VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

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

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ANALYTICAL STUDY AND EXPOSURE TO *FUSARIUM* MYCOTOXINS

ESTUDIO ANALÍTICO Y DE EXPOSICIÓN A MICOTOXINAS DE *FUSARIUM*

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Presentada per: Dirigida per:

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El **Dr. Jordi Mañes Vinuesa**, Catedràtic de Universitat de l'àrea de Nutrició i Bromatologia i la **Dra. Houda Berrada Ramdani**, Professora Titular de l'àrea de Toxicologia,

CERTIFIQUEN QUE:

El llicenciat en Ciència i Tecnologia dels Aliments **Yelko Rodríguez Carrasco** ha realitzat baix la seua direcció el treball **"Estudi analític i d'exposició a micotoxines de *Fusarium*"**, i autoritzen la seua presentació per a optar al títol de Doctor per la Universitat de València.

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

Burjassot (València), Abril 2015.

Dr. Jordi Mañes

Dra. Houda Berrada

Aquest treball ha sigut plasmat en 10 articles, publicats o que es publicaran en les següents revistes:

1. *Multi-mycotoxin analysis in wheat semolina using an acetonitrile-based extraction procedure and gas chromatography–tandem mass spectrometry. Journal of Chromatography A, 1270 (2012) 28 – 40. Impact factor: 4.258*
2. *Quantitative determination of trichothecenes in breadsticks by gas chromatography-triple quadrupole tandem mass spectrometry. Food Additives & Contaminants: Part A, 31 (2014) 1422 – 1430. Impact factor: 2.341*
3. *A survey of trichothecenes, zearalenone and patulin in milled grain-based products using GC–MS/MS. Food Chemistry, 146 (2014) 212 – 219. Impact factor: 3.259*
4. *Occurrence of Fusarium mycotoxins and their dietary intake through beer consumption by the European population. Food Chemistry, 178 (2015) 149 – 155. Impact factor: 3.259*
5. *Determination of mycotoxins in bee pollen by gas chromatography–tandem mass spectrometry. Journal of Agricultural and Food Chemistry, 61 (2013) 1999 – 2005. Impact factor: 3.107*
6. *Exposure estimates to Fusarium mycotoxins through cereals intake. Chemosphere, 93 (2013) 2297 – 2303. Impact factor: 3.499*
7. *Development of a GC–MS/MS strategy to determine 15 mycotoxins and metabolites in human urine. Talanta, 128 (2014) 125 – 131. Impact factor: 3.511*
8. *Exposure assessment approach through mycotoxin/creatinine ratio evaluation in urine by GC–MS/MS. Food and Chemical Toxicology, 72 (2014) 69 – 75. Impact factor: 2.610*
9. *Preliminary estimation of deoxynivaleniol excretion through a 24h pilot study. Toxins, 7 (2015) 705-718. Impact factor: 2.480*
10. *Mycotoxin multi-biomarker method in human urine based on salting-out liquid-liquid extraction and gas chromatography-tandem mass spectrometry. Journal of Chromatography A (under review).*

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"Es de importancia para quien desee alcanzar una certeza en su investigación, el saber dudar a tiempo."

Aristóteles (384 AC-322 AC)

"Si no conozco una cosa, la investigaré."

Louis Pasteur (1822-1895)

"Soy de los que piensan que la ciencia tiene una gran belleza. Un científico en su laboratorio no es sólo un técnico: es también un niño colocado ante fenómenos naturales que le impresionan como un cuento de hadas."

Marie Curie (1867 – 1934)

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Moltíssimes gràcies a tots i per tot.

Yelko

List of abbreviations

AcN	acetonitrile
AF/AFs	aflatoxin/aflatoxins
ATA	*Alimentary Toxic Aleukia*
AOAC	Association of Official Analytical Chemists
APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
BEA	beauvericin
BSA	N,O-bis(trimethyl silyl)acetamide
bw	body weight
C8	octyl silica
C18	octadecyl silica
CE	collision energy
Crea	creatinine
DAS	diacetoxyscirpenol
DLLME	dispersive liquid-liquid microextraction
DOM-1	deepoxy-deoxynivalenol
DON	deoxynivalenol
DON-GlcAs	deoxynivalenol glucuronides
Dt	dwell time
d-SPE	dispersive solid-phase extraction
EC	European Commission
ECD	electron capture detector
EDI	estimated daily intake
EFSA	European Food Safety Authority
EI	electron impact ionization
ENN	enniatins
EU	European Union
FAO	Food and Agriculture Organitzation
FB/FBs	fumonisin/fumonisins
FUSA	fusaproliferin
FUS-X	fusarenon-X
GC	gas chromatography
HFB	heptafluorobutyryl derivatives

HPLC	high performance liquid chromatography
HT-2	HT-2 toxin
IAC	immunoaffinity columns
IARC	International Agency for Research on Cancer
IT	ion trap
JECFA	Joint Expert Committee on Food Additives
RSD_r	intra-day relative standard deviation
RSD_R	inter-day relative standard deviation
LC	liquid chromatography
LLE	liquid-liquid extraction
LOD/LODs	limit of detection/limits of detection
LOQ/LOQs	limit of quantitation/limits of quantitation
m/z	mass-to-charge ratio
ME	matrix effect
MeOH	methanol
MIPs	molecular imprinted polymers
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSPD	matrix solid phase dispersion
n.d.	non detected
NEO	neosolaniol
NIV	nivalenol
NMR	Nuclear Magnetic Resonance
OTA	ochratoxin A
PAT	patulin
PDI	probable daily intake
PLE	pressurized liquid extraction
PMTDI	provisional maximum tolerable daily intake
PSA	primary secondary amine
PTV	programmable temperature vaporization
q	qualifier transition
Q	quantifier transition
QqQ	triple quadrupole
QuEChERS	quick, easy, cheap, effective, rugged and safe

r	correlation coefficient
R^2	coefficient of determination
RASFF	Rapid Alert System for Food and Feed
REC	recovery
RP	reverse phase
RSD	relative standard deviation
SALLE	salting-out liquid-liquid extraction
SCF	Scientific Committee on Food
S/N	signal to noise ratio
SPE	solid-phase extraction
SRM	selected reaction monitoring
SSE	signal suppression/enhancement
t_R	retention time
T-2	T-2 toxin
TCs	trichothecenes
TDI	tolerable daily intake
TFA	trifluoroacetyl derivatives
TMCS	trimethylchlorosilane
TMS	trimethylsilyl ether derivatives
TMSI	N-trimethyl silyilmidazole
TOF	time-of-flight
UGT	UDP- glucuronosyl-transferase
UHPLC	ultra high permormance
WHO	World Health Organization
ZAN	zearalanone
ZON	zearalenone
ZON-GlcAs	zearalenone glucuronides
α-ZAL	α-zearalanol
α-ZOL	α-zearalenol
β-ZAL	β-zearalanol
β-ZOL	β-zearalenol
$^{13}C_{15}$-DON	isotopic-labeled deoxynivalenol
3-ADON	3-acetyldeoxynivalenol
15-ADON	15-acetyldeoxynivalenol

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RESUMEN

Las micotoxinas son metabolitos secundarios de determinadas especies fúngicas que se encuentran habitualmente en los alimentos. El Reglamento No. 1881/2006 de la Comisión Europea, parcialmente modificado por posteriores Reglamentos, establece los contenidos máximos de micotoxinas para diferentes productos alimenticios. La introducción de estas normas pone de manifiesto el interés por el conocimiento de valores relativos a la concentración de estos tóxicos para poder evaluar riesgos y adoptar las medidas necesarias para proteger la salud de los consumidores. Los productos agrícolas contaminados, especialmente cereales y sus derivados, son la principal fuente de micotoxinas en la dieta de la población mundial. Es por ello que en la presente Tesis Doctoral se han desarrollado procedimientos analíticos rápidos, precisos y selectivos basados en la cromatografía de gases acoplada a espectrometría de masas en tándem (GC-MS/MS) con analizador de triple cuadrupolo para detectar y cuantificar micotoxinas de *Fusarium* en distintas matrices alimentarias, principalmente en las de elevado consumo. Las metodologías propuestas se validaron de acuerdo con las recomendaciones recogidas en las guías europeas obteniendo resultados satisfactorios. Se detectó la presencia de micotoxinas en un porcentaje importante de las muestras analizadas siendo los productos a base de trigo los que presentaron una mayor incidencia (67,6%); no obstante, las concentraciones halladas fueron inferiores a los contenidos máximos permitidos. También se detectó la presencia simultánea de

micotoxinas en el 10,8% de las matrices alimentarias analizadas (*n = 418*) siendo la combinación más frecuente deoxinivalenol y la toxina HT-2. Con los resultados obtenidos se llevó a cabo una evaluación de la exposición combinando los datos de contaminación y los datos de consumo. En general se obtuvieron ingestas diarias de micotoxinas inferiores a las ingestas tolerables, y por tanto la exposición a estos contaminantes alimentarios no supondría un riesgo importante para el consumidor medio. Especial atención hay que prestar a grupos de población de menor edad ya que para los niños se obtuvieron datos de exposición a micotoxinas significativamente mayores, como consecuencia de un mayor consumo de cereales y menor peso corporal.

Por otra parte, se desarrolló y validó un método de análisis para la determinación de micotoxinas y metabolitos en orina humana. Para ello, se aplicó una hidrólisis enzimática con β-glucuronidasa para separar los conjugados de azúcares y micotoxinas, productos formados como consecuencia de la fase II del metabolismo (principal vía de detoxificación de micotoxinas como el deoxinivalenol) y así identificar y confirmar la presencia de estos contaminantes alimentarios en orina. Los resultados mostraron presencia de micotoxinas en el 69% de las muestras analizadas (*n = 55*) siendo el deoxinivalenol la toxina más frecuente. La tasa de conjugación de esta micotoxina obtenida experimentalmente fue superior al 70%.

Además se evaluó la exposición a las micotoxinas a partir de las concentraciones obtenidas de las muestras de orina de donde se dedujo una ingesta de deoxinivalenol superior al 50% de la ingesta diaria tolerable provisional e incluso casi una décima parte de los sujetos expuestos sobrepasó la ingesta tolerable. Se obtuvieron así valores de exposición mayores a los calculados a partir del análisis individual de las matrices alimentarias. Finalmente, teniendo en cuenta la correlación entre los niveles de ingesta y excreción de micotoxinas y a fin de reducir la incertidumbre asociada a la evaluación a través del análisis de alimentos, se propone llevar a cabo estudios de exposición a micotoxinas utilizando biomarcadores de exposición en orina.

SUMMARY

Mycotoxins are secondary metabolites produced by fungal species which can usually be found in foodstuffs. Regulation (EC) 1881/2006, partially amended by other Regulations, sets maximum contents of some mycotoxins in different foodstuffs allowing to evaluate risks and take actions to protect public health. Contaminated crops such as cereals and cereal-based products are the main source of mycotoxins and they represent one of the most consumed commodity. Therefore, in this PhD Thesis rapid and selective analytical procedures based on gas chromatography tandem mass spectrometry (GC-MS/MS), with triple quadrupole mass analyzer (QqQ), were developed in order to determine *Fusarium* mycotoxins in several food matrices, mainly in those widely consumed. Proposed methodologies were validated according to the European guidelines recommendations and satisfactory results were obtained. Occurrence of mycotoxins was found in an important percentage of samples. Wheat-based products showed the highest incidence of mycotoxins (67.6%); nonetheless, the mycotoxin levels found were lower than the maximum permitted contents. Co-occurrence of mycotoxins was also found in 10.8% of analyzed food matrices (*n = 418*), being deoxynivalenol and HT-2 toxin the most frequent combination found. An exposure assessment was carried out by combining food consumption data and the results of mycotoxin contamination here obtained. In general terms, mycotoxin daily intakes were lower than tolerable daily intakes and thus, the exposure to these food contaminants would not imply a

risk for the average consumer. However, special attention should be paid to susceptible population groups such as children since they show a higher exposure due to their wide cereal consumption and their lower body weight.

On the other hand, an analytical method for determining mycotoxins and metabolites in human urine was developed and validated. Moreover, an enzymatic hydrolysis by β-glucuronidase was applied to liberate mycotoxins from Phase II metabolism products. The cleavage of DON glucuronide by β-glucuronidase hydrolysis allowed the total DON quantitation. Results showed occurrence of mycotoxins in 69% of analyzed samples (*n = 55*) being deoxynivalenol the most frequent toxin. Conjugation rate here experimentally obtained was greater than 70%.

The exposure to mycotoxins was also assessed throughout the obtained concentration from the analyzed urine samples. It was obtained a deoxynivalenol intake above 50% of the provisional tolerable daily intake in over 50% of the individuals. One tenth of exposed subjects exceeded the safety levels. So, exposure data obtained by urine analysis were higher than those calculated by food analysis. Finally, taken into account the correlation between intake and excretion of mycotoxins, and in order to reduce the uncertainty associated to the assessment through the analysis of food, mycotoxin biomarkers in urine are suggested to carry out exposure studies.

1. General introduction

Introducción general

1. INTRODUCCIÓN GENERAL

1.1. Preámbulo

El término *micotoxina* deriva de las palabras griegas *mikes* (hongo) y *toxicum* (veneno). Las micotoxinas se definen como metabolitos fúngicos secundarios, de bajo peso molecular (Pm <800 Da), producidos por hongos filamentosos cuya ingestión, inhalación o absorción cutánea puede generar distintas enfermedades y trastornos conocidos como *micotoxicosis* la severidad de las cuales dependerá de la toxicidad de la micotoxina, del grado de exposición, de la edad y del estado nutricional tanto del hombre como de los animales (Bennett and Klich, 2003).

La micotoxicosis más antigua descrita en humanos es el ergotismo, también conocido como *fuego de San Antonio*, una patología descrita en la Edad Media y causada por el consumo de productos de origen vegetal contaminados por alcaloides ergóticos producidos por el hongo *Claviceps purpurea* el cual desencadenó diversas epidemias que devastaron Europa Occidental (Soriano, 2007). El caso más importante de micotoxicosis humana por tricotecenos se describió como una enfermedad endémica de algunas zonas de Rusia en el año 1932 provocando altas tasas de mortalidad (60% de los afectados). En un principio se pensó que la enfermedad tenía un origen infeccioso e incluso que podía deberse a una deficiencia vitamínica por lo que se confundió con enfermedades como la escarlatina, difteria, pelagra e incluso escorbuto, pero fue finalmente en el 1943 cuando se denominó como “leucopenia tóxica hemorrágica” más

conocida como ATA (*Alimentary Toxic Aleukia*), causada por la contaminación de los cultivos por la toxina T-2; toxina producida por el hongo *Fusarium sporotrichoides* (Ramos, 2011). El descubrimiento de las aflatoxinas, en la década de los 60 del siglo XX, supuso un punto de inflexión en el estudio de la micotoxicología, cuando miles de crías de pavo y otros tipos de aves murieron en Inglaterra a causa de una enfermedad la cual fue acuñada como "enfermedad X del pavo" (*Turkey "X" disease*) debida al consumo de harina de cacahuete contaminada por toxinas de *Aspergillus flavus* (Ramos, 2011).

Desde entonces, se han llevado a cabo importantes investigaciones para conocer la presencia y la toxicidad de las micotoxinas en diversas matrices alimentarias así como se ha impulsado el desarrollo de estrategias de detoxificación de éstos compuestos tóxicos para garantizar la seguridad alimentaria (Garcia, et al., 2009).

1.2. Generalidades

De entre los principales mohos productores de micotoxinas cabe destacar aquellos englobados dentro de los géneros *Aspergillus*, *Penicillium*, *Fusarium*, *Claviceps* o *Alternaria*, que bajo condiciones determinadas (temperatura, humedad, actividad del agua, pH, composición del sustrato, etc.) pueden colonizar y posteriormente contaminar con micotoxinas, los alimentos y los piensos (Tabla 1) (Bennett and Klich, 2003).

Tabla 1: Condiciones de temperatura y de actividad de agua (a_w) para el crecimiento de los principales hongos micotoxigénicos y su toxinogénesis.

Especie	**Crecimiento**		**Toxinogénesis**		
	temperatura	a_w mínima	micotoxina	a_w mínima	a_w óptima
Aspergillus flavus	24°C a 37°C	0,78	AFs	0,80 - 0,82	0,95 - 0,99
Aspergillus parasiticus		0,80		0,83	0,98
Aspergillus ochraceus	24°C a 37°C	0,77	OTA	0,80 - 0,90	0,95 - 0,99
Penicillium verrucosum	20°C a 32°C	0,80		0,80 - 0,85	0,90 - 0,99
Penicillium expansum	23°C a 27°C	0,83	PAT	-	0,98
Fusarium verticillioides	25°C a 37°C	0,90	FBs	-	0,97
Fusarium proliferatum		0,90		-	0,97
Fusarium sporotrichioides	15°C a 27°C	0,90	ZON y TCs	0,95	0,97 - 0,99
Fusarium graminearum		0,88		0,95	0,97 - 0,99
Fusarium culmorum		0,87		0,95	0,97 - 0,99

Estos hongos normalmente se relacionan con distintas zonas geográficas, siendo la distribución más frecuente del género *Aspergillus* en zonas tropicales, la de *Fusarium* en climas fríos y la de *Penicillium* en las zonas templadas (Paterson and Lima, 2010).

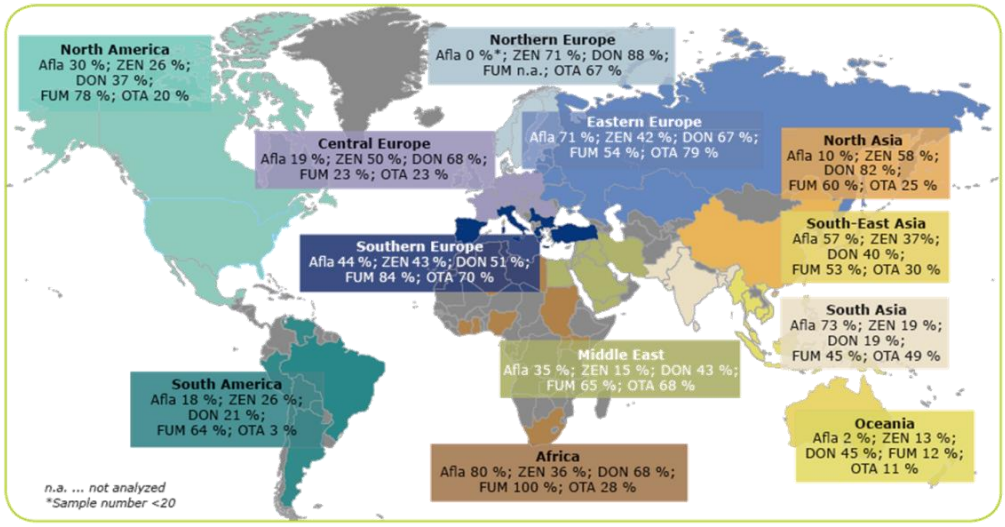


Figura 1: Incidencia de las principales micotoxinas según regiones.

La invasión por estos hongos puede producirse durante la precosecha (en campo) o en las etapas postcosecha (almacenamiento, transporte y procesamiento), generando graves pérdidas económicas y problemas de salud entre los seres humanos y en el ganado (FAO, 2001).

Es de destacar que una misma cepa toxigénica puede producir diferentes micotoxinas y una micotoxina puede ser sintetizada por diferentes hongos. Estos metabolitos poseen una estructura química y una actividad biológica muy variada. De acuerdo a la literatura, existen

descritas alrededor de 400 micotoxinas, de las cuales las más importantes por sus efectos adversos sobre la salud humana y los animales son: aflatoxinas, ocratoxina A, fumonisinas, tricotecenos, zearalenona y patulina (Bräse et al., 2009). La Tabla 2 muestra la combinación más frecuente entre alimentos contaminados y el tipo de micotoxina.

Puesto que son contaminantes naturales, no pueden ser eliminadas completamente sin dañar los alimentos, motivo por el que se han desarrollado programas de inocuidad alimentaria para evaluar los niveles de varias micotoxinas en distintas matrices. No obstante esto, la presencia de micotoxinas a lo largo de la cadena alimentaria sigue siendo un problema muy importante de salud pública (Shephard, 2008). Cabe en este punto mencionar que la Organización para la Agricultura y la Alimentación (FAO) considera que al menos el 25% de las cosechas mundiales están contaminadas por micotoxinas (FAO/WHO, 2001). Las micotoxinas son de los contaminantes alimentarios que mayor número de notificaciones presentan según los informes publicados por el Sistema de Alerta Rápida para Alimentos y Piensos (RASFF) (RASFF, 2014). Esta tendencia se viene manteniendo en el tiempo ajustándose a las fluctuaciones naturales de estos contaminantes. La Tabla 3 recoge las notificaciones totales por micotoxinas desde el año 2004. En la tabla 4 se muestra una comparativa del número de notificaciones registradas en los últimos años para micotoxinas, microorganismos patogénicos y residuos de plaguicidas.

Tabla 2: Combinación de alimentos más frecuentemente contaminados por micotoxinas y principales especies productoras

Micotoxina	Género	Especie	Alimentos contaminados más frecuentemente
Aflatoxinas	*Aspergillus*	*A. flavus, A. parasiticus, A. bombycis, A. pseudotamarii, A.ochraceoroseus*	Cacahuete, maíz, trigo, arroz, colza, semilla de algodón, nueces, huevos, leche, queso, higos
Ocratoxina A	*Aspergillus*	*A. ochraceus, A. melleus, A. niger, A.sulphureus, A. carbonarius*	Trigo, cebada, avena, maíz, legumbres secas, cacahuete, queso, café, pasas, uvas, frutos secas, vino
Patulina	*Penicillium*	*P. verrucosum*	Frutas, especialmente manzanas, zumo de frutas
	Aspergillus	*A. clavi, A. terreus*	
	Penicillium	*P. expansum, P. carneum, P. paneum, P. sclerotigenum*	
	Paecylomyces	*P. varioti*	
	Byssochlamys	*B. fulva, B. nívea*	

Fumonisinas	*Fusarium*	*F. verticillioides, F. proliferatum, F. anthophilum, F. dlamini, F. napiforme*	Maíz (salvado, harina, cereales de desayuno, fórmulas infantiles, palomitas, maíz tostado, maíz dulce), cerveza, espárragos, higos secos
Tricotecenos tipo A	*Fusarium*	*F. sporotrichioides, F. poae, F. equiseti*	Trigo, maíz, cebada, avena, pan, cereales de desayuno, cerveza
Tricotecenos tipo B	*Fusarium*	*F. graminearum, F. culmorum*	
Zearalenona	*Fusarium*	*F. graminearum, F. culmorum, F. cerealis, F. semitectum*	Maíz, avena, sorgo, trigo

Tabla 3: Notificaciones anuales de micotoxinas según los informes de la RASFF.

Año	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013
Notificaciones	880	996	878	760	933	669	688	635	538	405

Tabla 4: Comparación de notificaciones totales según peligros (RASFF).

Peligros	Año		
	2011	2012	2013
Micotoxinas	635	528	405
Microorganismos patogénicos	599	592	774
Residuos de plaguicidas	363	447	452

1.3. Principales micotoxinas

1.3.1. Micotoxinas producidas por hongos del género *Aspergillus* y/o *Penicillium*

1.3.1.1. Aflatoxinas

Las aflatoxinas (AFs) químicamente se definen como cumarinas sustituidas, conteniendo anillos de bifurano y configuración tipo lactona (Figura 2).

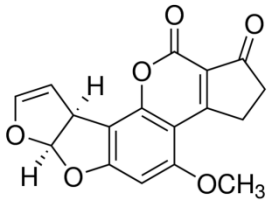


Figura 2: Aflatoxina B_1

Producción

Las AFs son producidas principalmente por las cepas de *Aspergillus flavus* y *Aspergillus parasiticus*. Las AFs más importantes se denominan B_1,

B_2, G_1, G_2 y M_1. La AFM_1 no es producida directamente por ningún hongo productor de AFs sino que es un derivado metabólico de la AFB_1 (Tantaoui El Araki, 2014). Por ello la AFM_1 no es considerada como un contaminante de cereales o piensos aunque sí presenta interés toxicológico y se encuentra en la leche.

A. flavus y *A. parasiticus* son comunes y especialmente abundantes en zonas tropicales y subtropicales con menor prevalencia en áreas templadas. El crecimiento de estos hongos es óptimo a 32°C aunque el intervalo de crecimiento se sitúa entre 10 y 43°C y una humedad relativa en torno al 90%. La producción de AFs tiene lugar a temperaturas entre 12 y 40°C con condiciones de pH en torno a 3,5 - 8,0 (siendo el pH óptimo 6,0) y una actividad de agua (a_w) óptima entre 0,95 y 0,99 (Sweeney and Dobson, 1998).

Presencia en alimentos

Los principales alimentos invadidos por hongos del género *Aspergillus* y la consiguiente producción de aflatoxinas son: cacahuetes, maíz, pistachos y semillas de algodón. También se ha detectado presencia de AFs en otras semillas oleaginosas (girasol y soja) así como en matrices tales como cereales, almendras, nueves, avellanas, pasas, higos secos, café, cacao y algunas especias como el pimentón y la pimienta (Weidenbörner, 2014).

Toxicología

Los síntomas de aflatoxicosis aguda incluyen necrosis hemorrágica hepatocelular, edema y letargo. También presenta efectos citotóxicos al inducir peroxidación lipídica produciendo daño oxidativo de los hepatocitos. La sintomatología de una intoxicación crónica por aflatoxinas no es muy específica e incluye disminución de peso corporal, menor producción de huevos y leche así como una mayor susceptibilidad frente a diversas enfermedades infecciosas debido a los efectos inmunosupresores ocasionados por estas toxinas. Las AFs presentan efectos carcinógenos y mutágenos. Por ello la *International Agency for Research on Cancer* (IARC) ha clasificado a las AFs dentro del grupo 1 (agentes carcinógenos para los seres humanos), con excepción de la AFM_1 la cual se sitúa dentro del grupo 2B (agente posiblemente carcinógeno para el ser humano) (IARC, 2015).

1.3.1.2. Ocratoxina A

La ocratoxina A (OTA) es un pentaquétido derivado de hidrocumarinas, unido a una molécula de L-β-fenilalanina por un enlace amida (Figura 3).

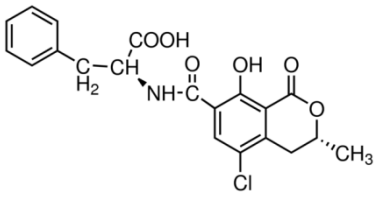


Figura 3: Ocratoxina A

Producción

Las principales especies productoras de OTA son *Aspergillus ochraceus* y *Penicillium verrucosum*. Otros hongos como *Aspergillus niger* y *Aspergillus carbonarius* también son especies relevantes en función de la matriz o el área geográfica (Weidenbörner, 2001).

A. ochraceus crece en un intervalo de temperaturas de 8 a 40°C, aunque de manera óptima entre 24 y 31°C. Así mismo este hongo crece en un intervalo de pH entre 3 y 10 siendo la a_w de crecimiento óptima entre 0,95 y 0,99. Por su parte *P. verrucosum* crece entre 0 y 31°C, aunque de manera óptima a 20°C y a un pH entre 6,0 y 7,0 siendo capaz de crecer a una a_w de 0,80 del mismo modo que *A. ochraceus* por lo que se consideran hongos xerófilos (Tantaoui El Araki, 2014).

