below 25 μg/kg. Besides, it was observed the simultaneous presence of two or more mycotoxins in a high percentage of the samples. Finally, an evaluation of the dietary exposure of the emerging *Fusarium* mycotoxins was performed in the Spanish population. The prevalence of ENs, BEA and FUS in cereal products suggests that the toxins may pose a health risk to Spanish population.

1. Introduction

Wheat is the most consumed cereal worldwide: global consumption of cereals (excluding beer) was 146.60 kg/*per capita* during 2007, and the global consumption for wheat reached about 66 kg/*per capita* (FAO, 2007). The Spanish population is one of the largest consumer of wheat in the world: the mean Spanish consumption of wheat (87.40 kg/*per capita*) was higher respect to the worldwide consumption in the year 2007 (FAO, 2007). Usually, wheat is ground to flour for the production of bread, pasta, biscuits and other products. Nowadays, pasta constitutes one of the most important wheat-based products of the Spanish economy: during the last years global consumption of pasta in Spain has increased around 6% between 2007 and 2011 (MAGRAMA, 2011).

Wheat-based products are one of the main sources of mycotoxins in both the human and animal diets. Surveillance studies have indicated that mycotoxin contamination is a world-wide problem (SCOOP, 2003), since it causes economic losses, both for the grain and for the marketing of foods and feeds, and is a potential threat to animal and human health. In 2006, the European Union proposed the maximum levels (MLs) for some mycotoxins in foodstuffs: aflatoxins, fumonisins and trichothecenes among other (Commission Regulation, 2006). For some *Fusarium* mycotoxins, MLs have been established mainly for cereals and cereal-based products, since usually *Fusarium* species are able to infect cereal crops. The contamination of mycotoxins in cereals is known to be affected by the local climate (rainfall, temperature or relative humidity), agricultural practices, harvest logistics, transport and storage conditions, and processing of products (Bakan et al., 2002). Specially, in the last few years the relationship between the influence of the agricultural practices (traditional or organic) and mycotoxin contamination have been discussed by many authors. In organic practices, chemical products (fungicides, pesticides, etc.) are no employed increasing the exposure of cereal grains to fungal colonization and to mycotoxin production. Some studies have supported this affirmation (ÁArco et al., 2009; Silva et al., 2009), but other studies have not observed significantly differences between organic and conventional products (González-Osnaya et al., 2007; Ariño et al., 2007). This fact can be due to the crop rotation used in organic and conventional agricultural practices, which prevents the transmission of plant diseases (Jestoi et al., 2004b).

Fusarium genus is probably the most prevalent toxin-producing fungi of the Northern temperate region. They are commonly found on cereals grown in the temperate regions of America, Europe and Asia (SCF, 2002). *Fusarium avenacum, Fusarium moniliforme, Fusarium proliferatum* and *Fusarium subglutinans* are the main producers of emerging *Fusarium* mycotoxins enniatins A, A1, B, B1 (ENA, ENA1, ENB and ENB1, respectively), beauvericin (BEA) and fusaproliferin (FUS) in various cereals, especially wheat, barley and maize (Jestoi, 2008). FUS is a bicyclic sesterterpene (Fig. 1) and enniatins (ENs) and BEA possess a cyclic hexadepsipeptide structure (Fig. 2). At the present, MLs have not been set for emerging *Fusarium*

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Risk assessment associated to the intake of the emerging *Fusarium* mycotoxins BEA, ENs and FUS present in infant formula of Spanish origin