Presencia en alimentos

La invasión con especies de mohos productores de OTA se ha detectado a nivel mundial. Las principales fuentes de este contaminante alimentario son cereales como trigo, cebada, maíz o arroz y sus productos derivados, bebidas alcohólicas como cerveza y vino (especialmente vino tinto), además de otras matrices como zumo de uva, pasas, legumbres, café, cacao y nueces (Weidenbörner, 2014).

Toxicología

La sintomatología por intoxicación a OTA incluye nefrosis y necrosis hepática con hemorragias multifocales en los principales órganos

(Kuiper-Goodman and Scott, 1989). Respecto a sus efectos crónicos, la OTA se ha clasificado como agente hepatotóxico, nefrotóxico, neurotóxico, inmunosupresor, citotóxico y genotóxico. Así mismo fue clasificada por la IARC en el grupo 2B como posible carcinógeno humano (IARC, 2015).

1.3.1.3. Patulina

La patulina (PAT) es una micotoxina incluida en un grupo de compuestos comúnmente conocidos como lactonas. Estructuralmente, la PAT es la 4-hidroxi-4H-furo[3,2-c]piran-26H)-ona, una lactona heterocíclica insaturada (Figura 4).

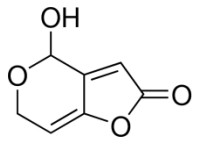


Figura 4: Patulina

Producción

La patulina es producida principalmente por *Penicillium expansum*; no obstante, también la pueden producir otras especies de *Penicillium*, *Aspergillus*, *Paecylomyces* y *Byssochlamys*. El intervalo de temperaturas para el crecimiento óptimo del hongo se sitúa entre 23 y 27°C y a un pH de 5,6. Sin embargo, la producción de toxina se puede dar incluso en condiciones de refrigeración. La a_w mínima para crecer es de 0,83 mientras que el crecimiento óptimo se alcanza en valores de a_w de 0,98 (Tantaoui El Araki, 2014).

Presencia en alimentos

Todas las especies anteriormente nombradas presentan capacidad de crecer sobre frutas, hortalizas, cereales, queso y ensilados de hierba y de maíz destinados a la alimentación animal. De toda esta variedad de alimentos sobre los que se puede desarrollar la micotoxina, se considera que son las frutas, particularmente la manzana y sus productos derivados (tales como zumo, puré y compota), la principal fuente de PAT (Weidenbörner, 2014).

Toxicología

Los efectos por una intoxicación aguda por PAT incluyen náuseas, vómitos y otros síntomas gastrointestinales que se acompañan de daños en el riñón. Se ha observado que la PAT tiene propiedades inmunotóxicas a elevadas concentraciones. La ingesta crónica de dosis elevadas de PAT produce trastornos inmunitarios, neurotóxicos y gastrointestinales muy graves (Soriano, 2007). No obstante, la IARC pone de manifiesto que no existe evidencia alguna que demuestre un efecto carcinógeno de esta micotoxina en animales de experimentación ni tampoco en humanos por lo que la clasifica dentro del grupo 3 (IARC, 2015).

1.3.2. Micotoxinas producidas por hongos del género *Fusarium*

1.3.2.1. Fumonisinas

Las fumonisinas (FBs) son aminopolioles cuya estructura principal consiste en una cadena lineal de 20 átomos de carbono, la cual tiene un

grupo amino en el carbono 2, junto con grupos metilo, hidroxilo y ácido tricarboxílico en diferentes posiciones a lo largo del esqueleto carbonado (Figura 5).

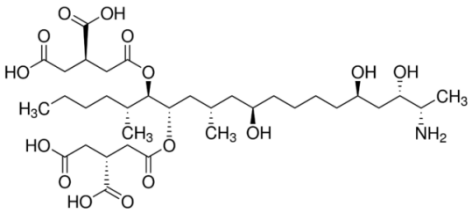


Figura 5: Fumonisina B_1

Producción

Las fumonisinas son producidas por varias especias del género *Fusarium*, aunque principalmente por *Fusarium verticillioides* y *Fusarium proliferatum*. Según los grupos químicos presentes a lo largo de la cadena lineal, las fumonisinas se pueden clasificar en cuatro series: fumonisinas A, B, C y P. No obstante, las toxinas pertenecientes a la serie B, y en el siguiente orden decreciente: B_1, B_2, B_3 y B_4, son las que mayoritariamente se aíslan en alimentos. La fumonisina más importante es la FB_1, ya que puede constituir hasta un 70 % de todas las FBs presentes en los alimentos (Rheeder, et al., 2002). Las condiciones óptimas descritas en la literatura para la producción de estas micotoxinas son temperaturas en un intervalo de 25 a 37°C y a_w de 0,97. *F. verticillioides* tolera valores de pH desde 3 a 9,5; no obstante, la formación de micotoxinas se ve favorecida por valores de pH que oscilan entre 3,4 y 5,5 (Tantaoui El Araki, 2014).

Presencia en alimentos

Las principales fuentes de FBs son cereales y derivados destacando el maíz como matriz alimentaria más susceptible de presentar contaminación por estos compuestos (Weidenbörner, 2014). Otras matrices donde se ha detectado la presencia de FBs son los piensos o la cerveza, como consecuencia de la utilización de sémola o jarabe de maíz como coadyuvante tecnológico.

Toxicología

La elevada similitud estructural de las FBs con los esfingolípidos determina su mecanismo de acción el cual se basa en la inhibición competitiva de la enzima ceramida sintasa provocando la perturbación del metabolismo de la ceramida y los esfingolípidos (Soriano, et al., 2005). La sintomatología de una intoxicación aguda por FBs incluye dolor abdominal, borborigmo y diarrea. A nivel crónico, existe una asociación probable entre el consumo de alimentos contaminados por FBs y cáncer de esófago e hígado motivo por el que han sido clasificadas por la IARC dentro del grupo 2B (IARC, 2015).

1.3.2.2. Tricotecenos

Los tricotecenos (TCs) son un complejo grupo de sesquiterpenoides químicamente relacionados, que comparten un núcleo tricíclico llamado tricodieno caracterizado por un doble enlace en la posición 9,10 y un grupo epóxido entre los carbonos 12 y 13 (Figura 6). Los

TCs se encuentran divididos en cuatro grupos (A, B, C y D) de acuerdo a los grupos sustituyentes funcionales. Los principales TCs tipo A incluyen a las toxinas T-2 y HT-2, diacetoxiscirpenol (DAS), neosolaniol (NEO), etc. y poseen en posición C-8 un grupo funcional distinto a cetona, mientras que los del tipo B se caracterizan por poseer un grupo cetona en posición C-8. Los tricotecenos tipo B engloban a micotoxinas como deoxinivalenol (DON) y nivalenol (NIV) y sus compuestos acetilados: 3-acetildeoxinivalenol (3-ADON), 15- acetildeoxinivalenol (15-ADON) y fusarenona X (FUS-X), siendo este grupo el más fitotóxico (Bennett and Klich, 2003). Como consecuencia, frecuentemente se asocia a la presencia de tricotecenos tipo B con la enfermedad conocida como *Fusarium head blight*, (FHB) (fusariosis de la espiga) (Eudes, et al., 2001).

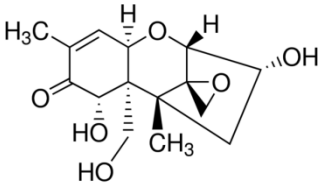
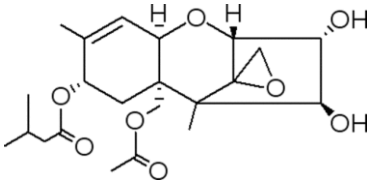
(A)   (B)

Figura 6: (A) deoxinivalenol y (B) toxina HT-2

Producción

Fusarium sporotrichioidies, *Fusarium poae* y *Fusarium equiseti* son las principales especies productoras de TCs tipo A. La producción de toxinas puede darse en un intervalo de temperaturas entre 15 y 27°C y con valores de a_w superiores a 0,95. Los TCs tipo B son producidos por especies de *Fusarium graminearum* y *Fusarium culmorum* cuyas temperaturas óptimas

de crecimiento son 25°C (a_w >0,88) y 21°C (a_w >0,87), respectivamente (Tantaoui El Araki, 2014).

Presencia en alimentos

Los cereales, en particular trigo pero también maíz, cebada, avena o arroz entre otros, son las principales fuentes de TCs en la dieta. El contenido de micotoxinas varía según su localización en el grano, siendo mayor en las partes más externas del cereal. Por ello en el salvado suelen encontrase valores significativamente más elevados de micotoxinas. De entre los TCs más comúnmente encontrados en alimentos cabe destacar el DON, y las toxinas T-2 y HT-2. Éstas últimas, presentan una toxicidad diez veces superior respecto al DON; no obstante su incidencia en los alimentos es menor. También en productos derivados de cereales tales como pan, cereales para el desayuno, alimentos para bebés, cerveza, etc. se detectan incidencias importantes de TCs (Weidenbörner, 2014).

Toxicología

La sintomatología asociada a una toxicidad aguda incluye náuseas, vómitos, pérdida de apetito, pérdida de peso y diarrea. La ingestión de altas dosis puede provocar lesiones en tejidos linfoides hemorragias intestinales severas así como destrucción celular en las membranas mucosas epiteliales del intestino (Creppy, 2002). Los TCs también se caracterizan por ser potentes inhibidores de la síntesis proteica en eucariotas y se han relacionado con enfermedades como la ATA (Marin, et al., 2013). Además de su actividad citotóxica, su actividad inmunosupresora

es destacable provocando una supresión de la resistencia a microorganismos patógenos. Todos los TCs clasificados por la IARC (DON, NIV, FUS-X y T-2) se encuentran dentro del grupo 3 (IARC, 2015).

1.3.2.3. Zearalenona

La zearalenona (ZON) es una micotoxina estrogénica no esteroidea. Químicamente es una lactona derivada del ácido β-resorcíclico (Figura 7).

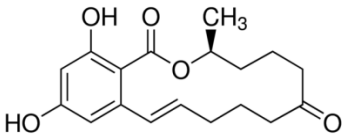


Figura 7: Zearalenona

Producción

Los principales hongos productores de zearalenona son *Fusarium graminearum*, *F. culmorum*, *F. cerealis* y *F. semitectum* (Tantaoui El Araki, 2014). Puesto que las principales especies productoras de ZON son similares a las productoras de TCs, las condiciones de crecimiento del hongo y producción de micotoxinas son idénticas. Por ello, la presencia simultánea de TCs y ZON en diversas matrices alimentarias es frecuente.

Presencia en alimentos

En particular el maíz y sus productos derivados son los alimentos que presentan mayor susceptibilidad por la invasión y subsecuente

producción de ZON. También se ha detectado contaminación por ZON, aunque en menor grado, en alimentos y derivados de cebada, avena, sorgo, trigo, arroz y mijo (Weidenbörner, 2014).

Toxicología

La toxicidad de la ZON y algunos de sus metabolitos, especialmente α-ZOL, está asociada con problemas reproductivos en algunos animales, y posiblemente en humanos, ya que tienen la capacidad de unirse de forma competitiva con los receptores estrogénicos. Recientemente, un estudio ha demostrado que la ZON puede estimular el crecimiento de carcinomas mamarios en humanos; no obstante la IARC no dispone de suficientes evidencias científicas por lo que esta toxina y sus metabolitos han sido clasificados dentro del grupo 3 (Zinedine, et al., 2007).

1.3.2.4. Micotoxinas emergentes

Dentro de las micotoxinas emergentes de *Fusarium* se incluyen la beauvericina (BEA), enniatinas (ENN) y fusaproliferina (FUSA). Estas toxinas son producidas por diferentes especies del género *Fusarium*, como *F. avenaceum*, *F. sporotrichioides*, *F. poea* o *F. langsethiae* entre otras. Son capaces de colonizar, y posteriormente producir micotoxinas, en matrices alimentarias altamente consumidas a escala mundial como son los cereales y sus productos derivados. La presencia de estos contaminantes alimentarios también ha sido detectada en otras matrices como nueces y frutos secos (Jestoi, 2008). Actualmente hay disponibles pocos estudios *in*

vivo (Manyes, et al., 2014) para conocer la toxicidad de las llamadas micotoxinas emergentes y los datos existentes sobre la actividad biológica se limitan principalmente a estudios *in vitro* los cuales han demostrado el carácter citotóxico de estas toxinas frente a diversas líneas celulares de animales y humanas (Prosperini, et al., 2013; Mallebrera, et al., 2014).

1.4. Legislación

Dada la creciente preocupación en los últimos años en materia de seguridad alimentaria con respecto a la presencia de micotoxinas en alimentos, las autoridades sanitarias han reconocido los potenciales riesgos para la salud de los animales y humanos que plantea la contaminación de alimentos por micotoxinas. Consecuentemente, la evaluación del peligro ocasionado por las micotoxinas ha conducido al Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) a la estimación de las dosis consideradas como seguras, tal como la ingesta diaria tolerable (*Tolerable Daily Intake*, TDI) o, en caso de escasez de datos fiables sobre las consecuencias de la exposición humana, a la estimación de la ingesta diaria tolerable máxima provisional (*Provisional Maximum Tolerable Daily Intake*, PMTDI) o la ingesta semanal tolerable máxima provisional (*Provisional Maximum Tolerable Weekly Intake*, PMTWI). Actualmente la Autoridad Europea de Seguridad Alimentaria (EFSA) ha establecido ingestas tolerables para OTA, PAT, la suma de FBs, ZON, NIV, la suma de DON y sus formas acetiladas (3-ADON y 15-ADON) y la suma de las toxinas HT-2 y T-2 (Tabla 8).

Por otro lado, no únicamente se han establecido límites máximos permitidos para distintas micotoxinas en varias matrices alimentarias sino también protocolos referentes a la toma de muestras y las especificaciones requeridas a los métodos analíticos para su determinación tal y como consta en el Reglamento (CE) 401/2006 (Commission Regulation (EC) No 401/2006) parcialmente modificado por el Reglamento (UE) No 178/2010 (Commission Regulation (UE) No 178/2010).

Tabla 8: Ingestas tolerables propuestas para las principales micotoxinas

Micotoxina	Ingesta tolerable	Referencia
OTA	0,12 µg/Kg p.c. semanales (0,017 µg/Kg p.c. diarios)	(EFSA, 2006)
PAT	0,4 µg/Kg p.c. diarios	(EFSA, 2002a)
$\Sigma(FB_1 + FB_2 + B_3)$	2 µg/Kg p.c. diarios	(EFSA, 2003)
Σ(DON + 3-ADON + 15-ADON)	1 µg/Kg p.c. diario	(EFSA, 2002b)
NIV	1,2 µg/Kg p.c. diarios	(EFSA, 2013)
Σ(HT-2 + T-2)	0,1 µg/Kg p.c. diarios	(EFSA, 2011a)
ZON	0,25 µg/Kg p.c. diarios	(EFSA, 2011b)

En el año 2006 entró en vigor el Reglamento (CE) No 1881/2006 (Commission Regulation (EC) No 1881/2006) sobre micotoxinas en alimentación humana derogando así al Reglamento 466/2001 hasta entonces vigente. En el actual Reglamento se fijó el contenido máximo de AFB_1, B_2, G_1, G_2 y M_1 en frutos secos, especias, cereales y derivados, como por ejemplo los alimentos infantiles, además de leche y preparados para

lactantes. Del mismo modo también se fijaron los contenidos máximos de OTA en cereales y derivados, vino, zumo de uva, pasas y especias. Respecto a PAT, el Reglamento establece límites máximos permitidos en zumos de fruta, productos derivados de las manzanas, bebidas fermentadas y alimentos infantiles. En lo que concierne a las micotoxinas de *Fusarium*, como DON, ZON, FB_1 y FB_2, el Reglamento fija sus contenidos máximos en cereales y derivados principalmente (Tabla 9).

Tabla 9: Contenidos máximos permitidos de micotoxinas según productos alimenticios.

Micotoxinas	Productos alimenticios	Contenido máximo (µg/Kg)
AFB_1	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad, alimentos dietéticos destinados a usos médicos especiales, cereales y productos a base de cereales, frutos secos, frutos de cáscara, especias y cacahuetes	0,1 – 8
AFM_1	Preparados para lactantes y preparados de continuación, alimentos dietéticos destinados a usos médicos especiales, leche cruda, leche tratada térmicamente y leche para la fabricación de productos lácteos	0,025 – 0,050
Suma de AFs	Cereales y productos a base de cereales, frutos secos, frutos con cáscara, café y cacahuetes	4 – 15

OTA	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad, alimentos dietéticos destinados a usos médicos especiales, cereales y productos a base de cereales, zumo de uva, vino, café y uvas pasas	0,5 – 10
Suma de FBs	Alimentos elaborados a base de maíz y alimentos infantiles para lactantes y niños de corta edad, alimentos a base de maíz y maíz no elaborado	200 – 2000
PAT	Alimentos infantiles distintos a los elaborados a base de cereales para lactantes y niños de corta edad, zumo de manzana y productos sólidos elaborados a partir de manzana, bebidas fermentadas elaboradas con manzanas o que contengan zumo de manzana, zumos de frutas y néctares	10 – 50
DON	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad, cereales y algunos productos a base de cereales, cereales no elaborados	200 – 1750
ZON	Alimentos elaborados a base de maíz para lactantes y niños, alimentos elaborados a base de cereales, distintos al maíz, para lactantes y niños, maíz y productos a base de maíz, maíz y otros cereales no elaborados	20 – 200

Hasta el momento, el Reglamento 1881/2006 de la Comisión, de 19 de diciembre de 2006 ha sido modificado parcialmente por:

- el Reglamento (CE) No 1126/2007 de la Comisión (Commission Regulation (EC) No 1126/2007), de 28 de septiembre de 2007 por lo que se refiere a las toxinas de *Fusarium* en el maíz y productos del maíz,
- por el Reglamento (UE) No 105/2010 de la Comisión (Commission Regulation (EU) No 105/2010), de 5 de febrero de 2010 por lo que se refiere a la OTA en determinados productos,
- por el Reglamento (UE) No 165/2010 de la Comisión (Commission Regulation (EU) No 165/2010), de 26 de febrero de 2010 por lo que respecta a los contenidos de las AFs en determinados productos,
- y por el Reglamento (UE) No 1058/2012 de la Comisión (Commission Regulation (EU) No 1058/2012), de 12 de noviembre de 2012 por lo que se refiere al contenido de AFs en higos secos.

1.5. Análisis de micotoxinas

La seguridad alimentaria es un motivo de preocupación creciente tanto para los consumidores como para los gobiernos y productores dada la globalización de los mercados en donde los alimentos son producidos y distribuidos a escala mundial. Aparte de ello, las alarmas alimentarias sufridas en las últimas décadas han generado un gran interés y preocupación poniendo de manifiesto la necesidad de establecer medidas adecuadas de control para tratar de garantizar la seguridad del

consumidor. En este sentido el establecimiento de límites reglamentarios ha impulsado el desarrollo y mejora de los procedimientos analíticos para la identificación y cuantificación de micotoxinas en alimentos, piensos y matrices biológicas. La Asociación de Químicos Analíticos Oficiales (*Association of Official Analytical Chemists*, AOAC) ha recomendado métodos normalizados de análisis para diferentes micotoxinas los cuales han sido validados en estudios interlaboratorios presentando un elevado grado de exactitud y precisión; no obstante algunos ofrecen unos límites de detección insuficientes para satisfacer las demandas cada vez más exigentes para la medición en niveles bajos (Tabla 10).

Tabla 10: Métodos oficiales para la determinación de micotoxinas (modificado de Pohland and Trucksess, 2001).

Micotoxina	Método	Producto	LOD (µg/Kg)
AFs	990.17	Maíz	1,5 - 10
OTA	991.44	Maíz y cebada	10
PAT	995.10	Zumo de manzana	20
FBs	995.15	Maíz	100 - 500
DON	986.18	Trigo	350
ZON	994.01	Trigo	1000

Diversos artículos de revisión han recopilado los métodos de análisis publicados en la última década para la determinación de micotoxinas (Shephard, et al., 2013a; Pereira, et al., 2014).

1.5.1. Muestreo

Es fundamental que el resultado de un análisis sea verdadero y representativo cuando se supervisa el nivel de micotoxinas para confirmar el cumplimiento de los valores reglamentarios. No obstante, la gran heterogeneidad en la distribución de los hongos toxigénicos productores de micotoxinas dificulta en gran medida el análisis de micotoxinas, y concretamente, la etapa de muestreo. Por ello la Unión Europea (UE) ha establecido protocolos de muestreo de algunos productos alimenticios para el análisis de micotoxinas en el Reglamento (CE) No 401/2006.

1.5.2. Extracción y purificación

Los procedimientos analíticos basados en técnicas cromatográficas o inmunoensayos normalmente requieren de una extracción de disolventes para liberar la micotoxina de la matriz a investigar, y de una posterior etapa de purificación del extracto para reducir el efecto de la matriz. Cuando la matriz es de naturaleza líquida, la extracción puede llevarse a cabo mediante un disolvente inmiscible con ella; no obstante, la extracción líquido-líquido no es actualmente una técnica de elección debido al elevado volumen de muestra y disolvente que se requieren, la baja selectividad junto a resultados de exactitud en ocasiones insuficientes y su difícil automatización. Si la matriz es de naturaleza sólida, suelen utilizarse distintas combinaciones de disolventes para la extracción. Habitualmente las mezclas de disolventes orgánicos con diferentes proporciones de agua o ácidos son más eficaces, ya que, pese a que la solubilidad de la mayoría de

las micotoxinas en agua es baja, las mezclas son más eficaces que el disolvente orgánico solo. La adición de sales y los cambios de pH pueden contribuir a mejorar el rendimiento de la extracción. Entre los disolventes más comunes para la extracción están las mezclas de acetonitrilo:agua y las de metanol:agua. Para aumentar la eficacia de la extracción se debe facilitar el contacto entre el disolvente, o mezclas de éstos, y la matriz durante un tiempo determinado. Para alcanzar dicho objetivo suelen emplearse agitadores (por ejemplo agitadores horizontales), o homogeneizadores tipo UltraTurrax.

En el procedimiento analítico, la fase de purificación de la muestra, o *clean up*, consiste en la separación de las micotoxinas de interés del resto de compuestos co-extraídos. El diseño de esta etapa depende en gran medida de la etapa posterior de cuantificación. Existen diversas técnicas de extracción y purificación utilizadas para el análisis de micotoxinas. Tal y como se ha comentado anteriormente, en la actualidad existe un interés creciente por la simplificación y miniaturización de las técnicas de análisis, con la consiguiente reducción de la cantidad de muestra utilizada en la extracción y del consumo de disolventes orgánicos, hecho que conduce al desarrollo de técnicas alternativas para la preparación de la muestra. A tal efecto, técnicas relacionadas con una extracción en fase sólida y técnicas de microextracción están siendo ampliamente utilizadas para el análisis de micotoxinas.

1.5.2.1. Extracción en fase sólida

Mediante la utilización de este tipo de extracción se consigue evitar los principales inconvenientes asociados a la extracción líquida-líquida convencional como son el elevado consumo de disolventes y los largos tiempo de análisis.

1.5.2.1.1. Extracción en fase sólida convencional

La extracción en fase sólida convencional (*Solid Phase Extraction*, SPE) se basa en la extracción y preconcentración de las micotoxinas disueltas en una matriz de naturaleza líquida mediante su retención selectiva en una fase estacionaria sólida apropiada, seguida de la elución de los analitos retenidos mediante un disolvente orgánico adecuado. Las fases estacionarias empleadas en la SPE están constituidas por partículas que presentan una gran afinidad por los analitos y generalmente se encuentran empaquetadas en el interior de pequeñas columnas, cartuchos cerrados o discos. La elección de la fase sólida depende de la polaridad de las micotoxinas y del tipo de matriz, siendo las columnas de mayor uso aquellas rellenas de sílice modificada.

Numerosos artículos han optimizado la SPE para el análisis de distintas micotoxinas tales como TCs, ZON, OTA o FBs (Romero-González, et al., 2009; Mariño-Repizo, et al., 2015).

1.5.2.1.2. Dispersión de matriz en fase sólida

Esta técnica se basa en la dispersión de una pequeña cantidad de muestra (generalmente 0,5 g) con una cantidad similar de fase sólida comercial como octadecilsílice (C18), octilsílice (C8), aminopropilsílice (NH_2), etc., hasta llevar a cabo una completa homogeneización. Tras ello, la mezcla se introduce en una columna de vidrio para su posterior elución con ayuda de un disolvente orgánico apropiado. A la columna de vidrio se le pueden añadir fases sólidas de polaridad diferente como silicato de magnesio (Florisil) o silicagel para purificar el extracto de la muestra (Capriotti, et al., 2013).

Por consiguiente, la dispersión de matriz en fase sólida (*Matrix Solid Phase Dispersion*, MSPD) es una técnica sencilla, de bajo coste, de bajo consumo de muestra y disolventes, rápida y selectiva, que se ha utilizado en los últimos años para la extracción y preconcentración de micotoxinas en diversas matrices (Rubert, et al., 2011; Blesa, et al., 2014).

1.5.2.1.3. Extracción con columnas MycoSep® y MultiSep®

Las columnas de limpieza multifuncional llamadas MycoSep® y MultiSep®, y desarrolladas por la compañía Romer Labs Incorporation, contienen, como material de empaque, una mezcla de adsorbentes específicos (carbón, celita, resinas de intercambio iónico, etc.) para las principales micotoxinas. Permiten la purificación del extracto un solo paso en 30 segundos. Se emplean para varias micotoxinas tales como AFs, OTA, ZON, FBs o TCs tipo A y B (Montes et al., 2012).

1.5.2.1.4. Extracción con columnas de inmunoafinidad

La utilización de columnas de inmunoafinidad (*Immunoaffinity Columns*, IAC) como técnica de purificación se ha visto incrementada debido a la facilidad del uso y la elevada selectividad que presenta frente a otras técnicas. Esta técnica consta generalmente de cuatro etapas consecutivas: acondicionamiento de la IAC, adición del extracto de la muestra en donde los analitos quedan unidos a los anticuerpos monoclonales fijados en la IAC, lavado para la eliminación de restos de matriz, y elución de los analitos por desnaturalización de los anticuerpos.

Actualmente existen IAC comerciales que permiten la extracción y purificación simultánea de micotoxinas como AFs, OTA, FBs, DON, ZON, T-2 y HT-2 (Li, et al., 2012; Desmarchelier, et al., 2014;). No obstante, sus principales desventajas radican en el hecho que las IAC sean de un solo uso unido a su relativamente elevado coste.

1.5.2.2. Método QuEChERS

Anastassiades, et al. (2003) desarrollaron un procedimiento analítico simple, rápido, seguro y válido para llevar a cabo un análisis multirresiduo que proporcionase unos resultados satisfactorios al cual denominaron QuEChERS, acrónimo de las palabras *Quick*, *Easy*, *Cheap*, *Effective*, *Rugged*, y *Safe*.

Esta metodología, consiste en una extracción basada en el reparto de los analitos entre una fase acuosa y un disolvente, seguido de un paso

de purificación de la fase orgánica mediante un proceso de dispersión en fase sólida (d-SPE). En la fase de extracción se emplea un disolvente orgánico, generalmente acetonitrilo, y posteriormente se adiciona sulfato de magnesio o sulfato de sodio para eliminar el agua presente debido a la humedad de la muestra y propiciar el reparto de los analitos hacia la fase orgánica. En casos concretos, se añade también alguna sal tipo cloruro sódico o acetato de sodio. En la etapa de purificación se lleva a cabo una d-SPE mediante la adición de PSA (*Primary and Secondary Amine*) a la mezcla de extracción para eliminar pigmentos polares, ácidos orgánicos y otros productos presentes en la matriz que pudieran interferir con los analitos. Generalmente la PSA se usa combinada con C18 para eliminar lípidos, esteroles y pigmentos.

El método QuEChERS originariamente se desarrolló para la determinación de plaguicidas en vegetales. No obstante, ha ido sufriendo modificaciones desde su primera aplicación en cuanto a los reactivos utilizados y las proporciones de los mismos, en función de los analitos y las matrices a estudiar. Actualmente es una técnica de preparación de muestras ampliamente utilizada para una gran variedad de compuestos como residuos de medicamentos veterinarios (Stubbings and Bigwood, 2009), residuos de plaguicidas (Wilkowska and Biziuk, 2011), o micotoxinas (Cunha and Fernandes, 2010; Rodríguez-Carrasco, et al., 2013).

1.5.2.3. Técnicas de microextracción

Siguiendo la tendencia de automatizar, simplificar y miniaturizar las técnicas de preparación de la muestra, la química analítica ha dado lugar al desarrollo y aplicación de técnicas de microextracción. Recientemente se han desarrollado multitud de técnicas y variantes con este objetivo revisándose en distintos trabajos (Martinis, et al., 2014; Maya, et al., 2014). De entre las múltiples opciones, en esta tesis se han abordado dos de ellas: la microextracción líquido-líquido dispersiva y la extracción líquido-líquido asistida por sales.

1.5.2.3.1. Microextracción líquido-líquido dispersiva

Rezaee, et al. (2006) introdujeron la microextracción líquido-líquido dispersiva (*Dispersive Liquid-Liquid Microextraction*, DLLME) como una novedosa alternativa a las técnicas de extracción.

Esta técnica se basa en la dispersión de un pequeño volumen de disolvente orgánico de extracción (extractante) mediante inyección en la muestra acuosa a alta velocidad junto con un segundo disolvente (dispersante), de manera que, tras agitar vigorosamente y centrifugar, los analitos quedan retenidos en el disolvente de extracción decantado. De este modo, el extractante se dispersa en la muestra en forma de microgotas, enturbiando la disolución y generando una gran superficie de contacto con los analitos, alcanzando el equilibrio casi de forma instantánea. La DLLME evita la volatilización y pérdida de los agentes de extracción, proporcionando una mejora en la sensibilidad y

reproducibilidad, así como una simplificación en el procedimiento de extracción.

El proceso extractivo se ve influenciado por factores como el volumen de muestra, volumen de extractante y dispersante o fuerza iónica entre otros.

La selección del tipo de disolventes es crucial para el desarrollo de una DLLME. Por un lado, el dispersante debe de ser miscible con la matriz de la muestra líquida, que generalmente es acuosa, siendo los disolventes más empleados el acetonitrilo, la acetona y el metanol. Por otro lado, el extractante debe tener la capacidad de extraer los analitos y de solubilizarse en el dispersante por ser insoluble o poco soluble en la matriz de la muestra. Además, la densidad del extractante debe de ser mayor a la de la matriz de la muestra para que las dos fases se puedan separar. En este sentido, los disolventes que comúnmente se emplean son en su mayoría disolventes clorados de carácter muy tóxico, aunque se utilizan pocos µL, tales como cloroformo, tetracloruro de carbono o diclorometano.

La DLLME ha sido aplicada como técnica de preparación de muestra para la determinación de residuos de plaguicidas, residuos de medicamentos veterinarios, aminas biógenas, bisfenol, clorofenoles, o micotoxinas en diversas matrices (Leong, et al., 2014).

1.5.2.3.2. Extracción líquido-líquido asistida por sales

Esta metodología se ha desarrollado como una alternativa debido a su simplicidad, rápido equilibrio de reparto, fácil purificación y condensación de extractos y mínimo gasto de disolventes. El fundamento de esta técnica conocida como SALLE (*salting-out assisted liquid-liquid extraction*) se basa en la adición de una elevada concentración de sales sobre la matriz acuosa con un disolvente orgánico miscible o parcialmente miscible que, por la solvatación de los iones de las sales con las moléculas de agua, da lugar a la separación de las dos fases miscibles (acuosa/orgánica) (Song, et al., 2013). En esta técnica las fuerzas intermoleculares débiles, como son los enlaces de hidrógeno entre moléculas y el agua, son interrumpidas fácilmente por la hidratación de los electrolitos. La fase orgánica separada contiene agua y sales incrementando así su capacidad de reparto, y por consiguiente la eficacia del proceso de extracción, con respecto al disolvente orgánico puro.

1.5.3. Técnicas de confirmación

La determinación de los niveles de micotoxinas exige procedimientos analíticos suficientemente sensibles y selectivos para detectar estos compuestos a niveles inferiores a los máximos permitidos. Las técnicas cromatográficas acopladas a espectrómetría de masas son las de elección para la identificación y cuantificación de micotoxinas.

1.5.3.1. Técnicas cromatográficas

1.5.3.1.1. Cromatografía gaseosa

La cromatografía gaseosa (*Gas Chromatography*, GC) es una técnica basada en la separación de compuestos en función de su volatilidad y afinidad por la fase estacionaria. Después de la derivatización de los grupos hidroxilo libres de las micotoxinas a formas trimetilsilil éteres (TMS), heptafluorobutiril ésteres (HFB) o trifluoroacetil ésteres (TFA), éstas llegan a ser lo suficientemente volátiles para el análisis por GC. Los detectores de GC más sensibles y específicos son los de detección de captura de electrones (*Electron Capture Detector*, ECD) y espectrometría de masas (*Mass Spectrometry*, MS).

Scott, et al. (1981) realizaron la primera aplicación de GC-MS para el análisis de DON en trigo y otros cereales con resultados satisfactorios. Posteriormente Jiao, et al. (1992) analizaron OTA por GC-MS mediante la formación del éster metílico *O*-metilocratoxina A. Actualmente, el uso más extendido de esta técnica se lleva a cabo para el análisis de tricotecenos del tipo A y B. En la tabla 11 se muestran algunos de los métodos de GC-MS publicados en los últimos cinco años para la determinación de micotoxinas.

1.5.3.1.2. Cromatografía líquida

La cromatografía líquida (*Liquid Chromatography*, LC) es una de las técnicas de separación más ampliamente utilizada debido a su versatilidad

Tabla 11: Métodos publicados en los últimos cinco años para la determinación de micotoxinas por GC-MS.

Año	Matriz	Nº compuestos	GC-MS		Referencia
			Ionización	LOD/LOQ (µg/kg ó µg/L)	
2011	cebada	6	EI (70eV) Q	0,013-1,91/0,05-8	(Ibáñez-Vea, et al., 2011)
2012	maíz	5	EI (70eV) Q	14-65/42-196	(Ferreira, et al., 2012)
2012	sémola de trigo	10	EI (70eV) QqQ	n.m./1.25-10	(Rodríguez-Carrasco, et al., 2012)
2012	maíz	1	IT	70/120	(Tran, et al., 2012)
2013	maíz, trigo	6	EI (70eV) Q	0,04-10/0,1-30	(Gregori, et al., 2013)
2013	trigo, arroz, maíz	10	EI (70eV) QqQ	0,6-5/1,25-10	(Rodríguez-Carrasco, et al., 2014a)
2014	orina	15	EI (70eV) QqQ	0,12-4/0,25-8	(Rodríguez-Carrasco, et al., 2014b)
2015	aceites	6	EI (70eV) QqQ	0,01-0,06/0,03-0,2	(Qian, et al., 2015)

n.m. not mentioned

y amplio campo de aplicación. Entre las ventajas más importantes que presenta la LC se encuentra la posibilidad de separar sustancias termolábiles, no volátiles, polares y apolares con aceptable resolución entre sustancias químicamente similares, de manera rápida y reproducible.

Para el análisis de micotoxinas se emplea la cromatografía en fase reversa (*reverse phase,* RP). La más común es la fase de octadecilsílice, que se utiliza junto con mezclas de disolventes polares como fases móviles (por ejemplo agua, metanol y acetonitrilo). Para el estudio de compuestos con estructura química y polaridades similares la elución isocrática es preferible mientras que la elución en gradiente es útil para el estudio de micotoxinas de diferente polaridad.

La asociación entre la cromatografía líquida y la espectrometría de masas se hizo posible gracias al desarrollo de técnicas de ionización a presión atmosférica (API). Las técnicas de API usadas en LC-MS son electrospray y APCI (ionización química a presión atmosférica).

La primera aplicación de la LC-MS en el análisis de AFs la realizó Rao and Anders, (1973), y desde entonces numerosos trabajos han sido publicados para la determinación de micotoxinas con propiedades físico-químicas muy dispares (Krska, et al., 2005). Algunos de los métodos publicados en los últimos cinco años para la determinación de micotoxinas por LC-MS se recogen en la Tabla 12.

Tabla 12: Métodos publicados en los últimos cinco años para la determinación de micotoxinas por LC-MS

Año	Matriz	Nº compuestos	LC-MS		Referencia
			Ionización	LOD/LOQ (µg/kg ó µg/L)	
2010	maíz, trigo, cebada	11	ESI (±) TOF	5-50/10-100	(Zachariasova, et al., 2010)
2010	maíz, trigo	9	ESI (±) QTrap	0,2-80/0,1-120	(Capriotti, et al., 2010)
2011	alimentos infantiles, leche	6	ESI (+) QqQ	2-9/6-25	(Beltrán, et al., 2011)
2011	cereales y derivados	1	ESI (+) QqQ	0,001/0,005	(Tam, et al., 2011)
2012	cereales y derivados	13	ESI (±) QqQ	n.m./0,25-85	(Serrano, et al., 2012)
2012	Maíz	11	ESI (±) QqQ	0,04-3,4/0,1-11	(Varga, et al., 2012)
2013	nueces y frutos secos	5	ESI (±) QqQ	0,02-0,15/0,1-0,5	(Tolosa, et al., 2013)
2013	semilla de loto	4	ESI (±) QqQ	0,003-0,007/0,01-0,02	(Liu, et al., 2013)
2014	alimentos infantiles	20	ESI (±) QqQ	0,05-5,5	(Juan, et al., 2014)
2014	Vino	36	ESI (±) QqQ	0,4-200/0,2-200	(Pizzutti, et al., 2014)
2015	Vino	1	ESI (±) QqQ	0,13/0,41	(Mariño-Repizo, et al., 2015)

1.5.3.2. Espectrometría de masas

La espectrometría de masas es una técnica basada en la ionización de la muestra y en la separación y registro de los iones producidos, según su relación masa-carga (*m/z*), en un sistema de vacío. La principal distinción entre los diversos espectrómetros de masas se encuentra en el tipo de analizador utilizado. Los analizadores de mayor relevancia para el análisis de contaminantes alimentarios son: trampa de iones (IT), tiempo de vuelo (TOF) y cuadrupolar (Q) (Hird, et al., 2014). Este apartado se centra exclusivamente en este último ya que la totalidad de los trabajos realizados en la presente Tesis se basan en el uso de analizadores triple cuadrupolo (QqQ).

Un analizador cuadrupolar consiste en cuatro barras o polos de sección cilíndrica o hiperbólica alineados paralelamente entre sí (Figura 8). Los iones de una determinada *m/z* siguen una trayectoria oscilante hasta el detector, a través del túnel formado por los cilindros, como consecuencia de la aplicación de voltajes variables de corriente continua (DC) y de radiofrecuencia (RF). De este modo, el cuadrupolo actúa como un filtro de iones, discriminándolos en función de su relación *m/z*.

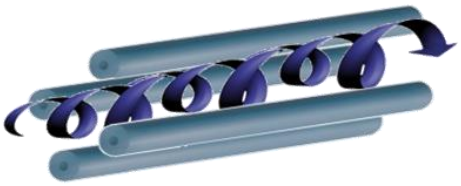


Figura 8: Esquema de un analizador cuadrupolar

Existen dos tipos de tándem (MS/MS): en el espacio (triple cuadrupolo e híbrido cuadrupolo-tiempo de vuelo) y el tándem en el tiempo (trampa de iones), así como combinaciones de ambos tipos de tándem en el tiempo y espacio (trampa lineal o el híbrido trampa de iones-tiempo de vuelo). La combinación de varios analizadores permite obtener información estructural y aumentar la especificidad de las determinaciones cuantitativas. En el triple cuadrupolo, el primer cuadrupolo actúa como un filtro que selecciona y separa las moléculas protonadas o desprotonadas en función del modo de ionización (positivo o negativo) utilizado. El tercer cuadrupolo actúa como analizador de masas y registra los productos obtenidos en el proceso de disociación al que son sometidos los iones seleccionados en el primer cuadrupolo, denominados iones precursores. Este proceso de disociación es inducido por un gas ionizado y acelerado en el segundo cuadrupolo, de forma que colisiona con las moléculas de analito provocando su fragmentación (Figura 9). Con analizadores QqQ se puede monitorizar una transición concreta (*Selected Reaction Monitoring*, SRM) de modo que se consigue aumentar de manera significativa la relación señal/ruido (*Signal to Noise*, S/N) al disminuir considerablemente el ruido químico repercutiendo en un notable aumento de la sensibilidad. Trabajar con transiciones específicas se traduce en métodos altamente selectivos, pudiendo utilizarse con finalidad confirmatoria.

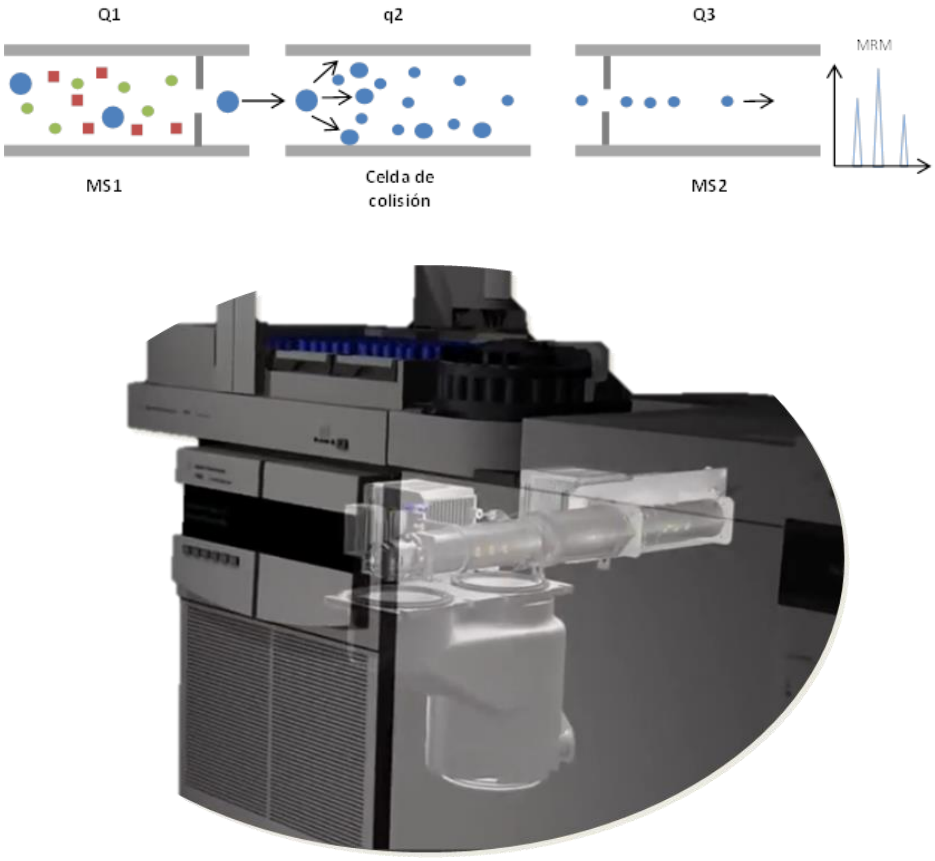


Figura 9: Esquema de un triple cuadrupolo

El desarrollo de la tecnología de espectrometría de masas ha permitido la puesta a punto de procedimientos analíticos para la determinación simultánea de varias micotoxinas. En este sentido el alto poder de resolución aportado por las técnicas cromatográficas junto con la elevada selectividad y sensibilidad suministrada por el espectrómetro de masas, convierte el acoplamiento de GC-MS o LC-MS en herramientas analíticas altamente poderosas para la identificación y cuantificación de

micotoxinas. No obstante, la gran diversidad química de las toxinas y los intervalos de concentración tan amplios y variados condicionan su determinación simultánea (Krska, et al., 2008). Por ejemplo, Varga, et al. (2013), desarrollaron un método semicuantitativo basado en cromatografía líquida de ultra resolución (UHPLC) acoplado a detección MS/MS para la determinación de 191 micotoxinas y otros metabolitos fúngicos en almendras, avellanas, cacahuetes y pistachos. Sin embargo, únicamente se obtuvieron resultados de validación satisfactorios para 65 analitos.

Así pues, a pesar de la elevada selectividad de la técnica, se encuentran dificultades en los métodos basados en LC-MS/MS como es la exaltación o supresión de la señal analítica como consecuencia del efecto que tienen interferentes presentes en la matriz en el momento de la ionización del analito. Aparte de esto, la adquisición y subsecuente mantenimiento de los equipos puede suponer grandes inversiones económicas para los laboratorios.

1.6. Evaluación de la exposición

El Libro Blanco sobre Seguridad Alimentaria establece que el análisis del riesgo debe ser la base de la política de la seguridad alimentaria, mediante sus tres componentes:

- evaluación del riesgo (asesoramiento científico y análisis de datos),
- gestión del riesgo (reglamentación y control)
- y comunicación del riesgo.

La evaluación toxicológica del riesgo producido por micotoxinas trata de identificar y estimar la probabilidad de que la exposición a las mismas produzca efectos nocivos. La metodología para su evaluación se aborda desde cuatro etapas:

- identificación del peligro,
- caracterización del peligro,
- evaluación de la exposición
- y caracterización del riesgo,

siendo esta última la integración de toda la información recabada en las etapas anteriores con objeto de estimar la probabilidad de aparición de efectos adversos para la salud en la población como consecuencia de la exposición.

Para evaluar la exposición se pueden adoptar en general tres enfoques: estudios de la dieta total, estudios selectivos de determinados alimentos, y estudios de porciones testigo (Kroes, et al., 2002). Tradicionalmente, los estudios de evaluación de la exposición a contaminantes alimentarios se han llevado a cabo combinando los datos de consumo de determinados alimentos con datos sobre la concentración de tóxicos en los mismos, y teniendo en cuenta el peso corporal de la población objeto de estudio (Marin, et al., 2013). No obstante, cabe destacar que la evaluación de riesgos está sujeta a incertidumbres relacionadas con los datos y con la selección del modelo apropiado. En este sentido, es esencial disponer de datos fiables sobre la ingesta de alimentos

para poder llevar a cabo una correcta evaluación de la exposición a partir de las concentraciones de los productos químicos presentes en los alimentos. Es importante conseguir datos detallados sobre el consumo de alimentos por parte del consumidor medio y de distintos grupos de población a fin de evaluar la exposición, sobre todo de los grupos sensibles (IPCS, 2009).

Por otro lado cabe además tener presente los compuestos conjugados, conocidos como micotoxinas enmascaradas, generados como consecuencia de los mecanismos naturales de detoxificación de algunas plantas y/o procesado de alimentos. Estas formas podrían ser hidrolizadas a sus precursoras en el tracto digestivo, pudiendo presentar efectos tóxicos comparables a los del compuesto original (Berthiller, et al., 2013).

Por consiguiente, para tratar de minimizar la incertidumbre asociada a la evaluación de la exposición a través del análisis de los alimentos, en los últimos años se ha recurrido cada vez más a la vigilancia directa de los tejidos y los líquidos corporales humanos a través del desarrollo de biomarcadores de exposición (Warth, et al., 2012a; Song, et al., 2013;), entendiendo como tal una sustancia exógena, su metabolito o el producto de una interacción entre un agente xenobiótico y alguna célula diana, que es medido a través de tejidos y fluidos biológicos y puede correlacionarse con la exposición a un compuesto específico.

1.7. Metabolismo y biomarcadores

El uso de marcadores biológicos, o biomarcadores, en toxicología tiene como principales objetivos medir la exposición a los agentes xenobióticos que producen enfermedades y predecir la respuesta tóxica que podría ocurrir (Arango, 2012). No obstante, para que ello ocurra la micotoxina debe ser bioaccesible y biodisponible. La bioaccesibilidad se refiere a la cantidad de toxina que se libera de la matriz alimentaria en el tracto gastrointestinal y, por consiguiente, puede ser absorbida. La biodisponibilidad hace referencia a la fracción de tóxico bioaccesible que alcanza la circulación sistémica a partir del tracto gastrointestinal y que está disponible para ejercer sus efectos adversos. Más allá de estos conceptos, cabe también tener en cuenta el metabolismo y biotransformación del tóxico. Este último concepto debe ser diferenciado del metabolismo humano considerando que algunas toxinas pueden también ser modificadas químicamente por microbiota y como consecuencia bioactivar o detoxificar el compuesto original.

En el desarrollo de biomarcadores la sensibilidad y la especificidad son parámetros esenciales (Crews, et al., 2001). La sensibilidad se relaciona con la posibilidad de detección del contaminante o de sus metabolitos cuando se encuentran en cantidades bajas; en este sentido los límites de detección de las técnicas analíticas juegan un papel fundamental. La especificidad se relaciona con la certeza de asociar el biomarcador únicamente al resultado evaluado y no a otros efectos no relacionados.

Se han propuesto en la literatura varios biomarcadores de exposición para algunas de las micotoxinas de mayor preocupación toxicológica. Por ejemplo, para la AFB_1 se ha propuesto la evaluación en orina de la AFM_1 y del aducto 8,9-dihidro-8-(N^7-guanil)-9-hidroxi-AFB_1, productos de su metabolismo mediada por el citocromo P450 (CYPs), así como el aducto albúmina- AFB_1 en plasma (Kensler, et al., 2011).

Con respecto a la OTA, la concentración de OTA en plasma y la concentración de OTA y de su metabolito de hidrólisis ocratoxina alfa (OTA-α) en orina son los biomarcadores más utilizados para evaluar la exposición a la misma (Muñoz, et al., 2010). Sin embargo, aún es necesario establecer la relación entre las concentraciones de estas toxinas en fluidos fisiológicos y la ingesta diaria de OTA (Gilbert, et al., 2001).

En cuanto a los biomarcadores de exposición de las toxinas de *Fusarium*, el incremento en el ratio esfinganina/esfingosina se propuso como indicador específico de la exposición a FBs. No obstante, un estudio publicado recientemente por van der Westhuizen, et al. (2010) concluyó que no existe una asociación clara entre dicho ratio y la exposición a fumonisinas. Por otra parte los niveles de FB_1 en su forma libre en orina mostraron una correlación con la ingesta de éstos contaminantes, validándose posteriormente como biomarcador de exposición (Shephard, et al., 2007).

En lo que concierne a la toxicocinética de DON, todavía no está completamente resuelta para humanos. Actualmente se conoce que en

mamíferos existen dos metabolitos principales: de-epoxideoxinivalenol (DOM-1), producto de detoxificación generado por las microbiota intestinal, y los glucurónidos del DON (DON-GlcAs) resultado de la fase II del metabolismo por acción de la uridinadifosfato glucuroniltransferasa (UGT), cuyo proceso de glucuronidación todavía es desconocido. Varios estudios han concluido que el metabolito DOM-1 no es mayoritario en humanos y que su relación como biomarcador queda pendiente de estudio (Turner, et al., 2010a). Por el contrario, estudios llevados a cabo en humanos han demostrado que un elevado porcentaje de DON (entre 70 – 90%) se excreta vía renal en forma de DON-GlcAs (Turner, et al., 2010b). En este sentido Meky, et al. (2003) desarrollaron y validaron un biomarcador de exposición para DON en orina basado en una hidrólisis enzimática de las formas DON-GlcAs con β-glucuronidasa para su posterior determinación como DON total (suma de DON en forma libre + DON liberado tras la hidrólisis). El método validado se correlacionó con la ingesta de DON vía consumo de cereales y alimentos a base de cereales. Posteriormente, el grupo de investigación de Warth, et al. (2011) desarrolló una metodología para cuantificar en orina los principales DON-GlcAs, deoxynivalenol-3-*O*-glucuronido (DON-3-GlcA) y deoxynivalenol-15-*O*-glucuronido (DON-15-GlcA), sin requerir hidrólisis previa y tras sintetizar, identificar y confirmar las estructuras por resonancia magnética nuclear (NMR) y MS. Recientemente, nuevas formas conjugadas han sido también identificadas (deoxynivalenol-7-*O*-glucuronido y deoxynivalenol-8-*O*-glucuronido) (Maul, et al., 2012; Warth, et al., 2012b). En cuanto a las micotoxinas 3-ADON y

15-ADON diversos estudios han sugerido que probablemente éstos compuestos son rápidamente deacetilados *in vivo* a DON tal y como se ha observado en cerdos (Eriksen, et al., 2003).

Finalmente, respecto a la ZON poca bibliografía hay referente a la ruta metabólica de esta micotoxina en humanos. Las principales biotransformaciones de la ZON en los animales son la hidroxilación, que conlleva la formación de los metabolitos α-ZOL y β-ZOL y la conjugación con el ácido glucurónico (ZON-GlcAs) (Pfeiffer, et al., 2010). También han sido descritos otros metabolitos de la ZON, concretamente ZAN, α-ZAL y β-ZAL. No obstante, estos compuestos también han sido detectados *per se* en cereales (Mizutani, et al., 2011). En términos de toxicidad, la actividad estrogénica de α-ZOL es diez veces superior que la de la ZON, mientras que β-ZOL presenta una menor afinidad por el receptor (Malekinejad, et al., 2006). Varios estudios han mostrado que la glucuronidación es una vía importante de detoxificación de ZON y todos sus metabolitos en humanos (Shephard, et al., 2013b; Solfrizzo et al., 2014). Consecuentemente, la evaluación de ZON-GlcAs en orina podría ser evaluado como un biomarcador de exposición a ZON.

1.8. Referencias

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2. Objectives

Objetivos

El **objetivo general** de la presente investigación consiste en la puesta a punto de un procedimiento analítico para la determinación simultánea de micotoxinas de *Fusarium*, y llevar a cabo una aproximación a la evaluación de la exposición.