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ABSTRACT

Forty-five samples of Spanish follow-up infant formula with different chemical compositions were analyzed determining the emerging *Fusarium* mycotoxins beauvericin (BEA), enniatins (ENs) (A, A_1, B, B_1), and fusaproliferin (FUS). The samples were extracted three times with ethyl acetate using an Ultra-turrax homogenizer. Mycotoxins were identified and quantified using a liquid chromatography (LC) coupled to a diode array detector (DAD). Results showed that the percentage of the samples contaminated with ENs and FUS were 46.6 and 20.0% respectively, whereas all analyzed samples were free of BEA. The ENs A and B were detected only in one sample with 149.6 and 39.4 mg/kg respectively. The ENB_1 was the more detected mycotoxin with levels ranging from 11.4 to 41.9 mg/kg. The ENA_1 was detected at levels ranging from 6.3 to 101.7 mg/kg. The minor *Fusarium* mycotoxin FUS was detected in a range variable from 0.7 to 1.7 mg/kg. Finally, dietary exposure of Spanish infants (between 6 and 12 months) to ENs, BEA and FUS, was estimated through the consumption of commercial follow-up infant formula by the calculation of the estimated daily intake (EDI). Considering the sum of the mycotoxins studied, the data evidenced that the EDI was 236.2 μg/kg bw/day.

1. Introduction

Mycotoxins are naturally occurring toxic secondary metabolites produced under appropriate favorable conditions by filamentous fungi, mainly *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. Among the mycotoxins produced by *Fusarium* spp., are included the enniatins A, A_1, B and B_1 (ENA, ENA_1, ENB and ENB_1), beauvericin (BEA) and fusaproliferin (FUS), which are a group of bioactive compounds called emerging mycotoxins or minor *Fusarium* mycotoxins (Meca, Zinedine, Blesa, Font, & Mañes, 2010). FUS is a bicyclic sesterterpene formed by five isoprenoid units, whereas BEA and enniatins (ENs) are cyclic hexadepsipeptides. In particular BEA is composed by of alternated D-α-hydroxyisovaleryl-(2-hydroxy-3-methylbutanoic acid) and N-methyl-L-phenylalanyl residues, whereas ENs are of three D-α-hidroxyisovaleryl and three N-methyl-L-amino acid residues (Jestoi, 2008). BEA and ENs show a similar chemical structures, and therefore can be assumed that present the same toxic dynamic actions: has been demonstrated that have cytotoxic effects in several cell lines (Ferrer, Juan-García, Font, & Ruiz, 2009; Fornelli, Minervini, & Logrieco, 2004; Kamyar, Rawnduzi, Studenik, Kouri, & Lemmens- Gruber, 2004); whereas FUS showed causes teratogenic effects on chicken embryos (Ritieni et al., 1997). Due to these toxic effects, the contamination by mycotoxins causes severe economic losses annually, and depends of several factors including climatic conditions, genetic susceptibility of cultivars to fungal infection, soil type and nutritional factors (Bakan, Melcion, Richard-Molard, & Cahagnier, 2002).

Several analytical methods have been described in the literature for the detection of minor *Fusarium* mycotoxins in several foods. The methods include the common steps applied in mycotoxin analyses: sample preparation using extraction of the analytes and removal of impurities using different types of columns. Sample analysis is carried out using high performance liquid chromatography (LC) with ultraviolet (UV) or mass spectrometric (MS) detection (Jestoi, Rokka, Rizzo, & Peltonen, 2005; Santini, Ferracane, Meca, & Ritieni, 2009). The presence of ENs, BEA and FUS in food commodities has been recently reported during the last decade in some European countries (Finland, Norway, Spain, Slovakia, Croatia, Switzerland and Italy), in USA, in South Africa and in Australia. Recently our research group has reported the contamination of cereals (maize, wheat and barley) and cereal products (breakfast cereals) available in Morocco and Spain (Mahnine et al., 2011; Meca et al., 2010; Sifou et al., 2011; Zinedine, Meca, Mañes, & Font, 2011). Mahnine et al. (2011) studied the presence of ENs, BEA and FUS in breakfast and infant cereals from Morocco, evidencing principally the presence of the ENB_1 and ENA_1.