Para alcanzar este objetivo se han planteado los siguientes **objetivos específicos**:

1. Desarrollo de un método de análisis multimicotoxina (*n = 18*) basado en cromatografía de gases acoplada a espectrometría de masas en tándem (GC-MS/MS) con analizador de triple cuadrupolo (QqQ).

2. Evaluación de técnicas extractivas como QuEChERS, dispersión de matriz en fase sólida, microextracción líquido-líquido dispersiva y extracción líquido-líquido asistida por sales para la extracción de micotoxinas en matrices alimentarias y biológicas.

3. Optimización de las distintas técnicas de preparación de muestras y validación de todas las metodologías desarrolladas siguiendo las guías europeas relativas al análisis de residuos y criterios de calidad para asegurar la fiabilidad de los resultados obtenidos.

4. Estimar la presencia de micotoxinas en distintas matrices alimentarias, principalmente aquellas de elevado consumo, como cereales y productos a base de cereales.

5. Evaluar la exposición de la población española a las micotoxinas objeto de estudio a través del consumo de los alimentos estudiados y comparando las ingestas estimadas con las ingestas diarias tolerables.

6. Estudio de un biomarcador de exposición para la identificación y cuantificación de micotoxinas y metabolitos en orina humana.

7. Evaluación de la exposición a micotoxinas en diferentes grupos de población a través de las concentraciones de estos contaminantes en orina.

8. Estudio preliminar para conocer la correlación entre los niveles de micotoxinas ingeridos a través de la dieta consumida y los niveles de toxinas y/o metabolitos encontrados en la orina.

The **general objective** of this research was to develop an analytical procedure for the simultaneous determination of *Fusarium* mycotoxins, as well as to carry out an exposure assessment approach.

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

1. To develop a multi-mycotoxin method (*n = 18*) based on gas chromatography coupled to tandem mass spectrometry (GC-MS/MS) with triple quadrupole mass analyzer (QqQ).

2. To evaluate several sample preparation procedures such as QuEChERS, matrix solid phase dispersion, dispersive liquid-liquid microextraction and salting-out liquid-liquid extraction for the extraction of mycotoxins in food matrices and urine.

3. To optimize and validate the proposed analytical strategies following the European guidelines laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs to ensure the reliability of the obtained data.

4. To estimate the occurrence of mycotoxins in several food matrices, mainly those highly consumed such as cereals and cereal-based products.

5. To assess the exposure to mycotoxins in the Spanish population through the data of consumption and by comparing the mycotoxin intakes with the established tolerable daily intakes.

6. To evaluate a biomarker of exposure in order to identify and quantify mycotoxins and metabolites in human urine.

7. To assess the exposure to mycotoxins in several population groups through the concentrations of these food contaminants in urine.

8. To carry out a preliminary study to evaluate the correlation between the levels of mycotoxin intakes from the diet and those calculated in urine.

3. Results

Resultados

3.1. Multi-mycotoxin analysis in wheat semolina using an acetonitrile–based extraction and gas chromatography–tandem mass spectrometry

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Multi-mycotoxin analysis in wheat semolina using an acetonitrile-based extraction procedure and gas chromatography–tandem mass spectrometry

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Keywords: QuEChERS, GC–MS/MS, Triple quadrupole, Wheat

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Abstract

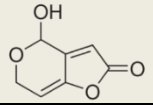
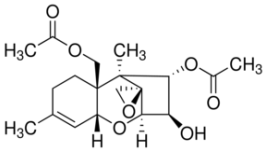
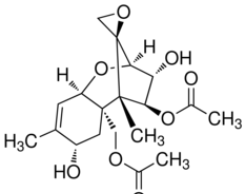
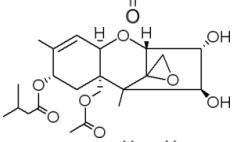
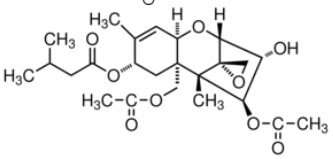
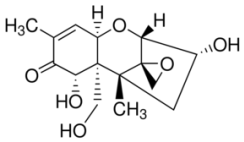
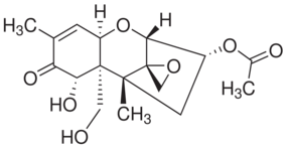
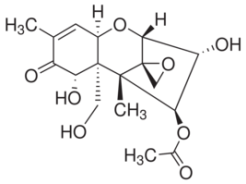
A new analytical method for the rapid and simultaneous determination of ten mycotoxins including patulin, zearalenone and eight trichothecenes (nivalenol, fusarenon-X, diacetoxyscirpenol, 3-acetyl deoxynivalenol, neosolaniol, deoxynivalenol, T-2 and HT-2) in wheat semolina has been developed and optimized. Sample extraction and purification were performed with a modified QuEChERS-based (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) procedure and determined by gas chromatography (GC) coupled to triple quadrupole instrument (QqQ). This is the first paper on the application of GC–QqQ-MS/MS to analysis of mycotoxins. Careful optimization of the gas chromatography–tandem mass spectrometry parameters was achieved in order to attain a fast separation with the best sensitivity allowing a total run time of 16 min. The validation was performed by analyzing recovery samples at three different spiked concentrations, 20, 40 and 80 μg kg^{-1}, with four replicates (*n = 4*) at each concentration. Recoveries ranged from 74% to 124% and the intra-day precision and inter-day precision, expressed as relative standard deviation, were lower than 13% and 17%, respectively for all studied compounds, except for zearalenone. Limits of quantification (LOQ) were lower than 10 μg kg^{-1} for all studied mycotoxins. Eight concentration levels were used for constructing the calibration curves which showed good linearity between LOQ and 100 times LOQ concentration levels (linear range). Matrix-matched calibration for applying the method in routine analysis is recommended for reliable quantitative results. The method

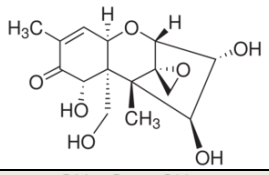
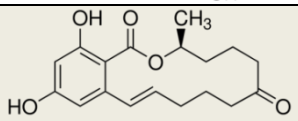
validated was successfully applied to fifteen wheat semolina samples detecting occurrence of mycotoxins at concentrations below the maximum permissible level.

1. Introduction

Food contamination with toxigenic molds has attracted increasing attention especially over the past decade. Most grain, such as wheat, maize and rice, can be infested by filamentous and microscopic fungi which can produce a wide range of mycotoxins. Mycotoxins are defined as secondary metabolites produced by a variety of fungi e.g. *Fusarium*, *Aspergillus*, *Alternaria*, *Penicillium*, *Claviceps*, etc. under appropriate circumstances. Some of the mycotoxins can have carcinogenic, mutagenic or teratogenic properties, and thus the occurrence of mycotoxins in agricultural commodities has been long recognized as a potential hazard for both human and animal health [1]. Usually, exposure is through consumption of contaminated food and feed, which causes diseases known as mycotoxicosis [2]. It was shown that toxin concentration and visible infection may not correlate in every case, and mycotoxins can be present in commodities without being able to detect fungi associated with the toxins and vice versa. Hence, urgent measures such as continuous monitoring and regulation of maximum mycotoxin levels in food products and commodities have been set in several countries [3]. A variety of *Fusarium* molds produce a number of different toxins, such as trichothecenes and zearalonene (Table 1).

Table 1: Chemical structures and main mold producers of the mycotoxins under study

Main mold producer	Mycotoxin	Chemical structure	Empirical formula	MW (g mol^{-1})
Aspergillus, Penicillium	PAT		$C_7H_6O_4$	154.12
Fusarium, *Trichoderma, Myrothecium, Stachybotrys, Cylindrocarpon, Trichothecium*	DAS		$C_{19}H_{26}O_7$	366.41
	NEO		$C_{19}H_{26}O_8$	382.40
	HT-2		$C_{22}H_{32}O_8$	424.48
	T-2		$C_{24}H_{34}O_9$	466.52
	DON		$C_{15}H_{20}O_6$	296.32
	3-ADON		$C_{17}H_{22}O_7$	338.35
	FUS-X		$C_{17}H_{22}O_8$	354.35

	NIV		$C_{15}H_{20}O_7$	312.32
Fusarium	ZON		$C_{18}H_{22}O_5$	318.36

MW: Molecular weight

Trichothecenes are a family of closed related sesquiterpenoids and according to their characteristic functional groups are divided into four groups (A–D). Type A trichothecenes are represented by HT-2 and T-2 toxins as well as by diacetoxyscirpenol (DAS) and neosolaniol (NEO). Type B trichothecenes is most frequently represented by deoxynivalenol (DON) and for its derivates 3-acetyldeoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON) together with other type B trichothecenes such as fusarenon-X (FUS-X) and nivalenol (NIV). They are immunosuppressive compounds which inhibit the synthesis of protein and may cause tissue necrosis, hemorrhage throughout the digestive tract, depression of blood regenerative processes in the marrow and spleen, changes in reproductive organs, diarrhea, vomiting, reduced feed intake, weight loss, abortion and death [4,5]. Regarding zearalenone (ZON), it is an estrogenic compound which causes hyperestrogenism and a variety of symptoms, such as decreasing fertility or increasing embryolethal resorptions in livestock and could be an important etiologic agent of intoxication in young children [6]. Other molds, as *Aspergillus* and *Penicillium*, also can colonize grains and produce mycotoxins. Patulin (PAT) is produced by different species of

Penicillium, *Aspergillus*, and *Byssochlamys* (Table 1). The most widespread among these fungi, *Penicillium expansum*, is known to invade fruits, berries, vegetables, bread and meat products [7]. Acute symptoms of patulin consumption can include agitation, convulsions, edema, ulceration, intestinal inflammation and vomiting. Chronic health effects of patulin were observed in experimental animals, while its effects on humans are not clear yet [8].

The importance of food quality control is widely recognized nowadays to assure the compliance of regulation of these products and guarantee the consumers health. The presence of mycotoxins in food is a matter of concern. For this reason, strict legislation exists at European Union (EU) level that establishes maximum levels. To ensure both regulatory levels, mass spectrometric determination and maximum levels fixed by EU, as well as to assess the toxicological risk for humans, the use of an analytical method that has been proved to generate reliable results is required. Additionally, there is a growing demand to reinforce the use of multi-mycotoxin methods to reduce time-frames and increase overall throughput [9]. In this way, for official food control, both gas chromatography and liquid chromatography coupled with mass spectrometry using different analyzers offer several advantages enabling analysis of complex samples. Over the last few years, liquid chromatography–tandem mass spectrometry (LC–MS/MS) has become the most extensively used analytical method for mycotoxins belonging to different chemical families enabling their efficient quantitative

determination in several food commodities. GC–MS methods offer a narrower analytical scope allowing simultaneously a very useful and relatively inexpensive analytical performance with some clear advantages as lower detection limits and greater selectivity [10–13]. In the multistage tandem mass spectrometric analysis, ion trap is selected for the fragmentation behavior studies acquiring information about the product ions [14,15] while GC-TOF is used as novel technique, for accurate mass measurements based on the extract masses [16]. The coupling of gas chromatography with a triple quadrupole mass spectrometer analyzer is a potent analytical tool for the determination of trace food contaminants in foodstuffs [17–19]. The use of triple quadrupole leads the possibility of adequate selection of precursor and ion products to reduce chemical noise in the chromatograms [20]. Special attention was devoted in this paper to the optimization of triple quadrupole operating parameters because to our knowledge there are a scarce detailed specification and discussion on settings these parameters.

The development of multi-mycotoxin methods with one common sample preparation and a single final determination is highly desirable. In this regard, the most critical step is the extraction that should allow good recoveries for all mycotoxins under investigation in a specific food matrix. The technique most frequently reported in the literature is solvent extraction but in many cases, is not selective enough to comply with the needs of food safety and regulations and is coupled with a clean up on a solid phase extraction (SPE) column [21]. Extraction techniques such as

matrix solid phase dispersion (MSPD) [22,23], pressurized liquid extraction (PLE) [24,25], solid-phase microextraction (SPME) [26], molecular imprinted polymers (MIPs) [27,28] and dispersive liquid–liquid microextraction (DLLME) [29] have been widely employed to achieve extraction and clean up during the determination of multiresidue and multiclass compounds in different matrices requiring less organic solvent and in general yielded satisfactory results.

Recently, QuEChERS extraction procedure has been frequently employed as a sample preparation methodology for multiresidue mycotoxin analysis, and has been modified and validated for the detection of a broad range of pollutants in food, including acidic and basic ones [25,30–33]. The original QuEChERS method developed by Anastassiades et al. [34] consists of initial extraction with acetonitrile, followed by partitioning after the addition of adequately mixed salts (anhydrous magnesium sulfate and sodium chloride), after which the extract is subjected to dispersive solid-phase extraction (d-SPE) followed by clean up with primary secondary amine and anhydrous magnesium sulfate. However, many modifications have been introduced, such as buffering the extraction medium with an acetate [35] or citrate buffer [36], or changes in the extraction solutions [37] in order to extract a wide range of compounds as was reported by Romero-González et al. [38] who optimized a QuEChERS-based extraction for more than 90 compounds. This technique has many advantages as the fastness and the high simple throughput, the

smaller volumes of no chlorinated organic solvents used and the good recoveries obtained.

To the best of our knowledge, up to now no paper has been reported on the application of GC–QqQ-MS/MS for the determination of mycotoxins in food samples. In this paper, a multi-mycotoxin method based on QuEChERS extraction and using GC–MS/MS analysis with triple quadrupole for detection, quantification and reliable identification of the analytes present in the samples, was developed and validated in order to attain the legal limits established by EU regulation for these mycotoxins [39–41]. An additional goal was to evaluate the occurrence of ten selected mycotoxins in wheat semolina samples commercialized in Spain.

2. Materials and methods

2.1. Chemical and reagents

Solvents (acetonitrile, hexane and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulphate was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18 was purchased from Phenomenex (Torrance, USA).

The derivatization reagent composed of BSA (N,O-bis(trimethyl silyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethyl silyilimidazole) (3:2:3) was purchased from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to

prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

2.2. Analytical standards

The standards of patulin, zearalenone and the type A and B trichothecenes:deoxynivalenol, 3-acetyldeoxynivalenol, diacetoxyscirpenol, nivalenol, fusarenon-X, neosolaniol, T-2 and HT-2 toxin were obtained from Sigma–Aldrich (St. Louis, USA). On the one hand, all stock solutions were prepared by dissolving 1 mg of the mycotoxin in 1 mL of pure methanol, obtaining a 1 mg mL^{-1} (1000 mg L^{-1}) solution. On the other hand, the stock solutions were diluted with acetonitrile in order to obtain the appropriate multicompound working standard solutions. All standards were kept at −20°C.

2.3. GC–QqQ-MS/MS conditions

Chromatographic determination was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, USA). The mass spectrometer was operated in electron impact ionization (EI, 70 eV). The transfer line and source temperatures were 280°C and 230°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data were acquired and processed using the Agilent Masshunter version B.04.00 software. Analytes were separated on a

HP-5MS 30 m × 0.25 mm × 0.25 µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode at 250°C in programmable temperature vaporization (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially 80°C, and the temperature was increased to 245°C at 60°C min^{-1}. After a 3 min hold time, the temperature was increased to 260°C at 3°C min^{-1} and finally to 270°C at 10°C min^{-1} and then held for 10 min.

2.4. Sampling

A total of fifteen wheat semolina samples were randomly purchased in supermarkets located in Valencia Metropolitan Area (Spain). All samples were homogenized using a laboratory mill and stored in a dark and a dry place into specific plastic food containers and analyzed within 2 days of sampling.

2.5. Sample preparation

2.5.1. Extraction and clean-up

A schematic flow diagram of the QuEChERS procedure employed is shown in Fig. 1A. 5 g of each homogenized sample were weighed into a 50 mL centrifuge tube and 25 mL of distilled water were added prior to be sonicated for 15 min. The main extraction involved the addition of 7.5 mL of acetonitrile, 4 g of MgSO4 and 1 g of NaCl. To induce phase separation and mycotoxins partitioning, the tube was shaken on a vortex for 30 s and centrifuged for 3 min at 4000 rpm. Then the upper layer was submitted to

a dispersive SPE clean up with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18. The tube was vortexed for 30 s and centrifuged for 1 min at 1500 rpm. After purification the extract was transferred into a vial and evaporated to dryness under nitrogen flow.

2.5.2. Derivatization

The dry extract was added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 250 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to an autosampler vial for the chromatographic analysis (Fig. 1B).

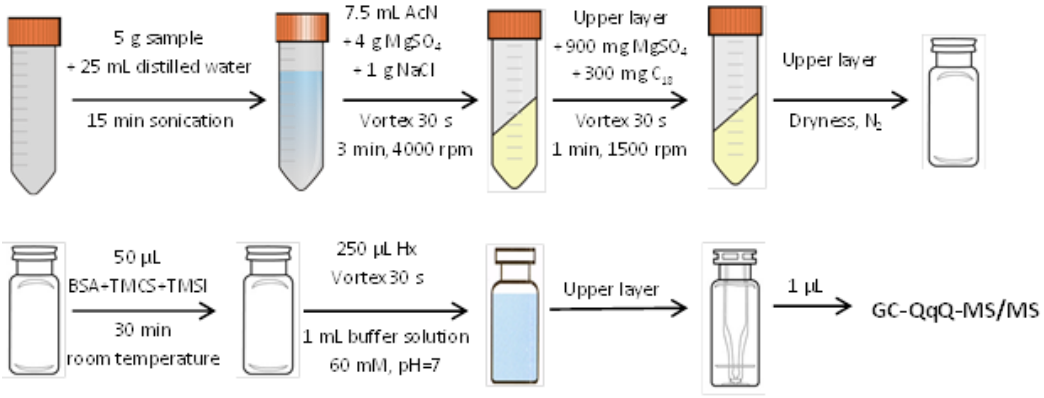


Figure 1: Schematic flow of the procedure followed in the proposed methodology. (A) Extraction and clean up step; (B) Derivatization step

2.6. Method validation

Two MS/MS transitions were acquired for each mycotoxin reaching four identification points with a defined MRM transitions ratio for the developed method as is indicated in the requirements for mass spectrometry [42, 43]. For each compound, the most abundant MRM transition was used for quantification while the other transition was used for confirmation. The criteria applied to confirm the identity of a mycotoxin were: (1) a signal for each of the two MRM transitions of the analyte had to be identical in the sample and in the standard or matrix-matched, obtaining four identification points for each analyte; (2) the peak ratio of the confirmation transition against quantification one should be within the established in Table 3; (3) the relative retention time of the analyte in both, sample and standard solution, should be as maximum difference of 0.1 min.

Linearity and matrix effects were studied using standard solutions and matrix-matched calibrations. Matrix-matched calibration curves were built by spiking blank samples with selected mycotoxins before extraction. Both external calibration curves and matrix-matched calibration curves were constructed by plotting peak applied to the calibration curves. Eight concentration levels were employed for constructing the calibration curves, values between LOQ and 100 times LOQ analyzing them in triplicate. To assess matrix effect the slope of wheat semolina matrix-matched (A) and the slope of external calibration (B) were calculated. Thus,

the ratio (A/B × 100) is defined as the matrix effect (%). A value of 100% indicated that there was no matrix effect. There was signal enhancement if the value was higher than 100% and signal suppression if the value was lower than 100%. Limit of quantification was defined as the concentration with a signal-to-noise ratio (S/N) of 10:1. This parameter was determined by analysis decreasing concentration of the spiked wheat semolina. The method's recovery and precision were calculated by the repeated analysis of wheat semolina samples at three concentrations levels, 20 µg kg^{-1}, 40 µg kg^{-1} and 80 µg kg^{-1}, and the relative standard deviation (RSD, %) of measurements of four replicates (*n = 4*), carried out in the same day (intra-day precision) and in four different days (inter-day precision), were obtained (Table 4).

3. Results and discussion

3.1. Optimization of extraction

The extraction was carried out according to the modified QuEChERS method [31] previously developed for the determination of five mycotoxins in breakfast cereals and flours as a starting point. Several improvements in the developed method were made in order to assure the extraction of the ten selected mycotoxin. The studied parameters were the effect of pH on extraction medium as well as the effect of solvent volume and the effectiveness of the clean up.

3.1.1. Effect of pH on extraction medium

Three buffer solutions were tested in order to check the behavior of the studied mycotoxins in the extraction procedure. The assayed buffer solutions were adjusted at pH = 3, pH = 6 and pH = 8. Additionally, distilled water was also tested. Triplicates of spiked samples at 80 μg kg^{-1} were extracted with 25 mL of each of these solutions following the procedure before mentioned. Results in terms of recovery and relative standard deviation are shown in Table 2. On the one hand, among the assayed buffer solutions, alkaline phosphate buffer showed, in general, more acceptable recoveries than when extraction in acidic medium was used due to the slight acidic nature of the studied mycotoxins [11]. This tendency is in agreement with the surveys carried out by Ferreira et al. [32] and Farhadi and Maleki [44]. On the other hand, it is necessary to bear in mind that the pH not only can affect mycotoxin extraction recovery but also the alkaline pH reduce the stability of patulin and zearalenone as reported in literature [32, 44–46]. Thus, it can justify the loss of analytic response in these mycotoxins when alkaline conditions were tested. Nonetheless, high recoveries for the assayed mycotoxins were obtained with distilled water. Data ranged from 83 to 111% with RSDs lower than 3% in all analytes except for zearalenone which showed a recovery of 63% and a RSD of 21% at 80 μg kg^{-1}. Despite that, the results obtained are in accordance with the Commission Regulation (EC) No. 401/2006.

Table 2: Study of the effect of pH in the medium, the volume of acetonitrile used and influence of the clean up as the optimization parameters in the extraction step of the studied mycotoxins. Results expressed as recoveries ± % RSDs (*n= 3*).

Compound	Buffered solution, %REC (RSD)			Distilled water %REC (RSD)	Volume of solvent, %REC (RSD)			Clean up, %REC (RSD)	
	pH=3	pH=6	pH=8		V=5 mL	V=7.5 mL	V=10 mL	V=7.5 mL	V=10 mL
PAT	76 ± 7	59 ± 10	50 ± 4	***96 ± 3***	non effective	***87 ± 10***	88 ± 17	***93 ± 1***	91 ± 7
DON	46 ± 4	43 ± 2	67 ± 1	***83 ± 1***		***77 ± 12***	69 ± 16	***86 ± 1***	83 ± 6
3-ADON	92 ± 1	84 ± 1	97 ± 1	***98 ± 1***		***78 ± 9***	95 ± 10	***86 ± 1***	89 ± 6
FUS-X	65 ± 3	59 ± 5	96 ± 1	***94 ± 1***		***89 ± 6***	99 ± 14	***88 ± 3***	92 ± 8
DAS	107 ± 1	86 ± 1	106 ± 1	***107 ± 2***		***99 ± 7***	102 ± 19	***95 ± 6***	106 ± 9
NIV	53 ± 1	52 ± 5	91 ± 2	***89 ± 2***		***91 ± 3***	81 ± 9	***92 ± 2***	98 ± 12
NEO	79 ± 1	73 ± 2	91 ± 1	***90 ± 2***		***85 ± 6***	96 ± 13	***82 ± 5***	94 ± 16
HT-2	93 ± 2	58 ± 2	89 ± 2	***85 ± 3***		***81 ± 7***	104 ± 29	***84 ± 7***	101 ± 17
T-2	118 ± 2	86 ± 2	114 ± 1	***111 ± 2***		***107 ± 5***	127 ± 31	***106 ± 5***	121 ± 15
ZON	13 ± 15	31 ± 9	22 ± 19	***63 ± 21***		***64 ± 40***	87 ± 8	***86 ± 11***	93 ± 14

3.1.2. Effect of solvent volume

The main extraction procedure involved the use of a solvent, such as acetonitrile, followed by the addition of several salts. Thus, different acetonitrile volumes (5 mL, 7.5 mL and 10 mL) were tested in order to optimize the minimum volume of solvent required. Furthermore, the optimization of this step represented an important improvement concerning the time-consumption in next steps. Table 2 shows the results expressed as recoveries and RSDs of a triplicate of measures when different solvent volumes were employed. Better recoveries were obtained with the high volumes tested. However, the use of 5 mL solvent to extract the mycotoxins was non-effective due to the very few volume obtained for the clean up step. Statistical analysis (Student's t-test) of repeated measures was applied to analyze the results obtained. No significant statistical differences for a confidence interval of 95% were found between the use of 7.5 mL and 10 mL as extraction volume. Moreover with 10 mL of acetonitrile, larger time-consuming was required when the subsequent extract is evaporated to dryness. Thus, 7.5 mL was chosen as the optimum solvent volume which accomplishes the criteria established in Commission Regulation (EC) No. 401/2006.

3.1.3. Influence of clean up step

In order to maximize the efficiency of the method developed, the efficacy of the clean up was probed. With this aim, an evaluation of this step was carried out by means of the absence and the presence of a

clean up which consisted of a mixture of $MgSO_4$ and C18. The results listed in Table 2 highlighted the usefulness of the clean up step regarding recovery values obtained.

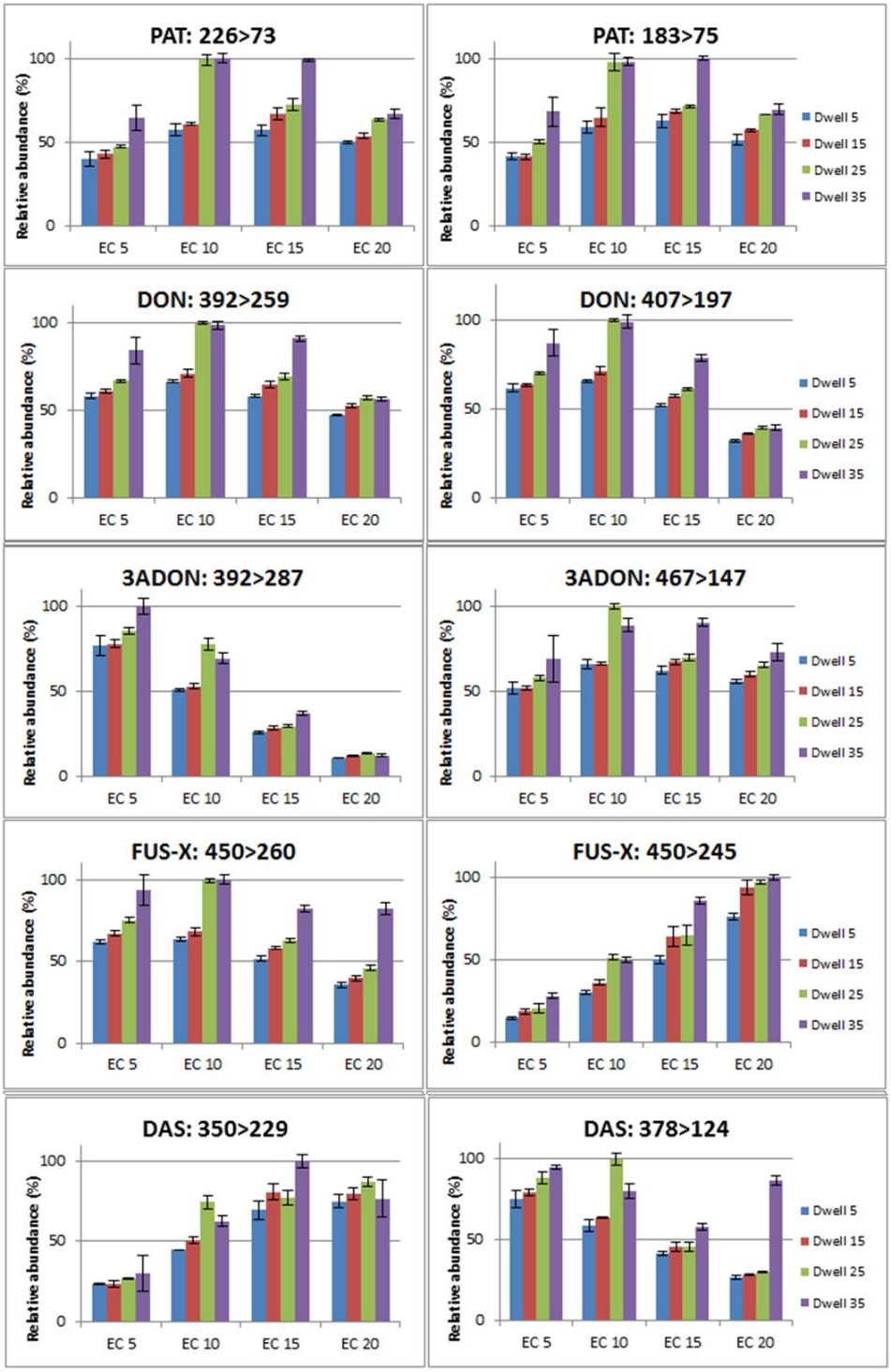
3.2. Optimization GC–QqQ-MS/MS conditions

To optimize MS/MS conditions, triple quadrupole mass spectrometer was performed for the fragmentation of mycotoxins. The relevant consideration included the choice of precursor ions, product ions, and optimization of parameters such as collision energies and dwell times (the measured time per mass) for the best response. Individual mycotoxin standards were injected in full scan mode in order to select the optimal precursor ions for the study of their fragmentation. Mass spectra in a range between 50 and 650 m/z were recorded in the electron ionization mode. With the aim of avoiding the problem of interferences from isobaric masses resulting from common molecule fragments, ions in the high mass range were preferred as the precursor ions. Once the precursor ions were selected, they were subjected to different values of collision energies (between 5 and 20 eV) to perform their fragmentation. The choice of product ions was usually made on the basis of the two most abundant fragments at the collision energy value where complete dissociation of the precursor ion occurred. In some cases, however, the second most intense ion transition was believed to be too weak or not sufficiently selective; thus, a different precursor ion which generated a more intense product ion was selected for qualitative purposes. Moreover, different dwell times

were tested, ranging from 5 to 35 ms, in order to obtain a good chromatographic peak shape maintaining satisfactory sensitivity for each compound due to for quantitative analysis the peak shape is very important, particularly when quantifications are done using peak areas. Fig. 2 shows the results obtained, in terms of relative abundance, with regards to the different binomial values of the collision energies and dwell times assayed for each selected transition. Taking into account these approaches, identifications can be made at lower levels when a more intense diagnostic ion transition is used. Once the final choices were made, the MRM conditions were tested in mixed sample extracts spiked or not with the ten mycotoxins in order to evaluate the sensitivity and selectivity of the method.

In this method, one precursor ion and two products ion were monitored (MS/MS transitions) and four identification points were achieved in compliance with Document No. SANCO 12495/2011 [43]. A complete MRM chromatogram of a spiked sample at 80 μg kg^{-1} is shown in Fig. 3. Confirmation of the detected analytes was ascertained by calculating the relative ion intensities between the area of the quantification transition and the area of the confirmation transition (ion ratio). The optimal collision energies and dwell times for each transition and the calculate ratio between quantification and confirmation transitions are listed in Table 3.

Figure 2: Optimization of the fragmentation of each transition depending on the binomial values of collision energy (CE) and dwell time employed.


PAT: 226>73
PAT: 183>75
DON: 392>259
DON: 407>197
3ADON: 392>287
3ADON: 467>147
FUS-X: 450>260
FUS-X: 450>245
DAS: 350>229
DAS: 378>124
Relative abundance (%)
0
50
100
EC 5
EC 10
EC 15
EC 20
Dwell 5
Dwell 15
Dwell 25
Dwell 35

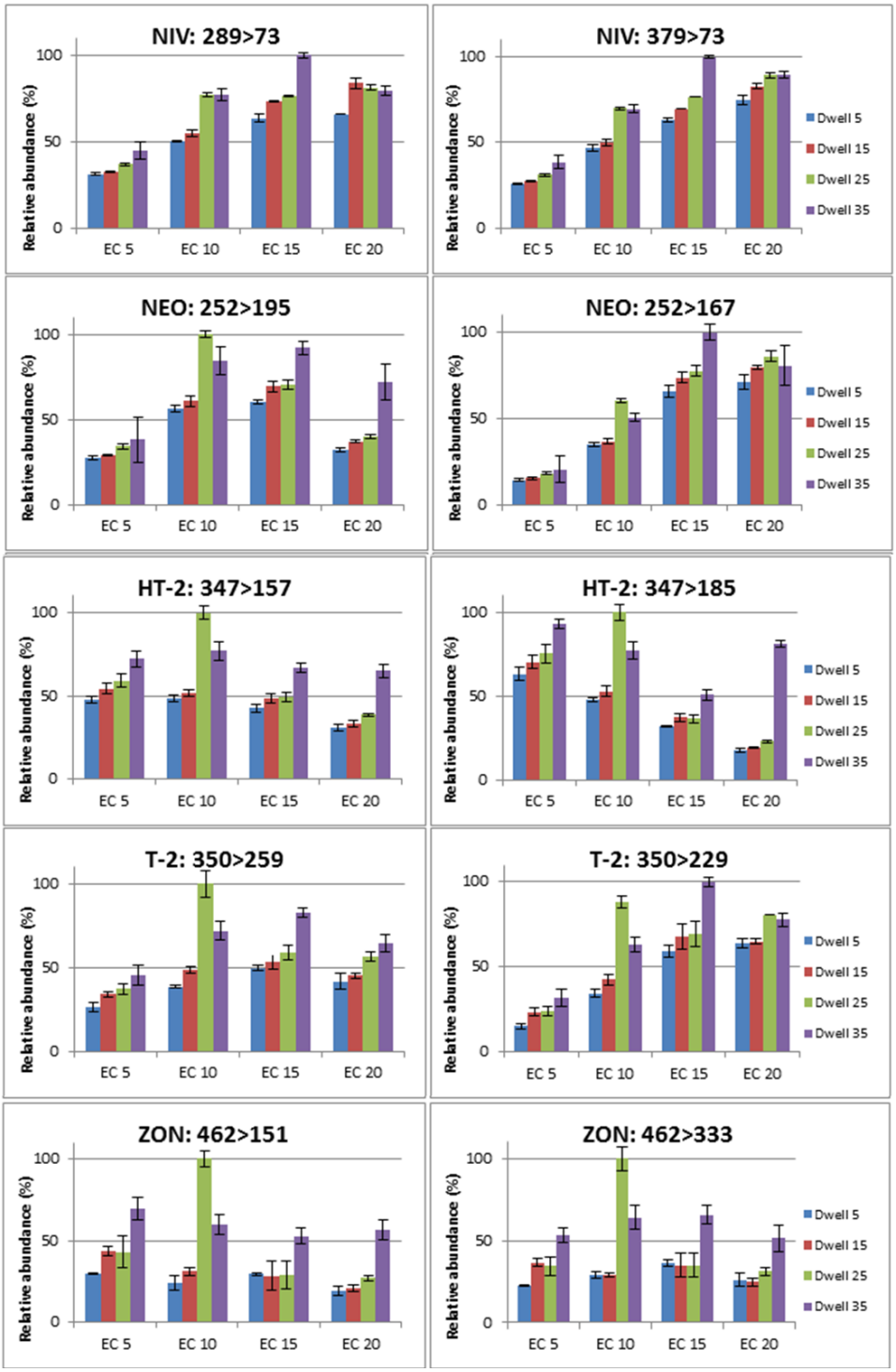

NIV: 289>73
NIV: 379>73
NEO: 252>195
NEO: 252>167
HT-2: 347>157
HT-2: 347>185
T-2: 350>259
T-2: 350>229
ZON: 462>151
ZON: 462>333
Relative abundance (%)
100
50
0
EC 5
EC 10
EC 15
EC 20
Dwell 5
Dwell 15
Dwell 25
Dwell 35

Table 3: Mass spectrometry parameters for the optimized GC-MS/MS method

Window (min)	t_R (min)	Compound	MRM_1 Quantifier	CE[a] (V)	Dt[b] (ms)	MRM_2 Qualifier	CE[a] (V)	Dt[b] (ms)	MRM_1/MRM_2 (% RSD)
5-6	5.21	PAT	226>73	10	35	183>75	15	35	88 ± 8
8-9	8.38	DON	392>259	10	25	407>197	10	25	42 ± 3
	9.42	3-ADON	392>287	5	35	467>147	10	25	48 ± 12
9.20-9.75	9.48	FUS-X	450>260	10	35	450>245	20	35	12 ± 7
	9.53	DAS	350>229	15	35	378>124	10	25	57 ± 10
9.75-10	9.89	NIV	289>73	15	35	379>73	15	35	30 ± 3
11-11.80	11.24	NEO	252>195	10	25	252>167	15	35	41 ± 4
14-15.20	14.66	HT-2	347>157	10	25	347>185	10	25	87 ± 8
	14.71	T-2	350>259	10	25	350>229	15	35	82 ± 6
15.50-16	15.72	ZON	462>151	10	25	462>333	10	25	70 ± 11

[a]: CE: Collision energy¸[b]: Dt: Dwell time

Further, the signal and thus the sensitivity decreased when several MRM transitions are measured in a particular time-window. A rapid decrease in peak intensity can be observed with increasing number of monitored transitions, which also corresponds to S/N. For that reason it was decided that a practical way to obtain a well-shaped chromatographic peaks and good sensitivity would be setting more retention-time-windows with fewer MRM transitions in each MRM segment.

3.3. Method performance

3.3.1. Linearity

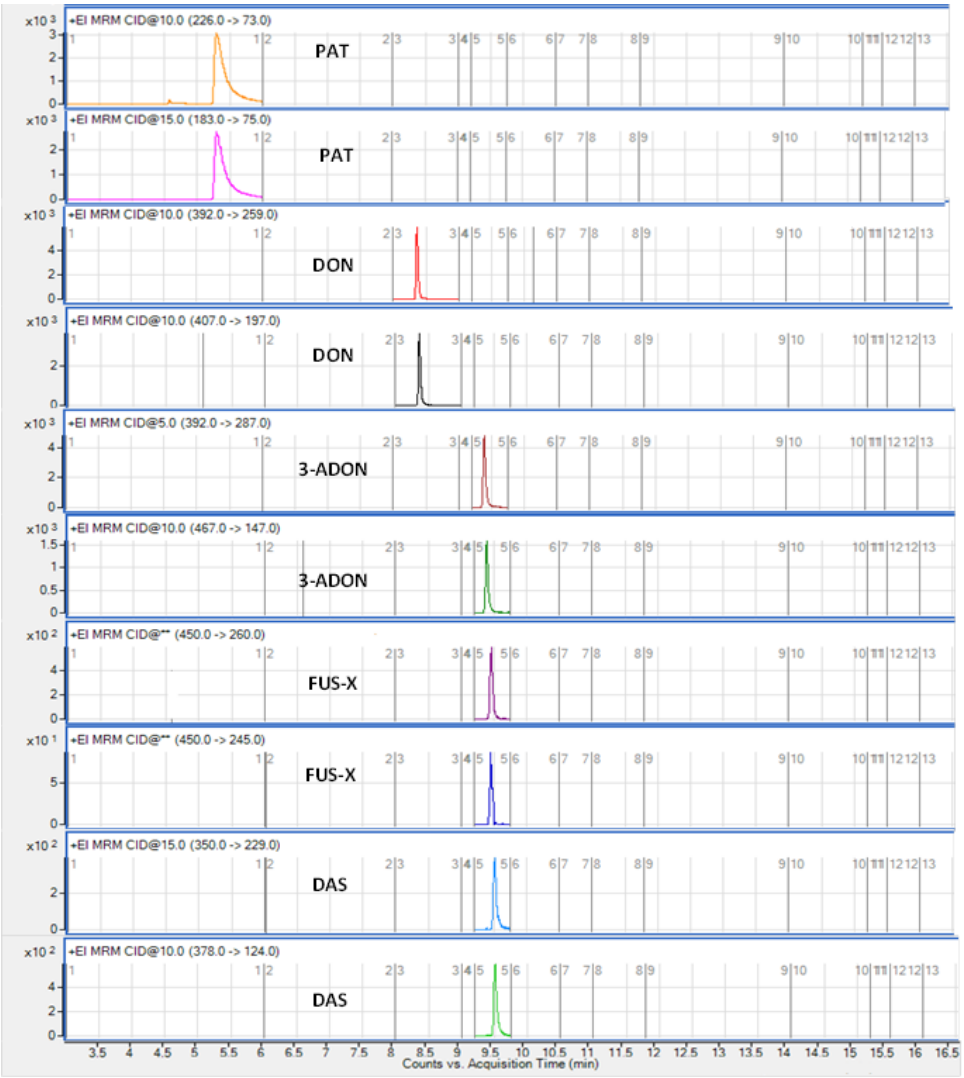
To assess the possible matrix effect on the chromatographic response, the slopes of the calibration in standard solutions with those obtained in matrix-matched standards were compared. The results obtained showed a signal suppression of the response for all analytes except for T-2 and HT-2 toxins which showed a slight enhancement in the analytical response. The results obtained demonstrated a good linearity for all derivatized mycotoxins studied within the tested range for the whole extraction and determination method (Table 4).

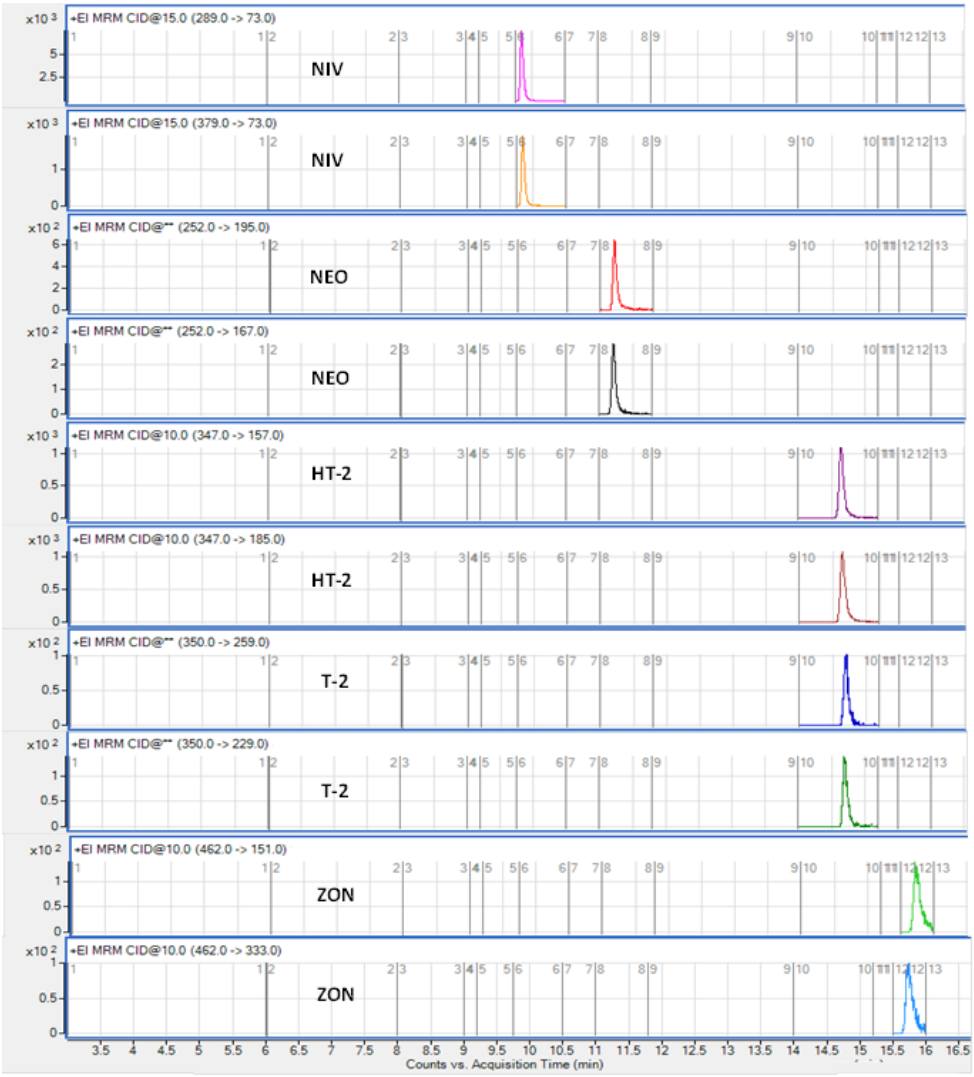
3.3.2. Recovery and precision

In general, recoveries were satisfactory (between 74% and 124% with RSDs < 13%) for all the compounds at three fortification levels (Table 4). The worst recoveries were obtained for zearalenone (ranging from 60%

to 67%); nevertheless these results were in a permitted range by Commission Regulation (EC) No. 401/2006.

Figure 3: Complete MRM chromatogram of a spiked sample at 80 µg kg^{-1}





The recovery results were very similar to those reported by Dermarchelier et al. [25] (55–112%), Sospedra et al. [30] (86–108%), Cunha-Fernandes et al. [31] (52–103%), Ferreira et al. [32] (61–118%), and Romero-González et al. [38] (70–120%).

The repeatability data expressed as intra-day precision was <13%. No apparent difference in repeatability results among the tested concentration levels was found. The inter-day precision was <17% for all studied mycotoxins except for zearalenone which was slightly upper than 20%, in spite of that, the repeatability obtained here lies also well within the commonly reported range Commission Regulation (EC) No. 401/2006.

3.3.3. Limit of quantification

Table 4 outlines LOQs of this method which were in a range from 1.25 to 10 μg kg^{-1}. The levels of LOQ obtained were lower than the maximum limits established by the EU for the legislated mycotoxins [40,41]. In addition, the LOQs of all studied mycotoxins were lower than those obtained by liquid chromatography determination even avoiding the derivatization step [1,47,48] and by GC–TOF-MS [16]. The high selectivity obtained with the GC-QqQ with the specific mass MS/MS transitions versus scanning a complete spectrum in TOF-MS could explain the results obtained.

3.4. Application of the optimized method to real wheat semolina samples

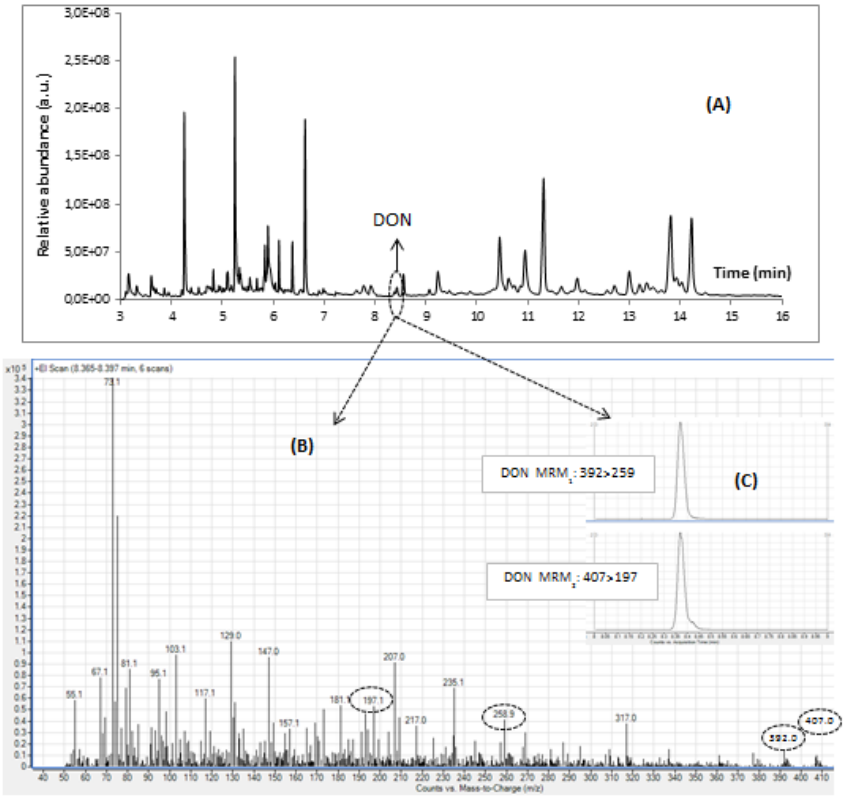
Once optimized and validated, the method was applied to the analysis of studied mycotoxins in 15 wheat semolina samples. The occurrence of mycotoxins and the detected levels in semolina are summarized in Table 5. Fig. 4 shows a chromatogram of a naturally contaminated wheat semolina sample.

Table 4: Analytical performance of the proposed method. Quantification was performed using matrix-matched calibration curves

Toxin	LOQ µg kg^{-1}	Recovery (%)			Precision (% RSDs)[b]						ME (%)	r^2
					Intra-day precision			Inter-day precision				
		20 µg kg^{-1}	40 µg kg^{-1}	80 µg kg^{-1}	20 µg kg^{-1}	40 µg kg^{-1}	80 µg kg^{-1}	20 µg kg^{-1}	40 µg kg^{-1}	80 µg kg^{-1}		
PAT	10	84	93	92	6	13	6	6	6	4	11	0.9902
DON	1.25	83	94	80	5	13	8	9	17	7	58	0.9625
3-ADON	1.25	78	105	94	13	7	8	5	1	5	59	0.9688
FUS-X	5	99	114	89	5	8	8	4	2	5	42	0.9755
DAS	5	106	114	101	9	8	6	6	1	6	92	0.9742
NIV	2.5	74	99	91	9	7	6	3	8	2	13	0.9798
NEO	5	103	117	86	11	4	6	5	3	4	101	0.9629
HT-2	2.5	86	116	84	12	7	8	6	2	2	124	0.9489
T-2	5	124	114	108	13	5	7	5	1	3	144	0.9623
ZON	10	60	67	64	9	10	9	21	18	22	90	0.9332

[a] Calculated in matrix-matched calibration; [b] RSD, relative standard deviation, (*n=4*)

Figure 4: GC-MS/MS chromatogram of a naturally contaminated wheat semolina at 55.4 µg kg^{-1} of DON showed in full scan mode (A), the mass spectrum of DON (B) and the characteristic transitions viewed in MRM mode (C)



The fifteen samples showed traces of deoxynivalenol with levels ranging from 5.8 to 55.4 µg kg^{-1}. Despite the high incidence of this mycotoxin the mean content of 17.3 µg kg^{-1} was well below the maximum legislated limit of 750 µg kg^{-1} in this type of food. The high incidence of DON found in the present study agrees with the previous studies carried

out in wheat samples. Cirillo et al. [49] found a 77% of the conventional Italian food samples and a 69% of the Italian organic food contaminated with DON at 65 and 38 μg kg^{-1}, respectively and the 84% of bread, pasta and cereal samples contaminated ranging from 7 to 93 μg kg^{-1} [50]. Similar levels of DON contamination were found by Schollenberger et al. [51] with an incidence of 92% in German bread samples and an average content of 134 μg kg^{-1}. In this way, Cano-Sancho et al. [47] found that the totally of the Spanish bread samples and the 74% of pasta analyzed were DON positive with a mean content of 242 μg kg^{-1} and 199 μg kg^{-1}, respectively. Similar results were found by González-Osnaya and co-workers [52] with an occurrence of DON in Spanish pasta samples of 62.6% ranging from 10.9 to 623.3 μg kg^{-1} and by Cunha and Fernandes [31] who reported a mean of 322 μg kg^{-1} in cereals from Portugal. Tanaka et al. [53] reported an incidence of 86% of the wheat Japanese samples analyzed with an average of 333 μg kg^{-1}, which were also according with the 272 μg kg^{-1} reported by Jestoi et al. [1] in Finnish grain samples and with the 274.6 μg kg^{-1} found in feedstuffs in Southern Europe by Griessler et al. [54].

A significant number of semolina samples tested were positive (33%) for HT-2 toxin with an average amount of 8.9 μg kg^{-1}. The HT-2 analyses were conducted by different research groups and similar amounts were found. Cano-Sancho et al. [47] reported an incidence of 10% in the samples analyzed with a mean content of 46 μg kg^{-1} and comparable results were reported by Jestoi and coworkers [1] with an average content of 40 μg kg^{-1}. Recently, Skrbic et al. [55] reported a mean contamination of

HT-2 at 9 μg kg^{-1} in the wheat samples analyzed. In the same way Lattanzio et al. [56] found in Italian cereal-based food samples an average of 3.4 μg kg^{-1}.

Data in Table 5 showed that the 20% of semolina samples were nivalenol positive with an arithmetic mean of all contaminated samples tested of 10.9 μg kg^{-1}. Jestoi et al. [1], Lattanzio et al. [56] and Tanaka et al. [53] reported different NIV occurrence in wheat samples with a mean contents of 150, 63.5 and 7 μg kg^{-1}, respectively. Referent to 3-ADON, scarce literature was reported regarding wheat contamination. An average of 17 μg kg^{-1} in the Finnish grain samples analyzed was reported by Jestoi et al. [1], while 3-ADON was quantified in only one semolina sample in the present study at 4.4 μg kg^{-1}.

3.5. Co-occurrence of mycotoxins in the samples

Fusarium fungi can produce a number of different mycotoxins, which can be produced simultaneously in a single matrix [57]. The number of positive samples for more than one mycotoxin was surprisingly high. One semolina sample showed simultaneous occurrence for DON, 3-ADON, NIV and HT-2 at 55.4 μg kg^{-1}, 4.4 μg kg^{-1}, 13.6 μg kg^{-1} and 6.7 μg kg^{-1}, respectively, whereas other four samples contained different amounts of DON, NIV and HT-2 toxins (Table 5). The results obtained suggest that the most common case of co-occurrence with the evaluated mycotoxins can be drawn by the simultaneous occurrence of DON and HT-2 toxin, as was previously elucidated in other publications [47, 55].