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Risk assessment of beauvericin, enniatins and fusaproliferin present in follow-up infant formula by *in vitro* evaluation of the duodenal and colonic bioaccessibility

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ABSTRACT

In this study, 72 samples of follow-up infant formula of Spanish origin were analyzed for the presence of the mycotoxins beauvericin (BEA), enniatins (ENs) (A, A_1, B, B_1), and fusaproliferin (FUS). The samples analyzed were extracted three times with ethyl acetate and then the mycotoxins were identified and quantified using a liquid chromatography (LC) coupled to a diode array detector (DAD).

The positive samples analyzed in this study were digested through a simulated gastrointestinal digestion model, which permit to simulate the physiological condition of the human gastrointestinal tract (duodenal and colonic compartments) in order to assess the bioaccessibility of the bioactive compounds contained in the follow-up infant formula, and to perform the approach to the risk assessment related to the intake of these bioactive compounds.

The EN B, was detected in 12 of the totally positive samples with a mean value of 32.3 ± 1.2 mg/kg, whereas the EN A was evidenced only in one sample with a value of 149.62 ± 2.36 mg/kg. The EN A_1 presented a mean contamination of 55.36 ± 2.1 mg/kg.

The duodenal bioaccessibility of the bioactive compounds present in the analyzed samples ranged from 0.37 to 22.41%, whereas employing the duodenal + colonic digestion the mean bioaccessibility of the mycotoxins studied ranged from 1.63 to 29.00%.

1. Introduction

Mycotoxins are secondary metabolites which are produced by several fungi mainly belonging to the genera: *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* (Sweeney & Dobson, 1998). Until now, approximately 400 secondary toxic metabolites produced by more than 100 molds have been reported.

The enniatins (ENs) are fungal metabolites produced by several *Fusarium* species (Ivanova, Skjerve, Eriksen, & Uhlig, 2006). They are cyclic depsipeptides, which are commonly composed of three D-α-hydroxyisovaleric acid residues linked alternatively to three L-configured *N*-methyl amino acid residues to give on 18-membered cyclic skeleton. ENs are described as phytotoxins, with antibiotic, herbicidal and insecticidal activities. They also have enzymatic inhibiting and oxidative phosphorylation properties (Tomoda et al., 1992). The cytotoxicity studies of ENs have been studied *in vitro* test assays in rodent, monkey, porcine, insect and human cell lines (Behm, Degen, & Follmann, 2009; Fornelli, Minervini, & Logrieco, 2004; Hyun, Lee, Lee, & Shin, 2009; Ivanova et al., 2006; Lee et al., 2008; Meca, Font, & Ruiz, 2011; Tonshin, Teplova, Andersson, & Salkinoja-Salonen, 2010; Vongvilai et al., 2004).

Fusaproliferin (FUS) is a recently described mycotoxin that is produced by two closely related species, *Fusarium subglutinans* and *Fusarium proliferatum* (Ritieni et al., 1995, 1997). Both species are important pathogens of maize (Kommedahl, Sabet, Burnes, & Windels, 1987; Ritieni et al., 1999) and other economically important crop plants and may be isolated together from the same plant (Moretti, Mulè, Ritieni, & Logrieco, 2007). FUS is toxic to *Artemia salina* (Moretti et al., 2007; Steenkamp, Wingfield, Desjardins, Marasas, & Wingfield, 2002), IARC/LCL 171 human B lymphocytes (Logrieco et al., 1996) and SF-9 insect cells (Ritieni et al., 1997) and has teratogenic and pathogenic effects on chicken embryos (Logrieco et al., 1996). Recently the occurrence of FUS in several naturally contaminated samples of corn was reported (Jestoi, 2008).