Table 5: Occurrence of the studied mycotoxins in naturally contaminated wheat semolina samples

Sample	Occurrence mycotoxin (µg kg^{-1})			
	DON	3-ADON	NIV	HT-2
1	6.2 ± 0.4	n.d.	n.d.	n.d.
2	9.4 ± 0.8	n.d.	n.d.	n.d.
3	12.2 ± 2.1	n.d.	10.3 ± 1.4	7.1 ± 0.9
4	6.2 ± 0.1	n.d.	n.d.	n.d.
5	52.2 ± 1.8	n.d.	n.d.	n.d.
6	16.8 ± 5.8	n.d.	n.d.	n.d.
7	14.7 ± 2.1	n.d.	n.d.	n.d.
8	8.6 ± 2.9	n.d.	n.d.	n.d.
9	8.9 ± 0.5	n.d.	8.8 ± 1.6	8.7 ± 1.9
10	55.4 ± 4.5	4.4 ± 0.1	13.6 ± 1.4	6.7 ± 0.2
11	13.9 ± 1.8	n.d.	n.d.	6.9 ± 0.1
12	7.4 ± 0.1	n.d.	n.d.	n.d.
13	5.8 ± 0.2	n.d.	n.d.	15.2 ± 2.4
14	26.1 ± 7.2	n.d.	n.d.	n.d.
15	16.9 ± 0.7	n.d.	n.d.	n.d.
% positive samples (*n = 15*)	100%	6.7 %	20 %	33.3 %
Mean (µg kg^{-1})	17.3	4.4	10.9	8.9

n.d. not detected

Interestingly, in most pasta and cereal-based food products, the co-occurrence of two or more mycotoxins was found. Additionally, several reports suggested that the mycotoxins could have synergistic effect *in vivo*; therefore, the knowledge of the occurrence of all mycotoxins should be considered a challenge in exposure assessment studies.

4. Conclusions

A rapid, sensitive and robust gas chromatography–triple quadrupole mass spectrometry method was developed for the determination of ten mycotoxins in wheat based food. Acceptable recoveries were obtained for all mycotoxins at three different spiked levels. Limits of quantification (from 1.25 to 10 μg kg^{-1}) were lower than the maximum limits established by EU. Furthermore, the GC–MS/MS optimized method offers good sensibility and selectivity, with structural information for unequivocal identification of target analytes. Finally, this method was applied to fifteen real samples and several amounts of four different mycotoxins were found. Considering the advantages of the proposed method, this could be applied for regular monitoring of mycotoxins in wheat-based food by routine laboratories. Thus, the relevance of analytical method for simultaneous monitoring of these toxins is confirmed and the need to include the co-occurrence maximum limits in the future legislative reports is suggested.

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3.2. Quantitative determination of trichothecenes in breadsticks by gas chromatography–triple quadrupole tandem mass spectrometry

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Quantitative determination of trichothecenes in breadsticks by gas chromatography-triple quadrupole tandem mass spectrometry

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Keywords: Breadsticks, Mycotoxins, Rrisk characterization, QuEChERS, GC-MS/MS

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Abstract

Breadsticks are pencil-sized sticks of dry bread widely consumed as a pre-meal appetiser. They are basically wheat-based snacks, which makes them a good matrix to evaluate mycotoxin contamination, since wheat is very susceptible to fungal attack. In this sense, the fast, selective and sensitive gas chromatography-triple quadrupole tandem mass spectrometry (GC-QqQ-MS/MS) method proposed here allows simultaneous determination of deoxynivalenol (DON), 3-acetyl deoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, neosolaniol, HT-2 and T-2 toxin in breadsticks after QuEChERS extraction and clean-up. The performance of the method was assessed with respect to European Commission Regulations by studying the selectivity and specificity, limit of detection (LOD), limit of quantitation (LOQ), linearity, matrix effect, accuracy, precision and trueness. Satisfactory results in terms of validation parameters were obtained for all selected mycotoxins (recovery range of 70 – 110%, RSD <10%, LOQ <40 μg kg^{-1}). The trueness of the method was supported by using certified reference material (DON 1062 ± 110 μg kg^{-1}). The method was successfully used to evaluate the occurrence of the studied *Fusarium* toxins in 61 breadstick samples. A total of 64% of the samples showed mycotoxin contamination, DON being the most frequently detected toxin. Nonetheless, mean levels obtained were far below the maximum levels permitted by European Union legislation. An additional goal was to carry out a risk characterisation approach to DON by comparing

probable daily intake and provisional maximum tolerable daily intake (PMTDI).

Introduction

Bakery products are one of the widest consumed food categories in the world. Among the wide range of this food category, a big portion is dry, shelf-stable foods such as biscuits, crackers and breadsticks. Typical ingredients include wheat flour, water, salt, either oil or fat, and yeast or a chemical raising agent (Ktenioudaki et al. 2012). The basic breadstick is a pencil-shaped stick of bread that has been rolled and baked to a crispy texture and seasoned lightly, usually with a little salt. They may also be combined with ingredients such as peanuts and sunflower seeds, cheese or chocolate. Overwhelmingly, the most popular varieties are made with bleached flour, although whole wheat and mixed grain versions are gaining in popularity. In fact, there is lot of interest in increasing the nutritional value of foods by means of redesigning healthier convenience foods which are consumed 'on the go'. This makes them an alternative source of nourishment to take along (Ktenioudaki et al. 2012). In this line, it has to be highlighted that the European savoury snacks market grew by 3.3% between 2007 and 2010, to reach a total of 2 billion kg in 2011. Moreover, the Spanish savoury market totaled 248.1 million kg in 2011, representing a compound annual growth rate of 1.7% since 2007, and it is forecast to have a volume of 270.9 million kg, an increase of 1.4% by 2016 (Datamonitor 2013).

Nonetheless, agricultural commodities, including wheat, are susceptible to fungal attack in the field, during drying and subsequent storage (Paterson & Lima 2010; Tirado et al. 2010). Fungi may produce as secondary metabolites diverse groups of naturally occurring toxic chemical substances, known as mycotoxins, compromising the safety of food. The *Fusarium* genus is a renowned producer of trichothecenes, considered a highly toxic class of mycotoxins that is subdivided into four groups (A–D). Among them, trichothecenes A, comprising T-2 and HT-2 toxins, and trichothecenes B, including deoxynivalenol, are the most common.

Food contaminated by mycotoxins has been associated with acute and chronic effects, especially hepatic, gastrointestinal and carcinogenic diseases (Juan-García et al. 2013). It has to be highlighted that mycotoxins are relatively stable to cooking and processing, thus food preparation procedures do not guarantee their removal (Kabak 2009). Consequently, the European Union has established maximum mycotoxin levels allowed in several food commodities in Commission Regulation EC No. 1881/2006 and EC No. 1126/2007. Currently, the maximum permitted level set for DON in bread including small bakery wares, pastries, biscuits, cereal snacks and breakfast cereals is 500 μg kg^{-1}. No other maximum limit has still been set in foodstuffs for any other trichothecene.

Monitoring of mycotoxin levels in food samples is imperative because of their widespread occurrence. For that reason, the development of sensitive, selective and reliable analytical multi-mycotoxin methods is of

major interest (Meneely et al. 2011). Sample preparation should provide clean extracts and be rapid, simple, cheap and environmental friendly as far as possible. Traditionally mycotoxins analyses were performed by the time- and solvent-consuming liquid–liquid extraction, which have been replaced by good alternatives such as pressurised liquid extraction, SPE, matrix solid phase dispersion (MSPD) and QuEChERS (Köppen et al. 2010).

MSPD includes sample homogenisation, cellular disruption, exhaustive extraction, fractionation and cleanup in a simple process in which a small amount of sample (0.1–5 g) is blended with the selected solid phase (such as C18, C8, silica, Florisil or alumina) followed by gravitational elution of compounds with a small volume of an appropriate solvent. When necessary, further purification with adsorbents can be performed.

QuEChERS consists in an acetonitrile partitioning and dispersive solid-phase extraction (d-SPE) method well known for its applicability in simultaneous analysis of a large number of compounds in a variety of food matrices. Moreover, the QuEChERS methodology presents some advantages, such as simplicity and effectiveness for complex samples cleaning-up. It generally involves two simple steps. First, the homogenised samples are extracted and partitioned using an organic solvent and salt solution, then a d-SPE technique is used for the supernatant.

As regards the determination of mycotoxins, HPLC and GC are the most widely applied techniques. In the last few years, the number of chromatographic methods coupled with MS/MS detection reported for

multiclass analysis of mycotoxins in food has increased considerably, complying with the requirements established in current legislation. More specifically, a high dynamic range and good performance reached in SRM mode make the triple quadrupole (QqQ) one of the most widely employed mass analyzer (Fernandes et al. 2013; Rodríguez-Carrasco et al. 2013).

Considering the above described situation about the determination of mycotoxins in foodstuffs, a method based on GC-QqQ-MS/MS for the simultaneous determination of eight *Fusarium* toxins belonging to the trichothecene mycotoxin group in breadsticks is proposed. To the best of our knowledge, this is the first study to provide detailed information about the occurrence and co-occurrence of *Fusarium* toxins in this widely consumed appetiser.

Material and methods

Chemical and reagents

Solvents (acetonitrile, hexane and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate (thin powder) was obtained from Alfa Aesar GmbH & Co (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, CA, USA).

The derivatisation reagent composed of BSA (N,O-bis(trimethyl silyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethyl silyilimidazole) (3:2:3) was purchased from Supelco (Bellefonte, PA, USA).

Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

The standards of the type A and B trichothecenes: deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), diacetoxyscirpenol (DAS), nivalenol (NIV), fusarenon-X (FUS-X), neosolaniol (NEO), T-2 and HT-2 toxin were obtain from Sigma-Aldrich (St. Louis, MO, USA). Individual stock solutions of all analytes were prepared at the same concentration (1000 mg L^{-1}) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (50 mg L^{-1}). All standards were stored in darkness and kept at –20°C until the GC-MS/MS analysis.

Certified reference material BRM 003004 (artificially contaminated wheat, DON 1062 ± 110 µg kg^{-1}) was purchased from Biopure Referenzsubstanzen GmBH (Tulln, Austria).

Samples

Sixty-one breadstick snacks were randomly purchased from supermarkets located in different regions of Valencia Metropolitan Area (Spain). All were homogenized (35 g) using a laboratory mill and kept at 4°C in the dark and in dry conditions.

A subdivision of the samples into six groups based on the ingredients was carried out. Samples were categorized as simple

breadsticks (*n = 21*), peanuts and sunflower seeds (*n = 14*), whole-grain (*n = 12*), cheese (*n = 7*), chocolate (*n = 5*) and soybean (*n = 2*) breadsticks.

Dispersive-based sample preparation

A preliminary study was performed in order to determine the extraction procedure that achieves the best results. In this sense, MSPD and QuEChERS methods were compared for the extraction of mycotoxins from breadsticks followed by determination by GC-MS/MS. As a starting point, a triplicate set of samples spiked at 100 µg kg^{-1} was extracted following the above-mentioned extraction methods. Recovery and precision data of both methods were compared.

QuEChERS

In brief, 5 g of homogenised sample were added to 25 mL of distilled water and sonicated for 15 min. The main extraction involved the addition of 8 mL of acetonitrile, 4 g of $MgSO_4$ and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the supernatant was submitted to d-SPE with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18 and centrifuged for 1 min at 1500 rpm. Finally the extract was evaporated to dryness under nitrogen flow.

Matrix solid-phase dispersion

The MSPD procedure described by Blesa et al. (2004) was taken as a starting point and optimised for the selected mycotoxins. Briefly, 1 g of sample was placed into a glass mortar (50 mL capacity) and gently blended

with 1 g of C18 for 5 min using a pestle to obtain a homogeneous mixture. The homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column with a coarse frit (No. 2) and covered with a plug of silanised glass wool at the top of the column, and eluted drop wise with 15 mL of acetonitrile/methanol (50/50, v/v) by applying a slight vacuum. Then the extract was transferred to a 25 mL conical tube and evaporated to dryness at 35°C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA).

Derivatisation

The dry extract was added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatised sample was diluted to 250 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7). Finally, the hexane layer was transferred to an autosampler vial for the chromatographic analysis.

GC-QqQ-MS/MS conditions

A GC system Agilent 7890 A coupled with an Agilent 7000 A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA) were used for MS/MS analysis. The mass spectrometer operated in electron-impact ionisation (EI, 70 eV). The transfer line and source temperatures were 280°C and 230°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as the

quenching gas, both at 99.999% purity and supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data were acquired and processed using Agilent Masshunter version B.04.00 software.

Separation was achieved on a HP-5MS 30 m × 0.25 mm × 0.25 µm capillary column. A total of 1 µL of the final clean extract of mycotoxins was injected in splitless mode at 250°C in a programmable temperature vaporisation (PTV) inlet employing helium as the carrier gas at a fixed pressure of 20.3 psi. The oven temperature programme was initially 80°C, and the temperature was increased to 245°C at 60°C min^{-1}. After a 3 min hold time, the temperature was increased to 260°C at 3°C min^{-1} and finally to 270°C at 10°C min^{-1} and then held for 10 min. Chromatographic analysis time was performed in 15 min. The GC-MS/MS parameters of the studied mycotoxins are shown in Table 1.

Method performance

The developed method was validated following SANCO 12495/2011. Linearity was first evaluated. Eight concentration levels for independent determination for three parallel sets between LOQ and 100 × LOQ were employed for linearity evaluation. Matrix-matched calibration curves were built by spiking blank sample extracts with selected mycotoxins at the same concentration levels than standard calibration curves.

Table 1: GC-MS/MS parameters for mycotoxin determination

MS/MS parameters	DON	3-ADON	FUS-X	DAS	NIV	NEO	HT-2	**T-2**
Quantitation transition (Q)	392>259	392>287	450>260	350>229	289>73	252>195	347>157	350>259
Collision Energy (V)	*10*	*5*	*10*	*15*	*15*	*10*	*10*	*10*
Dwell time (ms)	*25*	*35*	*35*	*35*	*35*	*25*	*25*	*25*
Confirmation transition (q)	407>197	467>147	450>245	378>124	379>73	252>167	347>185	350>229
Collision Energy (V)	*10*	*10*	*20*	*10*	*15*	*15*	*10*	*15*
Dwell time (ms)	*25*	*25*	*35*	*25*	*35*	*35*	*25*	*35*
Ion ratio (Q/q ± RSD, %)	42 ± 3	48 ± 12	12 ± 7	57 ± 10	30 ± 3	41 ± 4	87 ± 8	82 ± 6
Retention time (min)	8.28	9.42	9.48	9.53	9.89	11.24	14.66	14.71

Table 2: MSPD and QuEChERS preliminary data for the studied mycotoxins

Extraction procedure	Sample weight	Extraction time	Recovery range (at 100 µg/kg)	Solvent volume	Equipment cost	Simultaneous operation
MSPD	1 g	3 h	46 - 89%	15 mL (AcN:MeOH)	Low	Up to 6 assemblies
QuEChERS	5 g	1.5 h	70 – 110%	8 mL (AcN)	Low	Up to 12 assemblies

Accuracy was verified by measuring the recoveries from spiked blank samples at 100 μg kg^{-1}. Precision (expressed as %RSD) of the method was determined by repeatability (intra-day precision) and reproducibility (inter-day precision). Intra-day variation was evaluated in six determinations in a single day, while inter-day variation was tested on three different working days within 20 days. Sensitivity was evaluated by LOD and LOQ values. LOD was determined as the analyte concentration that produced a peak signal of three times the background noise from the chromatogram regarding SRM2, confirmation transition. LOQ was determined as the analyte concentration that produced a peak signal of 10 times the background noise from the chromatogram regarding SRM1, quantification transition.

Exposure assessment

For the exposure assessment based on a deterministic approach, the probable daily intake (PDI, μg kg^{-1} bw day) was determined by multiplying the mean concentration by the consumption data. Exposure assessment was calculated for children and adults assuming an average body weight (bw) of 25 kg and 70 kg, respectively. The ratio PDI/PMTDI expressed as a percentage (% of relevant PMTDI) was used for health risk characterisation.

Results and discussion

The effectiveness of the clean-up of the breadstick extract was compared for both dispersive-based sample preparation methods. The

most time-consuming step of the single sample preparation procedure is the evaporation under nitrogen flow. In this sense, the most time-consuming and most laborious method was MSPD. Despite both methods requiring a dryness step to derivatise, the MSPD volume to evaporate was significantly higher than those obtained by QuEChERS. Therefore, QuEChERS extraction is the fastest and less laborious method. In addition, it is possible to assemble up to 12 samples in the QuEChERS methodology, whereas in MSPD only a simultaneous operation up to six assemblies can be carried out.

The vast majority of recovery results exceeded 80% in QuEChERS, whereas a range from 46% to 89% was obtained by the MSPD method. In terms of repeatability (expressed as %RSD), all analytes gave RSDs < 15% in QuEChERS, while up to 37% was obtained in MSPD. Taking into account the above results and considering the advantages offered by QuEChERS compared with MSPD, it was chosen to complete the validation process. The differences between methods as well as the demands on chemicals, material and time are illustrated in Table 2.

Chromatographic analysis time was performed in 15 min, which meets the requirement for a high throughout determination. Moreover, the developed analytical procedure offers reliable results in terms of sensibility and sensitivity (Table 3).

Table 3: Performance characteristics of the proposed method in breadsticks

Mycotoxin	LOD (µg/kg)	LOQ (µg/kg)	REC (%)	Intra-day precision (% RSD) (*n=6*)	Inter-day precision (% RSD) (*n=3*)	ME (%)
DON	0.6	1.25	116	3	9	18
3-ADON	5	10	92	1	5	50
FUS-X	20	40	100	4	10	38
DAS	5	10	80	3	12	54
NIV	2.5	5	93	4	9	9
NEO	5	10	86	3	10	47
HT-2	1.25	2.5	80	1	7	50
T-2	20	40	73	5	11	67

ME: matrix effect. The ratio (A/B x 100) is defined as the absolute matrix effect expressed as percentage where (A) is the matrix-matched calibration slope and (B) the slope of the standard calibration in solvent

QuEChERS validation

The performance of the method was assessed to meet European Commission regulations by studying the selectivity and specificity, LOD, LOQ, linearity, matrix effect, accuracy, precision and trueness.

Selectivity and specificity were assessed by recognizing the quantitation (Q) and confirmation (q) transitions of each mycotoxin studied at values close to the LOQ in the presence of the other evaluated mycotoxins. There were neither interferences resulted from the presence of the other mycotoxins nor from the presence of the matrix. Additionally, the ratio between both transitions (Q/q) demonstrated the good correlation between peak areas and concentration of trichothecenes (Table 3).

Correlation coefficients higher than 0.990 were obtained for all mycotoxins in the concentration range studied (from LOQ to 100 × LOQ). The LODs and LOQs obtained (from 0.6 to 20 μg kg^{-1} and from 1.25 to 40 μg kg^{-1}, respectively) were lower than the maximum limits established by Commission Regulation (EC) No. 401/2006) showing the suitability of the developed method for the determination of trace amounts of *Fusarium* toxins in the food matrix studied. The method sensitivity is similar to other surveys, as shown in Table 4.

The matrix effect (ME), defined as the ratio between the slopes of the matrix-matched extract and the slopes of external calibration, was calculated. A value of 100% indicated that there was no matrix effect. There was signal enhancement with a value higher than 100% and signal suppression with values lower than 100%. ME values from 9% to 67% were obtained for the studied mycotoxins (Table 3) and matrix-matched calibrations were used for quantitation purpose.

Recovery studies for the whole procedure were carried out by spiking blank samples with the working standard solutions at 100 μg kg^{-1} to correct the matrix signal suppression. Satisfactory results in terms of recoveries were found (70 – 110%) (Table 3). The results obtained are according to recoveries accepted by the Commission Regulation (EC) No. 401/2006. Precision studies showed that the method was repeatable ($n = 6$) (RSD < 5%) and reproducible ($n = 3$) (RSD <12%).

Table 4: Comparison of the sensitivity obtained by several authors after MSPD and QuEChERS extraction and chromatography techniques coupled to mass spectrometry determination

Mycotoxin	Commodity	Extraction procedure	Confirmatory technique	LOQ (µg/kg)	Reference
DON	wheat flour		GC-MS	25	(Cunha &Fernandes, 2010)
	wheat semolina	QuEChERS	GC-MS/MS	1	(Rodríguez-Carrasco et al.,2012)
	wheat flour		LC-MS	10	(Sospedra et al.,2010)
	wheat		UHPLC	50	(Zachariasova et al., 2010)
			LC-MS/MS	4	(Zhang et al., 2013)
	wheat flour	MSPD	LC-MS/MS	45	(Rubert et al., 2012)
3-ADON	wheat	QuEChERS	UHPLC	50	(Zachariasova et al., 2010)
	wheat semolina		GC-MS/MS	1	(Rodríguez-Carrasco et al.,2012)
	wheat flour	MSPD	LC-MS/MS	18	(Rubert et al., 2012)
FUS-X	wheat flour	QuEChERS	GC-MS	30	(Cunha &Fernandes, 2010)
	wheat semolina		GC-MS/MS	5	(Rodríguez-Carrasco et al.,2012)
	wheat flour	MSPD	LC-MS/MS	30	(Rubert et al., 2012)
DAS	wheat semolina	QuEChERS	GC-MS/MS	5	(Rodríguez-Carrasco et al.,2012)
	wheat flour		LC/MS	5	(Sospedra et al.,2010)
		MSPD	LC-MS/MS	6	(Rubert et al., 2012)

NIV	wheat flour	QuEChERS	GC-MS	40	(Cunha &Fernandes, 2010)
	wheat semolina		GC-MS/MS	3	(Rodríguez-Carrasco et al.,2012)
	wheat flour		LC/MS	100	(Sospedra et al.,2010)
	wheat		UHPLC	100	(Zachariasova et al., 2010)
	wheat flour	MSPD	LC-MS/MS	110	(Rubert et al., 2012)
	wheat			75	(Rubert et al., 2011)
NEO	wheat semolina	QuEChERS	GC-MS/MS	5	(Rodríguez-Carrasco et al.,2012)
	wheat flour	MSPD	LC-MS/MS	45	(Rubert et al., 2012)
HT-2	wheat semolina	QuEChERS	GC-MS/MS	3	(Rodríguez-Carrasco et al.,2012)
	wheat		UHPLC-MS/MS	3	(Romero-González et al.,2011)
	wheat flour		LC/MS	18	(Sospedra et al.,2010)
	wheat		UHPLC	30	(Zachariasova et al., 2010)
			LC-MS/MS	4	(Zhang et al., 2013)
	wheat flour	MSPD	LC-MS/MS	10	(Rubert et al., 2012)
	wheat				(Rubert et al., 2011)
T-2	wheat semolina	QuEChERS	GC-MS/MS	5	(Rodríguez-Carrasco et al.,2012)
	wheat		UHPLC-MS/MS	5	(Romero-González et al.,2011)
	wheat flour		LC/MS	4	(Sospedra et al.,2010)
	wheat		UHPLC	10	(Zachariasova et al., 2010)
			LC-MS/MS	2.5	(Zhang et al., 2013)
	wheat	MSPD	LC-MS/MS	6	(Rubert et al., 2012)
	wheat			5	(Rubert et al., 2011)

The trueness of the method was supported by certified reference material (BRM003004). The analysis of the certified reference material showed a mean value of 1025 ± 23 μg kg^{-1} (*n = 6*) when matrix-matched calibration was used. These calculated concentrations were satisfactory according to the certificated values 1062 ± 110 μg kg^{-1}. Considering the results of the proposed method, this could be applied for regular monitoring of mycotoxins in wheat-based food by routine laboratories.

SRM chromatograms of a blank breadstick sample spiked at 100 μg kg^{-1} of the multi-mycotoxin working solution (A), a certificated reference material (wheat artificially contaminated by DON at 1032 ± 110 μg kg^{-1}) (B), and a naturally contaminated breadstick sample with DON and HT-2 at 42 and 18 μg kg^{-1} (C), respectively, are shown in Figure 1.

Quantitation of mycotoxins in snack samples

The proposed QuEChERS-based procedure followed by GC-MS/MS determination was used to evaluate the mycotoxins occurrence in 61 breadstick samples (Figure 2). Results showed that 64% of samples were contaminated with at least one mycotoxin. DON was the mycotoxin detected in higher amount. Despite its considerable incidence, the concentration range found (from 30 to 60 μg kg^{-1}, mean = 32 ± 8 μg kg^{-1}) was much lower than the maximum limit established in the current legislation for this type of matrix (500 μg kg^{-1}).

Figure 1. SRM chromatogram of a blank breadstick with a chocolate sample spiked at 100 µg kg^{-1} of the multimycotoxin working solution (A); certificated reference material (wheat artificially DON contaminated at 1032 ± 110 µg kg^{-1}) (B); and breadstick with chocolate naturally contaminated with DON (1) and HT-2 toxin (7) at 42 and 18 µg kg^{-1}, respectively (C).

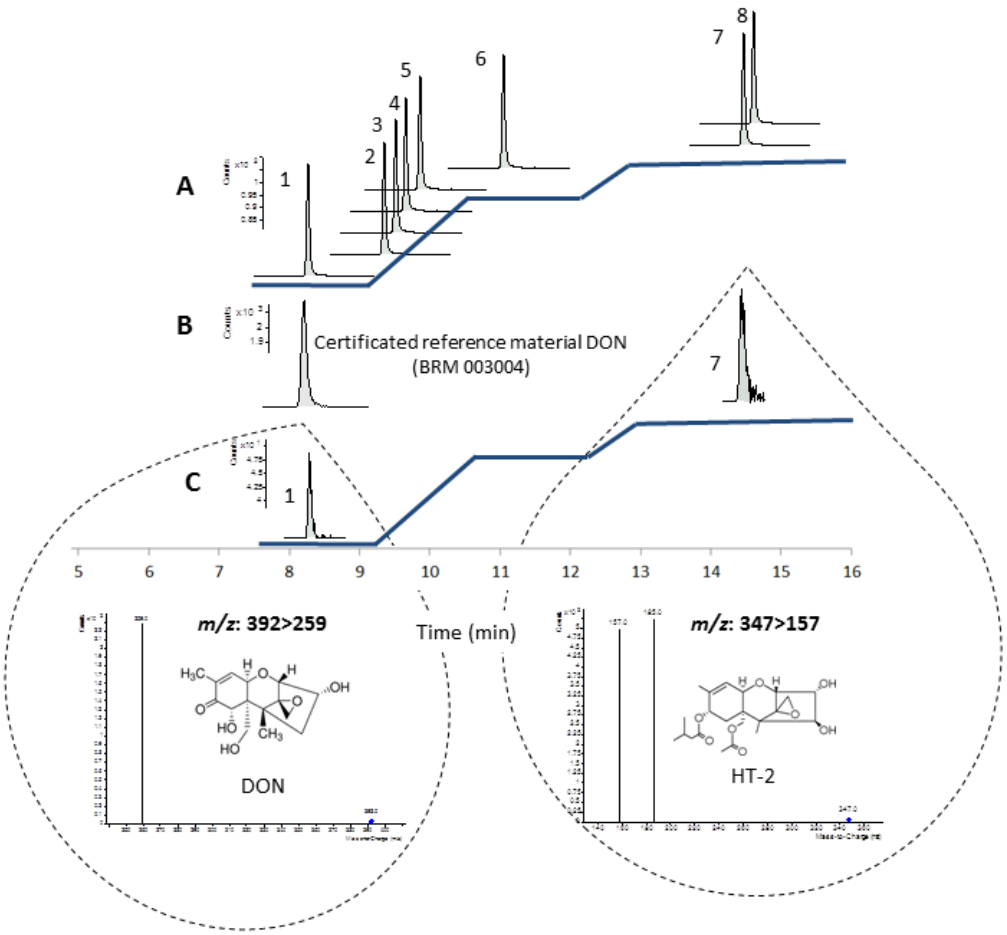
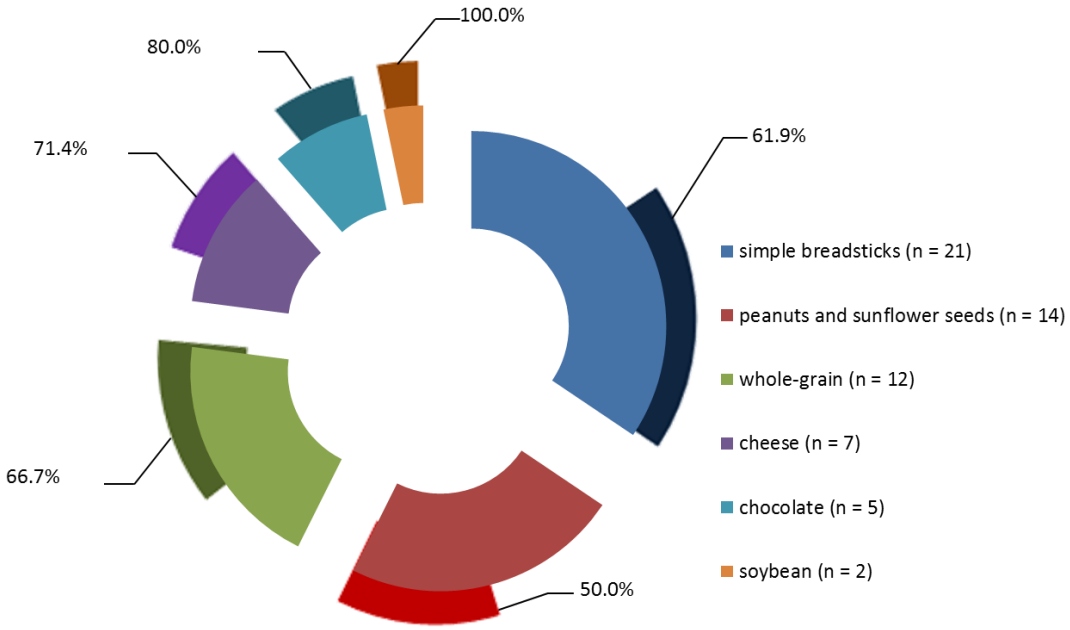


Figure 2. Incidence of mycotoxins contamination (%) in breadstick samples analysed.



Taking into account the subdivision of the samples, breadstick samples with cheese, chocolate and soybean were the samples with the highest occurrence of DON, with incidences of 71.4%, 80% and 100%, respectively. Furthermore, two out of five samples containing chocolate showed co-occurrence of mycotoxins. The *Fusarium* toxins found were DON and HT-2 toxin, which were quantified at 32 and 35 µg kg^{-1} of DON and at 17 and 22 µg kg^{-1} of HT-2, respectively. As regards the other types of breadstick samples, incidences of DON of 50.0%, 61.9% and 66.7% were found in peanuts and sunflower seeds, simple and whole grain, respectively. NEO was also found in one whole-grain sample at 19 µg kg^{-1}.

No other studied *Fusarium* toxins were found in any of the samples analysed. Recently, Tolosa et al. (2013) reported the occurrence of *Fusarium* toxins in nuts, dried fruits and dates from Valencia, and thus the results obtained in the breadsticks combined with other ingredients could be justified. In this way, Sirot et al. (2013) reported DON contamination in bread and dried bread products at mean value of 132.1 μg kg^{-1}. Similar values were obtained by González-Osnaya et al. (2011) in Spanish bread samples with a maximum concentration of 146.6 μg DON kg^{-1} and in a recent study carried out in 34 fibre-enriched bread samples by De Boevre et al. (2013) where an average DON content of 34 μg kg^{-1} was found.

A comparison of all whole-grain and simple breadstick samples was carried out in order to investigate the amount of mycotoxins in fibre-rich samples. Eight out of 12 whole-grain breadsticks were DON contaminated (66.7%), whereas 13 out of 21 simple breadsticks contained DON (61.9%). In spite of the low DON contamination, the mean levels were similar being 36 ± 5 and 40 ± 10 μg kg^{-1} for whole-grain and simple breadsticks, respectively. Statistical analysis (Student's t-test) of repeated measures was applied to analyse the results obtained. No significant statistical differences for a confidence interval of 95% were found between whole-grain and simple breadsticks. These results are in line with the study conducted by Vrček et al. (2014) who compared the nutritional value and food safety of organically and conventionally produced wheat flours. Although average concentrations of detected mycotoxins were higher in conventional than in organic flours, this difference was not significant.

Similarly, Edwards (2009) and Ok et al. (2011) did not find statistically significant differences in the trichothecene content in the analysed cereals obtained from different agricultural practices.

DON intake

A deterministic analysis was performed in a first attempt to assess the dietary exposure of DON by breadsticks. Official consumption data were not available and the authors have estimated that the complete pack acquired from a supermarket is consumed on a daily basis (35 g). As specified in the exposure assessment section, the PDI was calculated. The PDIs obtained were 0.045 and 0.016 μg kg^{-1} bw day for children and adults, respectively. The PDI was compared with DON PMTDI (1 μg kg^{-1} bw day) as an approach to DON risk characterization (SCF 2002). Results showed that the intake of the breadsticks amounted to 1.6% DON PMTDI in adults and 4.5% DON PMTDI in children.

Conclusions

The GC-QqQ-MS/MS method proposed in this paper allowed the unambiguous identification of all mycotoxins studied below μg kg^{-1} levels, fulfilling the requirements established by the European Union and satisfactory results in terms of recovery were reached by QuEChERS-based sample preparation. Considering the advantages of the proposed method, this could be applied for regular monitoring of mycotoxins in wheat-based food by routine laboratories.

As regards occurrence of mycotoxins, a considerable number of contaminated breadstick samples (64%) contained at least one mycotoxin, but at concentrations lower than the European Union maximum level. Nonetheless, it has to be highlighted that several cereal-based commodities eaten daily should be considered for exposure assessment studies.

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3.3. A survey of trichothecenes, zearalenone and patulin in milled grain-based products using GC–MS/MS

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A survey of trichothecenes, zearalenone and patulin in milled grain-based products using GC–MS/MS

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Abstract

An analytical protocol based on QuEChERS and gas chromatography–tandem mass spectrometry (GC–MS/MS) was successfully applied for the determination of trichothecenes, patulin and zearalenone in 182 milled grain-based samples. The analytical method was validated following the SANCO 1495/2011 document. LOQs were lower than 10 µg kg^{-1} for the selected mycotoxins. Recoveries of fortified cereals ranged between 76 – 108% and 77 – 114% at 20 and 80 µg kg^{-1}, respectively, with relative standard deviation lower than 9%. More than 60% of the analysed samples showed deoxynivalenol contamination, followed by HT-2 toxin and nivalenol with frequencies of 12.1% and 10.4%, respectively. Co-occurrence of mycotoxins was also detected in major cereals. A risk characterisation was carried out based on probable daily intake (PDI) and tolerable daily intake (TDI). Despite PDI of the average consumers was below TDI, special attention should be paid in high consumers as well as other susceptible population.

1. Introduction

Cereal foods are an important source of nutrition, specifically in the diet of infants and young children and are one of the first solid food eaten. Among the cereals commercialised, wheat is the most consumed cereal worldwide followed by rice and maize. The latest published data by the Food and Agricultural Organisation (FAO) reported a global consumption for wheat of 66 kg/per capita. It has to be noted that the Spanish population is one of the largest consumer of this cereal in the world, with an average consumption of 86.10 kg/per capita. Rice consumption in Spain (11.5 kg/per capita) is twice higher than the European (5.5 kg/per capita). As regards maize, an average global consumption of 17.1 kg/per capita was reported by FAO. Lately, there is also a considerable interest in the consumption of alternative crops (oat, rye, soy, barley or tapioca among others), as potential ingredients for healthy food production and for special dietary use (diabetes, coeliac disease, phenylketonuria, etc.) (FAO, 2009).

These agricultural commodities are susceptible to fungal attack in the field, during drying and subsequent storage (Juan, Moltó, Lino, & Mañes, 2008). Fungi may produce as secondary metabolites diverse groups of naturally occurring toxic chemical substances, known as mycotoxins (Bennett & Klich, 2003). In fact, the FAO has estimated that one-quarter of the world's food crop is contaminated, to some extent, with mycotoxins (JECFA, 2001). These fungal metabolites can have toxic effects that range

from acute to chronic symptoms, and thus they can represent a serious threat to both human and animal health (Richard, 2007). In this way, maximum tolerable concentrations of some mycotoxins were laid down for several foodstuffs by European legislative institutions to protect consumers from health risk associated with the intake of mycotoxins (EC No. 1126/2007; EC No. 1881/2006). It should be considered that when the mycotoxins have a similar structure or are from the same fungal origin, it is likely to expect that the mode of action of these mycotoxins and the toxicity profiles will be quite similar. Thus, co-occurrence of mycotoxins is an important parameter to contemplate in risk assessment studies. Therefore, validated analytical methods allowing simultaneous quantitation of mycotoxins in several commodities become necessary.

Mycotoxin analysis is generally a multi-step process which includes toxin extraction from the matrix, usually with mixtures of water and polar organic solvents, extract clean up and finally detection and quantitative determination. According to scientific literature, the most common extraction solvents employed are methanol/water and acetonitrile/water. Other sample preparation procedures such as pressurized liquid extraction, solid phase extraction, matrix solid phase dispersion or microextraction techniques have also been successfully applied in mycotoxin analysis. A QuEChERS (acronym of Quick, Easy, Cheap, Effective, Rugged and Safe) extraction procedure has been favorably applied for a satisfactory mycotoxins extraction in the latest years (Meneely, Ricci, van Egmond, & Elliott, 2011). The final separation and detection of compounds of interest

is usually achieved by either chromatographic techniques followed by various detection methods or by immunochemical methods. While immunochemical methods rely on specific antibodies for each mycotoxin, chromatographic techniques can separate a huge number of analytes. Both gas chromatography and liquid chromatography coupled with mass spectrometry offer several advantages enabling the use of multimycotoxin methods to reduce time-frames and increase overall throughput. Liquid chromatography–tandem mass spectrometry has become the most extensively used analytical method for mycotoxins belonging to different chemical families enabling their efficient and quantitative determination in several food commodities. Gas chromatography–mass spectrometry offers also an analytical scope allowing simultaneously a very useful and relatively inexpensive analytical performance with some clear advantages as lower detection limits and greater selectivity (Koeppen et al., 2010; Rodríguez-Carrasco, Font, Mañes, & Berrada, 2013).

In order to assess the exposure, reliable data on the occurrence of mycotoxins in different commodities is needed (Meneely et al., 2011; Rodríguez-Carrasco, Ruiz, Berrada, & Font, in press). Thus, the objective of this study was to obtain data on the occurrence of patulin, zearalenone and the type A and B trichothecenes: deoxynivalenol, 3-acetyl deoxynivalenol, diacetoxyscirpenol, nivalenol, fusarenon-X, neosolaniol, T-2 and HT-2 toxins from 182 cereal-based and gluten-free food products highly consumed as well as to evaluate the potential contribution of the selected food matrices to the dietary exposure to mycotoxins.

2. Materials and methods

2.1. Samples

A wide range of brands and retailers, including supermarkets and smaller shops located in different regions of Spain, were randomly purchased to ensure that the survey was a representative study from April to July, 2012. A total of 182 samples (250 g) including wheat (*n = 119*), rice (*n = 23*), maize (*n = 17*), spelt (*n = 8*), oat (*n = 8*), soy (*n = 4*) and tapioca (*n = 3*) were analysed. All samples were homogenised using a laboratory mill and kept at 4°C under dark and dry conditions. Most samples collected are categorised as minute pasta (such as alphabet, little threads, melon seeds, little stars, couscous, little shells, semolina, etc.) requiring less than 8 min for cooking.

2.2. Chemicals and reagents

Solvents (acetonitrile, hexane and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate was obtained from Alfa Aesar GmbH & Co (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18 was purchased from Phenomenex (Torrance, USA).

The derivatization reagent composed of BSA (N,O-bis (trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilylimidazole) (3:2:3) was purchased from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium hydrogen phosphate,

used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

The standards of patulin (PAT), zearalenone (ZON) and the type A and B trichothecenes: deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), diacetoxyscirpenol (DAS), nivalenol (NIV), fusarenon-X (FUS-X), neosolaniol (NEO), T-2 and HT-2 toxin were obtain from Sigma–Aldrich (St. Louis, USA). Individual stock solutions of all analytes were prepared at the same concentration (1000 mg L^{-1}) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (50 mg L^{-1}). All standards were stored in darkness and kept at -20°C until the GC–MS/MS analysis.

2.3. Sample preparation

Sample preparation was performed according to a previous study (Rodríguez-Carrasco, Berrada, Font, & Mañes, 2012). In brief, 5 g of homogenised sample were added to 25 mL of distilled water and were sonicated for 15 min. The main extraction involved the addition of 7.5 mL of acetonitrile, 4 g of $MgSO_4$ and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18 and centrifuged for 1 min at 1500 rpm. Finally the extract was evaporated to dryness under nitrogen flow.

The dry extract was added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatized

sample was diluted to 250 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the diluted derivatized sample was added with 1 mL of phosphate buffer (60 mM, pH 7) and the upper layer (hexane phase) was transferred to an autosampler vial for the chromatographic analysis.

2.4. GC–MS/MS analysis

A GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, USA) were used for MS/MS analysis. The mass spectrometer operated in electron impact ionisation (EI, 70 eV). The transfer line and source temperatures were 280°C and 230°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent Masshunter version B.04.00 software.

The separation was achieved on a HP-5MS 30 m x 0.25 mm x 0.25 µm capillary column. One microlitre of the final clean extract of mycotoxins was injected in splitless mode at 250°C in programmable temperature vaporisation (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially 80°C, and the temperature was increased to 245°C at 60°C min^{-1}. After a 3 min hold time, the temperature was increased to 260°C at 3°C min^{-1} and finally to 270°C at

10° min^{-1} and then held for 10 min. Mass spectrometric parameters of studied mycotoxins are described in Table 1.

3. Results and discussion

3.1. Analytical performance

The analytical method was in-house validated with respect to selectivity, linearity, LOQ, precision and accuracy according to SANCO 12495/2011 document (SANCO, 2011). Linearity, plotted as MS response area against concentration estimated for the matrix-matched standards, and matrix effects were studied using standard solutions and matrix-matched calibrations. Calibration curves were built at eight different mycotoxin levels, from LOQ to 100 times LOQ. Each level was prepared in triplicate. To assess the possible matrix effect on the chromatographic response, the slopes of the calibration in standard solutions with those obtained in matrix-matched standards were compared. The results obtained showed slight signal suppression (<100%) of the response for all analytes except for T-2 and HT-2 toxins which showed a slight enhancement in the analytical response (>100%).

The results obtained justified the use of matrix-matched calibration for quantification in order to compensate matrix effect (Table 2). A good linearity (R^2 > 0.99) for all derivatized mycotoxins studied within the tested range was obtained in all food matrices evaluated. For the recovery analysis, one wheat, rice and maize-based sample, previously analysed to assure the absence of studied mycotoxins, were used as a

representative of the samples. The recovery experiments were carried out at two different concentration levels in quintuplicate (*n = 5*) in the same day as well as in three different days (*n = 3*). The repeatability (intra-day precision) and reproducibility (inter-day precision), expressed as the relative standard deviation (RSD), were calculated from the analysis of cereal samples spiked with the selected analytes at each selected fortification level. Recoveries of fortified cereals ranged between 76–108% and 77–114% at 20 and 80 μg kg^{-1} respectively, in the three food matrices evaluated. Intra-day precision <9% was achieved for both spiked levels while an inter-day precision lower than 14% was obtained. LOQ was defined as the concentration with a signal-to-noise ratio (S/N) of 10:1. LOQs obtained were in a range from 1.25 to 10 μg kg^{-1} for the studied mycotoxins being lower than the maximum limits established by the EU (Table 2).

3.2. Occurrence of mycotoxins in samples

One hundred and eighty-two samples of cereal-based and gluten-free food products were analysed. The results of the occurrence of the studied mycotoxins by commodity are presented in Table 3.

With regard to wheat-based cereals samples, 79.8% showed DON contamination with an average content of 12.7 μg kg^{-1} being DON the most frequently mycotoxin found. HT-2, NIV and 3-ADON were also found with incidences of 16.8%, 13.4% and 3.4% and average contents of 16.8, 13.4 and 3.4 μg kg^{-1}, respectively.

Table 1: Optimized GC-MS/MS parameters of the selected mycotoxins

MS/MS parameters	PAT	DON	3-ADON	FUS-X	DAS	NIV	NEO	HT-2	T-2	ZON
MRM (Q)	226>73	392>259	392>287	450>260	350>229	289>73	252>195	347>157	350>259	462>151
CE (V)	*10*	*10*	*5*	*10*	*15*	*15*	*10*	*10*	*10*	*10*
Dt (ms)	*35*	*25*	*35*	*35*	*35*	*35*	*25*	*25*	*25*	*25*
MRM (q)	183>75	407>197	467>147	450>245	378>124	379>73	252>167	347>185	350>229	462>333
CE (V)	*15*	*10*	*10*	*20*	*10*	*15*	*15*	*10*	*15*	*10*
Dt (ms)	*35*	*25*	*25*	*35*	*25*	*35*	*35*	*25*	*35*	*25*
Ion ratio **(Q/q ± RSD, %)**	88 ± 8	42 ± 3	48 ± 12	12 ± 7	57 ± 10	30 ± 3	41 ± 4	87 ± 8	82 ± 6	70 ± 11

CE: Collision energy; Dt: Dwell time

Table 2: Limits of quantification (LOQ), matrix effects, average recoveries and RSD at different spiked concentrations obtained from wheat, rice and maize.

Toxin	Wheat-based Recovery (RSD %)		ME	LOQ	Rice-based Recovery (RSD %)		ME	LOQ	Maize-based Recovery (RSD %)		ME	LOQ
	20 µg/Kg	80 µg/Kg	(%)	µg/Kg	20 µg/Kg	80 µg/Kg	(%)	µg/Kg	20 µg/Kg	80 µg/Kg	(%)	µg/Kg
PAT	82 (6)	93 (7)	78	10	78 (5)	97 (4)	71	10	86 (6)	81 (4)	89	5
DON	85 (4)	87 (4)	83	1.25	83 (7)	82 (7)	88	1.25	94 (8)	96 (6)	85	1.25
3-ADON	79 (7)	94 (8)	76	1.25	89 (2)	90 (1)	79	2.5	90 (2)	85 (8)	95	1.25
FUS-X	97 (5)	90 (5)	87	5	85 (5)	77 (4)	90	1.25	104 (6)	97 (3)	76	2.5
DAS	106 (2)	114 (2)	94	5	102 (9)	101 (9)	82	10	108 (9)	102 (6)	89	2.5
NIV	76 (6)	78 (9)	69	2.5	98 (7)	87 (7)	75	5	89 (7)	84 (8)	75	1.25
NEO	101 (4)	98 (5)	73	5	85 (4)	105 (3)	63	5	83 (1)	98 (5)	96	5
HT-2	88 (8)	94 (7)	114	2.5	93 (2)	91 (7)	77	2.5	89 (5)	79 (7)	108	2.5
T-2	102 (5)	99 (3)	122	5	92 (6)	106 (5)	104	2.5	96 (7)	93 (8)	116	5
ZON	78 (8)	82 (6)	65	10	76 (8)	80 (9)	109	5	81 (7)	77 (9)	78	10

Other *Fusarium* toxins, such as T-2, FUS-X and NEO were detected in only one wheat-based sample. Neither PAT, nor DAS, nor ZON were found in any of the samples analysed. As far as other surveys are concerned, Table 4 reviews some recent surveys focused on the presence of mycotoxins reported in different countries. The high occurrence of DON found in the analysed samples are in agreement with the data accessed. They also highlighted the natural occurrence of HT-2 and T-2 toxins as well as the presence of NIV contamination in an important percentage of the samples assayed. Comparable results were also reported by González-Osnaya, Cortés, Soriano, Moltó, and Mañes (2011) who evaluate the presence of DON and T-2 toxin in pasta commercialised in Spain. DON was reported in 69.2% of the samples analysed in a range of concentration from 18 to 201.5 µg kg^{-1} while T-2 toxin was detected in 7.7% in a range from 28.7 to 115.9 µg kg^{-1}. A study carried out by Tanaka et al. (2009) in wheat samples reported a high incidence of DON and NIV contamination with incidences of 86% and 77% and mean values of 333 and 7 µg kg^{-1}, respectively. Likewise, an investigation on the occurrence of DON reviewed by Schollenberger, Jara, Suchy, Drochner, and Müller (2002) showed that there were wheat flour samples highly contaminated exceeding the maximum permitted level of 750 µg kg^{-1} established in the European Union (EU). Moreover, the frequency of occurrence of DON was very high with almost 100%. Similar occurrence data were reported in a study carried out by Leblanc, Tard, Volatier, and Verger (2005) in pasta samples for DON, NIV and ZON but with average amounts of 15, 15 and 5 µg kg^{-1}, respectively.

Schothorst et al. (2005) carried out a survey to determine trichothecenes in duplicate diets of infants between the ages of 8 and 12 months. DON was detected in all samples analysed, while combined HT-2 and T-2 was detected in 69% of the samples with average contents of 5.75 and 0.27 μg kg^{-1}, respectively. 42% of the samples were contaminated with NIV at 0.33 μg kg^{-1} whereas DAS was reported only in one sample (1.4%) at 0.90 μg kg^{-1}.

Concerning rice-based cereals samples, DON and NEO were detected with an occurrence of 13.0% and 4.3%, and average contents of 5.0 and 15.5 μg kg^{-1}, respectively. The other studied mycotoxins were not found in these samples (Table 3). Mycotoxin contamination in rice is often found lower than in wheat or maize. However, Leblanc et al. (2005) reported a frequency of contamination of 75% for DON with an average amount of 58.8 μg kg^{-1} in rice and semolina samples. Moreover, an incidence of 100% was reported for NIV and ZON in the same samples with mean contents of 15 and 5 μg kg^{-1}, respectively. Nevertheless, a survey in rice-based cereals carried out by Lombaert et al. (2003) did not find DON, NIV and HT-2 toxin. In spite of that, ZON was found at 1 μg kg^{-1} in one out of seven samples analysed which was according with the results reported by Maragos (2010) who found 2.9 μg kg^{-1} of ZON in rice samples.

Relating to maize-based cereals, 29.4% of the samples analysed showed presence of DON at 10.5 μg kg^{-1}, whereas HT-2 toxin and NIV were found in minor incidence both with an occurrence of 5.9% and average

contents of 6.4 and 4.9 μg kg^{-1}, respectively. With regard to ZON, one out of the seventeen samples analysed, presented trace amount of this mycotoxin. None other studied mycotoxins were found in maize-based samples (Table 3). Concerning surveys focused on mycotoxin occurrence in maize samples, Cunha and Fernandes (2010) did not find any studied *Fusarium* toxin in maize samples whereas Cerveró, Ángeles Castillo, Montes, and Hernández (2007) determined and quantified DON in commercial corn-based food in Spain. The frequency of DON-positive samples (*n = 25*) was 68% and the levels detected ranged from 29 to 195 μg kg^{-1}. Nevertheless, the presence of DON was not detected in any of the samples analysed by Marina Martins, Almeida, Marques, and Guerra (2008). Maragos (2010) reported a similar incidence of ZON with a mean content of 1.24 μg kg^{-1} in Spanish maize samples but did not find presence of this mycotoxin in Portuguese maize samples.

Regarding occurrence of mycotoxin in the rest of samples, spelt showed the highest DON contamination with a frequency of 62.5% and a mean value of 25.6 μg kg^{-1} followed by tapioca and soy with incidences of 33.3% and 25.0% and average amounts of 18.3 and 34.8 μg kg^{-1}, respectively. Moreover, in two out of eight spelt samples were also detected NIV at 42.1 μg kg^{-1} and ZON at 15.8 μg kg^{-1}, whereas HT-2 and T-2 toxins were quantified in oat-based cereals in 12.5% and 37.5% at 14.4 and 42.5 μg kg^{-1}, respectively.