The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body for absorption. The release of contaminant in the gastrointestinal tract,

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Degradation study of enniatins by liquid chromatography–triple quadrupole linear ion trap mass spectrometry

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ABSTRACT

Enniatins A, A_1, B and B_1 (ENs) are mycotoxins produced by *Fusarium* spp. and are normal contaminants of cereals and derivate products. In this study, the stability of ENs was evaluated during food processing by simulation of pasta cooking. Thermal treatments at different incubation times (5, 10 and 15 min) and different pH (4, 7 and 10) were applied in an aqueous system and pasta resembling system (PRS). The concentrations of the targeted mycotoxins were determined using liquid chromatography coupled to tandem mass spectrometry. High percentages of ENs reduction (81–100%) were evidenced in the PRS after the treatments at 5, 10 and 15 min of incubation. In contrast to the PRS, an important reduction of the ENs was obtained in the aqueous system after 15 min of incubation (82–100%). In general, no significant differences were observed between acid, neutral and basic solutions. Finally, several ENs degradation products were identified using the technique of liquid chromatography–triple quadrupole linear ion trap mass spectrometry.

1. Introduction

The *Fusarium* genus is the most prevalent toxin-producing fungi of the Northem temperate region (SCF, 2002). Several *Fusarium species as avenacum*, *moniliforme*, *proliferatum* and *subglutinans* are producers of some minor *Fusarium* mycotoxins called enniatins (ENs). These bioactive compounds are cyclic hexadepsipeptides formed by alternating of the D-α-hydroxy-isovaleric acid (HyLv) and different N-methylamino acid residues as valine (Val) and isoleucine (Ile). The ENs are classified as ionophoric compounds, forming stable molecules with a "sandwich" structure with alkali metals or alkaline earth metals, across human cell membranes (Jestoi, 2008).

In vitro studies have demonstrated that ENs evidenced cytotoxic activity in different cell lines, including rodent (V79), lepidopteran (SF-9), monkey (Vero) and human cells (Caco-2, Hep-G2, HT-29) (Behm, Degen, & Föllmann, 2009; Fornelli, Minervini, & Logrieco, 2004). Generally, the contamination levels by ENs evidenced in cereals collected in Mediterranean area is higher than the data evidenced in Central and Northern European regions, probably due to the different clime condition of these two different parts of the continent (Santini, Meca, Uhlig, & Ritieni, 2012). ENs have been detected in processed products containing essential cereals for adult and infant nutrition, such as breakfast cereals, rice, pasta, infant formula, bread mill and other derived products (Jestoi, 2008; Serrano, Font, Mañes, & Ferrer, 2013a).

Several studies have been published related to the mitigation strategies of mycotoxins in food, focalized principally on the reduction of the trichothecenes, fumonisins (FBs), aflatoxins (AFs), ochratoxin A (OTA), patulin (PAT) and zearalenone (ZEA) during food processing (Abramson, House, & Nyachoti, 2005; Bullerman & Bianchini, 2007; Cramer, Königs, & Humpf, 2008; Kushiro, 2008; Park, Scott, Lau, & Lewis, 2004; Ryu, Hanna, Eskridge, & Bullerman, 2003). At the moment, only two studies are available in the scientific literature on the thermal degradation of the minor *Fusarium* mycotoxins. In particular, Meca, Ritieni, and Mañes (2012) studied beauvericin (BEA) stability during several heat treatments, in a model system and also in homemade crispy bread, obtaining percentages of degradation variables from 20% to 90%. Vaclavikova et al. (2013) determined ENs levels during beer and bread production, concluding that ENs concentrations were reduced during breadmaking (from 71% to 79% in milling and from 50% to 60% in baking), whereas these mycotoxins were not detected in the final beer.

Nevertheless, other studies have focused on the identification of the mycotoxins degradation products formed after the treatments, as well as in the evaluation of the toxicity of these new identified compounds. Meca, Luciano, Zhou, Tsao, and Mañes (2012) studied the stability of BEA in a solution model and in wheat flour using allyl isothiocyanate (AITC) as a reactant. Two reaction products between the bioactive compounds employed in this study were identified by LC–MS-LIT, corresponding to BEA conjugates

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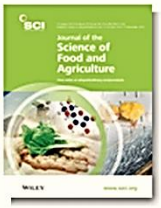

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