Table 3: Occurrence of mycotoxins in cereal-based and gluten-free food products tested

Commodity		PAT	DON	3-ADON	FUS-X	DAS	NIV	NEO	HT-2	T-2	ZON
Wheat-based cereals	Incidence	0/119	95/119	4/119	1/119	0/119	16/119	1/119	20/119	1/119	0/119
	Positive samples (%)		79.8	3.4	0.8		13.4	0.8	16.8	0.8	
(*n=119*)	Mean value (µg kg^{-1})	n.d.	12.7	4.6	10.8	n.d.	19.2	15.5	10.2	13.7	n.d.
	Maximum level found (µg kg^{-1})		83.2	5.3	10.8		53.6	15.5	28.2	13.7	
Rice-based cereals	Incidence	0/23	3/23	0/23	0/23	0/23	0/23	1/23	0/23	0/23	0/23
	Positive samples (%)		13.0					4.3			
(*n=23*)	Mean value (µg kg^{-1})	n.d.	5.0	n.d.	n.d.	n.d.	n.d.	15.5	n.d.	n.d.	n.d.
	Maximum level found (µg kg^{-1})		5.5					15.5			
Maize-based cereals	Incidence	0/17	5/17	0/17	0/17	0/17	1/17	0/17	1/17	0/17	1/17
	Positive samples (%)		29.4				5.9		5.9		5.9
(*n=17*)	Mean value (µg kg^{-1})	n.d.	10.5	n.d.	n.d.	n.d.	4.9	n.d.	6.4	n.d.	<LOQ
	Maximum level found (µg kg^{-1})		22.1				4.9		6.4		<LOQ

Spelt-based cereals	Incidence	0/8	5/8	0/8	0/8	0/8	2/8	0/8	0/8	0/8	2/8
	Positive samples (%)	n.d.	62.5	n.d.	n.d.	n.d.	25.0	n.d.	n.d.	n.d.	25.0
(*n=8*)	Mean value (µg kg^{-1})		25.6				42.1				15.8
	Maximum level found (µg kg^{-1})		56.8				61.8				17.7
Oat-based cereals	Incidence	0/8	0/8	0/8	0/8	0/8	0/8	0/8	1/8	3/8	0/8
	Positive samples (%)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.5	37.5	n.d.
(*n=8*)	Mean value (µg kg^{-1})								14.4	42.5	
	Maximum level found (µg kg^{-1})								14.4	67.2	
Soy-based cereals	Incidence	0/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
	Positive samples (%)	n.d.	25.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(*n=4*)	Mean value (µg kg^{-1})		34.8								
	Maximum level found (µg kg^{-1})		34.8								
Tapioca-based cereals	Incidence	0/3	1/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3
	Positive samples (%)	n.d.	33.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(*n=3*)	Mean value (µg kg^{-1})		18.3								
	Maximum level found (µg kg^{-1})		18.3								
Total (*n=182*)	**Overall incidence (%)**	**n.d.**	**60.4**	**2.2**	**0.5**	**n.d.**	**10.4**	**1.1**	**12.1**	**2.2**	**1.6**

Table 4: Occurrence of the mycotoxins studied in latest reported surveys

Country	Commodity	Toxin	*N*	% (+)	Mean (μg kg^{-1})	Maximum (μg kg^{-1})	Reference
Spain	wheat	ZON	14	64	22.80	38.61	Ibáñez-Vea et al. (2011)
	maize	ZON	21	43	5.42	17.93	
Portugal	wheat	DON	7	57.1	-	434	Cunha and Fernandes (2010)
Italy	wheat	DON	57	17.5	7.7	17.3	Lattanzio et al. (2008)
		NIV		1.7	63.5	63.5	
		HT-2		43.8	3.4	14.1	
		T-2		10.5	1.3	1.9	
Germany	wheat	T-2	130	85	0.21	1.9	Gottschalk et al. (2009)
		HT-2		94	1.6	22	
		DAS		3.1	0.01	0.25	
		NEO		24	0.02	0.40	
		DON		100	57	1163	
		3-ADON		52	0.57	15	
		NIV		67	6.8	96	
		FUS-X		5.4	0.02	0.93	
	oat	T-2	98	100	4.2	34	
		HT-2		99	10	51	
		DAS		60	0.05	0.38	
		NEO		92	0.37	2.7	
		DON		74	2.8	55	
		3-ADON		28	0.43	8.2	
Tunisia	wheat	DON	65	83	-	54	Bensassi et al. (2010)
Croacia	maize	DON	40	85	-	1792	Pleadin et al. (2012)
		ZON		87.5	-	511	
Serbia	wheat	DON	15	86.7	325	976	Skrbic et al. (2012)
		ZON		33.3	4.6	21.1	
		T-2		26.7	4.1	26.9	

Bahrain	rice	ZON	10	80	-	0.7	Musaiger et al. (2008)
South Korea	rice	NIV	65	35	26.9	45	Ok et al. (2011)
		DON		15	23.8	31.7	
		FUS-X		15	9.1	15	
	maize	NIV	25	52	48.8	129.4	
		DON		96	114	491.9	
		FUS-X		24	8.7	19.1	
		3-ADON		28	4.1	6.8	
	wheat	NIV	38	21	24.7	31.8	
		DON		79	35.4	199.5	
		FUS-X		5	9	9.5	
		3-ADON		13	4.3	4.9	
Malasya	rice	DON	40	20	-	34.92	Soleimany et al. (2012)
		ZON		12.5	-	51.1	
		T-2		7.5	-	55.35	
		HT-2		2.5	-	48.18	
	wheat	DON	20	25	-	18.62	
		ZON		20	-	12.74	
		T-2		15	-	34.45	
		HT-2		15	-	40.11	
	oat	DON	5	60	-	16.48	
		T-2		20	-	35.56	
		HT-2		20	-	31.22	
Japan	wheat	DON	56	86	333	2248	Tanaka et al. (2009)
		NIV		77	7	27	
Canada	oat	DON	53	66	52	90	Lombaert et al. (2003)
	soy	DON	8	100	116	240	
		ZON	30	76.7	18.4	35	
	rice	ZON	7	14.3	1	1	

Relating to mycotoxin contamination in minor cereals, very scarce data were reported in literature. The studies in oat samples carried out by Gottschalk, Barthel, Engelhardt, Bauer, and Meyer (2009) as well as by Soleimany, Jinap, and Abas (2012) showed a high mycotoxin incidence. Nevertheless, mean contents reported were comparable with those found in the present work related to HT-2 and T-2 toxins. A high number of Canadian soy samples analysed by Lombaert et al. (2003) were positive for DON and ZON but with average amounts of 116 and 18.4 μg kg^{-1}, respectively, whereas only one out of four soy samples analysed in the present work was DON contaminated at 34.8 μg kg^{-1}.

Summarizing, none of the samples assayed contained toxins at non-permitted level for all mycotoxins according to the maximum limits established by the EU legislation. Despite that, it has to be highlighted that 64.8% of the samples showed mycotoxin contamination with at least one mycotoxin. Results by commodity indicated that wheat-based samples showed the highest mycotoxin contamination by one mycotoxin with an incidence of 79.8% followed by maize-based samples and rice-based samples with a percentage of contaminations of 29.4% and 13.0%, respectively. Results by mycotoxin showed that DON represents the most critical contaminant with the highest percentage of positive samples in the seven types of food matrices studied with an overall incidence of 60.4% followed by HT-2 toxin and NIV with incidences data of 12.1% and 10.4%, respectively. Fig. 1 shows a multiple reaction monitoring (MRM)

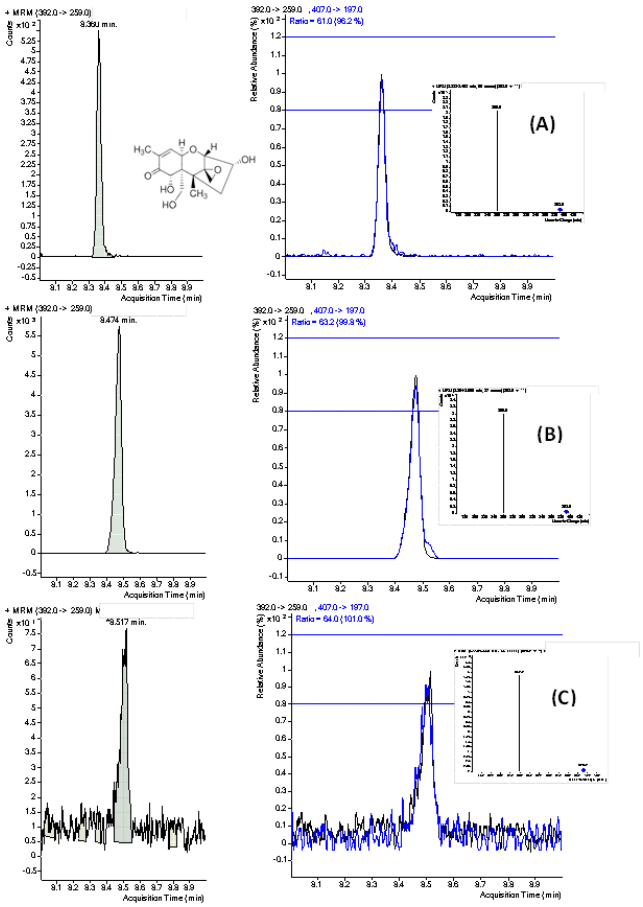
chromatogram of naturally contaminated wheat, maize and rice with DON at 83.2, 17.9 and 4.8 μg kg^{-1}, respectively.

3.3. Co-occurrence of mycotoxins in the studied food matrices

The co-occurrence of mycotoxins is listed in detail in Table 5. With regard to seven commodities investigated, 10.1% of the wheat-based samples and 4.4% of rice-based samples showed simultaneous presence of two mycotoxins, while maize-based samples did not show co-occurrence of two mycotoxins. Concerning the samples contaminated with two mycotoxins simultaneously, DON and HT-2 were detected in 8 samples whereas DON and NIV were detected in 4 samples at maximum sum concentrations of 61.5 and 56.5 μg kg^{-1}, respectively. 8.4% of wheat-based samples and 5.9% of maize-based samples were contaminated with three mycotoxins being the most common combination DON, HT-2 and NIV.

The highest sum concentration of this mycotoxin combination was 72.5 μg kg^{-1}. On the other hand, the triad of mycotoxins DON, 3-ADON, NIV and DON, HT-2, FUS-X were detected in two samples at sum concentrations of 51.4 and 28.2 μg kg^{-1}, respectively. Moreover, two wheat-based samples showed contamination with four mycotoxins simultaneously at sum concentrations of 80.1 and 90.9 μg kg^{-1}, respectively. In addition, co-occurrence of five mycotoxins was found in one wheat-based sample with sum concentrations of 77.7 μg kg^{-1}. None co-occurrence of mycotoxins were found in the other commodities studied.

Figure 1: MRM chromatograms and ion ratios obtained from naturally contaminated (A) wheat-, (B) maize- and (C) rice-based products with deoxynivalenol at 83.2, 17.9 and 4.8 µg kg^{-1}, respectively



Some researchers have affirmed that non-acetylated forms predominate over the acetylated forms (DON > 3-ADON and 15-ADON, NIV > FUS-X), except for T-2 (T-2 > HT-2) (Langseth & Rundberget, 1999). In the samples analysed, DON and NIV were present in higher concentrations

than 3-ADON and FUS-X, respectively, but HT-2 toxin was present at higher concentration and incidence than T-2 toxin.

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

Co-occurrence	number of samples	ΣC_{min} ($\mu g\ kg^{-1}$)	ΣC_{max} ($\mu g\ kg^{-1}$)
2 mycotoxins			
DON-HT-2	8	12.3	61.5
DON-NIV	4	13.4	56.5
3 mycotoxins			
DON-3-ADON-NIV	1	51.4	51.4
DON-HT-2-NIV	8	26.4	72.5
DON-HT-2-FUS-X	1	28.2	28.2
4 mycotoxins			
DON-3-ADON-HT-2-NIV	2	80.1	90.9
5 mycotoxins			
DON-3-ADON-HT-2-T-2-NIV	1	77.7	77.7

Results have shown that a great number of samples were contaminated with several *Fusarium* toxins. Although produced by different *Fusarium* species, the toxins DON, NIV and HT-2 toxin seemed to co-occur in cereals more frequently than others. The results indicate that infection by one *Fusarium* species make the grain more susceptible also to other *Fusarium* species as previously elucidated by Langseth and Rundberget (1999). In fact, DON and HT-2 toxins occurred together in 76% of the co-contaminated samples and DON and NIV were frequent co-

contaminants (64%). Even, the simultaneous contamination with DON, HT-2 and NIV, were detected in 44% of the co-contaminated samples.

3.4. Probable daily intake of mycotoxins

The tolerable daily intake is the result of hazard analysis (both identification and characterisation) and can vary between different assessment bodies. A form of risk characterisation can be done by calculating a percentage of PDI over TDI derived by a particular assessment body, which is called percent of relevant TDI.

Wheat-based food products were the most consumed based on the Food Balance Sheet reported by FAO and the most contaminated taken into consideration the results reported in the present work. For these statements, a study of risk characterisation of mycotoxins was carried out. In Spain, an average adult (70 kg) consumes around 235.9 g of wheat-based products per day (FAO, 2009). The mean mycotoxins concentrations found in wheat-based food products analysed in this study were 12.7, 4.6, 10.8, 19.2, 15.5 and 23.9 μg kg^{-1} and the probable daily intake were 0.043, 0.016, 0.036, 0.065, 0.052 and 0.081 μg kg^{-1} bw day^{-1} for DON, 3-ADON, FUS-X, NIV, NEO and for the sum of HT-2 and T-2 toxins.

These values are considerable lower than the TDI established by the Scientific Committee on Food of the European Commission (1 μg kg^{-1} bw day^{-1} for DON and its acetylated derivatives, 0.7 μg kg^{-1} bw day^{-1} for NIV and 0.1 μg kg^{-1} bw day^{-1} for the sum of HT-2 and T-2). Based on the data exposed in this paper, it could be stated that wheat-based products

analysed contribute from 4.3 to 82% of the TDI of DON and the sum of HT-2 and T-2, respectively. These results suggest that the current exposure levels of these toxins might not pose a health risk for the average consumer, although mycotoxins intake by certain populations consuming high amounts of cereals and cereals-based products might be of importance. Thus, the reported data reflects the necessity to take a vigilant attitude to guarantee food safety.

4. Conclusions

The QuEChERS–GC–MS/MS method has been validated for the three major cereals consumed worldwide, obtaining satisfactory results in terms of accuracy and precision as well as being in compliance with the European legislation purposed for the validation procedures. In an application of the methodology, 113 out of 182 randomly collected samples analysed showed mycotoxin contamination being DON the most common mycotoxin followed by HT-2 toxin and NIV. Nevertheless, none of the samples exceeded the maximum limit levels established in the EU legislation. Despite that, co-occurrence of mycotoxins in a relatively important number of samples was found; thus, constant monitoring throughout the cereals production chain is necessary in order to minimize health risks and to comply with trade requirements. Risk characterization showed a percent of relevant TDI from 4.3 to 82% for DON and the sum of HT-2 and T-2, respectively. Results showed values of PDI below TDI for

average consumers; nonetheless high consumers (children, ethnic groups or vegans) could exceed safety levels.

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3.4. Occurrence of *Fusarium* mycotoxins and their dietary intake through beer consumption by the European population

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Occurrence of *Fusarium* mycotoxins and their dietary intake through beer consumption by the European population

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Abstract

Since cereals are raw materials for production of beer and beer-based drinks, the occurrence of mycotoxins in these commodities are frequently reported. The occurrence of fourteen mycotoxins in 154 beer samples was topic of investigation in this study. The analyses were conducted using QuEChERS extraction and gas chromatography-tandem mass spectrometry determination. The analytical method showed recoveries for vast majority of analytes ranged from 70% to 110%, relative standard deviations lower than 15% and limits of detection from 0.05 to 8 µg/L. A significant incidence of HT-2 toxin and deoxynivalenol (DON) were found in 9.1% and 59.7% of total samples, respectively. The exposure of European population to mycotoxins through beer consumption was assessed. No toxicological concern was associated to mycotoxins exposure for average beer consumers. Despite that, for heavy beer drinkers, the contribution of this commodity to the daily intake is not negligible, approaching or even exceeding the safety levels.

1. Introduction

Mycotoxins are toxic secondary metabolites produced naturally by filamentous fungi, such as *Aspergillus, Penicillium* and *Fusarium*. Most mycotoxins are immunosuppressive agents and some are classified as carcinogens, hepatotoxins, nephrotoxins, or neurotoxins. They have been reported in several kinds of food, especially in globally consumed cereals such as wheat, rice, maize and barley (Marroquín-Cardona, Johnson, Phillips & Hayes, 2014). DON, the most commonly detected *Fusarium* mycotoxin in cereal grains, was related to deleterious health effects like anorexia, weight loss, malnutrition, endocrine dysfunction and immune alterations (Pestka, 2010) but is non-classifiable as carcinogen to humans (IARC, 1993).

As co-occurrence of mycotoxins in cereals for beer production is frequently reported, their levels should be contemplated in exposure estimates studies. Mycotoxin contamination could occur at various stages of the brewing process and would be transmitted from malt into beer as a consequence of their thermal stability and relatively good water solubility (Kostelanska, Zachariasova, Lacina, Fenclova, Kollos & Hajslova, 2011; Inoue, Nagatomi, Uyama & Mochizuki, 2013). Moreover, during brewing process some mycotoxins can be converted to its metabolites as previously reported Mizutani, Nagatomi & Mochizuki (2011).

The investigation on a formation/release of DON conjugate during processing of contaminated barley was initiated by studies conducted by

Berthiller et al. (2013) who characterize the main DON plant metabolite. However, very scarce literature concerning the toxicokinetics of deoxynivalenol-3-glucoside (D3G) is available. Up to now, D3G seems to be resistant to acidic conditions and thus it is unlikely that D3G can be hydrolyzed into its parent compound in the stomach of mammals (Berthiller et al., 2011).

Important enzymes related to the hydrolysis of plant glucosides such as β-glucosidase, are expressed in human liver and gut. Nonetheless, some naturally occurring glucosides, including D3G, cannot be cleaved by the human cytosolic β-glucosidase. In that sense, only partial hydrolysis was observed upon incubation with several pure cultures of intestinal bacteria as reported Berthiller et al. (2011) and thus, only a fraction of D3G will be bioavailable. In this line, the digestibility and absorption of D3G in *in vitro* models was recently investigated and they reported that no evidence was found for D3G hydrolysis to DON in the digestion model representing the upper part of the gastrointestinal tract. Moreover, it was shown that bioavailability of D3G in humans may be low as compared to DON since Caco-2 cells did not absorb D3G, in contrast to DON (De Nijs et al., 2012).

Currently, there is no regulation for *Fusarium* toxin levels in beer. Maximum limits are set only for raw materials used for production of this commodity (EC No 1881/2006). A provisional maximum tolerable daily intake (PMTDI) of 1 μg/kg bw was established for the sum of DON and its

acetylated forms by the Scientific Committee on Food (SCF, 2002). SCF has also established TDI for other mycotoxins frequently reported in cereals and cereals-based foods. For instance, a TDI for the sum of HT-2 and T-2 toxin of 0.1 µg/kg bw was set (SCF, 2011a) and a temporary tolerable daily intake (t-TDI) of 1.2 µg/kg bw was established for nivalenol (SCF, 2013). In 2000 the SCF established a t-TDI of 0.2 µg/kg bw for zearalenone. However, in 2011 the SFC concluded that a TDI of 0.25 µg/kg bw can be established based on recent data in the most sensitive animal species (SCF, 2011b).

This widely popular fermented drink may, under certain conditions, contribute significantly to intake of mycotoxins, approaching or even exceeding the safety levels when consuming a regular diet. The latest data available from Food and Agricultural Organization (FAO) reported an annual *per capita* consumption of beer exceeding 70 litres in 46.4% of European countries, with a maximum of 142.8 litres in Ireland (FAO, 2011). Hence, exposure of consumers to mycotoxins through beer should not be underestimated, particularly in case of heavy drinkers (Warth et al., 2012).

Therefore, development of reliable analytical multi-mycotoxin methods becomes necessary. Sample preparation method still remains the bottleneck in the entire protocol because of the wide range of properties of the several mycotoxins and food matrix. Sample preparation techniques such as SPE (Romero-González, Martínez Vidal, Aguilera-Luiz & Garrido Frenich, 2009), immunoaffinity columns (Bertuzzi, Rastelli, Mulazzi, Donadini & Pietri, 2011), QuEChERS (Tamura, Uyama & Mochizuki, 2011) or

enzyme-linked immunosorbent assay (Kuzdraliński, Solarska & Muszyńska, 2013) have been employed in multi-mycotoxin analysis in beer yielding in general satisfactory results. Analytical procedures based on chromatographic principles such as gas chromatography coupled to mass spectrometry (GC-MS) (Scott et al., 1993) and/or liquid chromatography coupled to mass spectrometry (LC-MS), (Malachova, Varga, Schwartz, Krska & Berthiller, 2012) for developing multi-mycotoxin methods in several foodstuffs have been widely employed. For instance Scott et al. (1993) developed a sensitive method for the determination of five *Fusarium* toxins in beer by capillary gas chromatography-mass spectrometry.

The acquisition of two selected reaction monitoring (SRM) transitions per compound by the triple quadrupole detector (QqQ) and the predetermined selected ions intensity ratio allowed the unequivocal confirmation of positive samples and accomplished the requirements set by the Commission Decision 2002/657/EC (2002/657/EC) as regards criteria and procedures for the validation of analytical methods satisfied by MS/MS. In this sense, high dynamic range and good performance reached in SRM mode make the triple quadrupole one of the most widely employed mass spectrometry analyzer (Rubert, Soler, Marín, James & Mañes, 2013). The here proposed methodology is presented as a sensitive and robust analytical tool for the simultaneous determination of fourteen mycotoxins by GC-MS/MS. Thus, this work serves as an update of the gas chromatography methods for the determination of mycotoxins in foodstuffs.

Robust and selective methods are highly desirable for the mycotoxin determination. These requirements can be achieved by both GC and LC coupled to mass spectrometry as recently reviewed Pereira, Fernandes & Cunha (2014). GC offers some benefits over LC methods such as lower instrument cost as well as lower maintenance, even though analysis of mycotoxins by GC requires derivatization. Ion sources used in LC-MS are also related to ion suppression or enhancement with strong consequences on the accuracy, precision and sensitivity of the methods (Ran, Wang, Han, Wu, Zhang & Shi, 2013).

Monitoring studies regarding evaluation of mycotoxins in several foodstuffs should be continuously conducted as recommended EFSA to collect and evaluate occurrence data on mycotoxins in food and feed. The work carried out by Varga, Malachova, Schwartz, Krska & Berthiller (2013) is one of the largest performed study of the occurrence of DON mainly in Austrian beers. One of the most pressing goals of the here proposed article is to investigate the occurrence data of not only deoxynivalenol but also other trichothecenes as well as other *Fusarium* toxins (e.g. zearalenone and its metabolites) in beers. In order to accomplish this, the objectives of this work were to develop an analytical strategy based on a QuEChERS-based extraction and gas chromatography-tandem mass spectrometry for the simultaneous determination of fourteen *Fusarium* toxins and metabolites in a total of 154 beer samples produced mainly in different European countries and to estimate the dietary intake of these mycotoxins among the European population.

2. Materials and methods

2.1. Chemical and reagents

Solvents (acetonitrile, hexane and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulphate was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18 was purchased from Phenomenex (Torrance, USA).

The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilylimidazole) (3:2:3) was purchased from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

2.2. Analytical standards

The standards of the type A and B trichothecenes: deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), diacetoxyscirpenol (DAS), nivalenol (NIV), fusarenon-X (FUS-X), neosolaniol (NEO), T-2 and HT-2 toxins, zearalenone (ZON) and its derivatives α-zearalenol (α-ZOL), β-zearalenol (ß-ZOL), α-zearalanol (α-ZAL), β- zearalanol (ß-ZAL) and zearalanone (ZAN) toxin were obtained from Sigma–Aldrich (St. Louis, USA). All stock solutions were prepared by dissolving 1 mg of the mycotoxin in 1 mL of pure methanol, obtaining a 1 mg/mL solution and

diluted with acetonitrile in order to obtain the appropriate multicompounds working standard solutions. All standards were kept at −20°C.

2.3. Sampling

A total of one hundred and fifty-four bottled commercial beer samples were randomly purchased from different retail outlets located in Valencia (Spain) from May to July 2013. None of the beers surpassed their expiration date. Until sample preparation, they were stored in a dark and cold environment at 4°C. The beer samples were classified based on both country of production and type of beer.

2.3.1. Country of production

The vast majority of beer samples (*n = 60*) were produced in Spain. Fifty-four samples were imported mainly from Europe. The imported beer originated from Germany (*n = 24*), Ireland (*n = 8*), Portugal (*n = 8*), Belgium (*n = 7*), Denmark (*n = 7*), Netherlands (*n = 7*), Czech Republic (*n = 6*), Great Britain (*n = 6*), United States of America (*n = 6*), France (*n = 5*), Mexico (*n = 5*) and Argentina (*n = 5*).

2.3.2. Type of beers

Samples were subdivided into 6 groups: non-alcoholic (*n = 17*) beers which contained no alcohol or an alcohol content < 1% Vol; shandy (*n = 14*) beers which contained mixtures of different ratios of beer and lemonade; light (*n = 16*) beers which contained an alcohol content between

1 and 3.5 % Vol; lager (*n = 58*) beers brewed with barley malt and exhibiting a light color; dark (*n = 24*) any beer exhibiting a darker, brownish color, regardless of the alcohol content and wheat beers (*n = 25*) brewed with wheat malt alone or in combination with different ratios of barley malt.

2.4. Mycotoxin extraction

Beer samples were processed using the following procedure. First, each bottle of beer sample was gently shaken and approximately 100 mL was degased by sonication for 15 min. Then, 5 mL of acetonitrile was added to 10 mL of sample and vigorously shaken for 30 s prior the addition of 4 g of anhydrous $MgSO_4$ and 1 g NaCl. The mixture was vortexed for 30 s and sonicated for 3 min prior to be centrifugated for 3 min at 3500 rpm. After the acetonitrile extract was submitted to a dispersive solid phase extraction (d-SPE) into a tube containing 900 mg $MgSO_4$ and 300 mg C18, it was vortexed for 1 min and then centrifuged for 1 min at 3500 rpm. Finally, the supernatant was collected and evaporated to dryness under a gentle nitrogen flow.

The dry extract was treated with 50 µL of BSA + TMCS + TMSI (3:2:3), and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 200 µL with hexane and mixed thoroughly on a vortex for 30 s. The hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7) and transferred to an autosampler vial for the chromatographic analysis.

2.5. GC-MS/MS analysis

The final extract (1µL) was injected in splitless mode at 250°C in programmable temperature vaporization (PTV) using an Agilent 7890A GC system coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA). The mass spectrometer was operated in electron impact ionization (EI, 70 eV). The source and transfer line temperatures were 230°C and 280°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as carrier gas at fixed pressure of 20.3 psi, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent MassHunter version B.04.00 software. Analytes were separated on a HP-5MS 30 m x 0.25 mm x 0.25 µm capillary column. The oven temperature program was initially 80°C, and the temperature was increased to 245°C at 60°C/min. After a 3 min hold time, the temperature was increased to 260°C progressively at 3°C/min and finally to 270°C at 10°C/min and then held for 10 min. The analysis was performed with a solvent delay of 3 min in order to prevent instrument damage.

The criteria established in Document No. SANCO 12495/2011 (SANCO, 2011) was achieved for quantitation purpose. For each analyte, two SRM transitions for each compound and compliance of the SRM ratio, defined as the relative ion intensities between the area of both the

quantitation (Q) and confirmation transition (q), were required. The most intense SRM transition was selected for quantitation purposes. The specific MS/MS parameters for each mycotoxin are shown in Table 1.

2.6. Method validation

A prior analysis of the samples was performed in order to ensure they did not contain any of the studied compounds. Then, blank samples were selected for spiking, calibration curves and recovery purposes. Calibration functions of both neat solvent standards and spiked samples were established by plotting peak areas versus analyte concentrations in the measured solutions and performing linear regression. Linear range was tested from 0.1 to 500 µg/L by spiking at eight concentration levels. Spiking at each level was carried out in triplicate. In order to reveal the presence of matrix effects, matrix-matched calibration prepared by spiking extracts of blank samples with mycotoxins at similar concentrations than the calibration built in neat solvent without any matrix were compared. The slopes of the resulting linear calibration functions were compared and the signal suppression/enhancement (SSE) due to matrix effects was determined according to equation. 1:

SSE (%) = Slope matrix-matched calibration/Slope standard x 100 (1)

A SSE of 100% indicates that no matrix effect occurred in the concentration range investigated. A SSE above 100% revealed signal enhancement, while a SSE below 100% signal suppression.

Table 1: GC-MS/MS parameters for the analytes under study.

Analyte	Retention time (min)	*Quantitation transition*			*Confirmation transition*			SRM Ratio (%)
		Q1	Q3	CE, eV (Dt, ms)	Q1	Q3	CE, eV (Dt, ms)	
DON	8.6	392	259	10 (25)	407	197	10 (25)	41.6
3-ADON	9.68	392	287	5 (35)	467	147	10 (25)	47.5
FUS-X	9.73	450	260	10 (35)	450	245	20 (35)	11.9
DAS	9.85	350	229	15 (35)	378	124	10 (25)	56.9
NIV	10.15	289	73	15 (35)	379	73	15 (35)	29.6
NEO	11.68	252	195	10 (25)	252	167	15 (35)	40.6
HT-2	14.73	347	157	10 (25)	347	185	10 (25)	86.7
T-2	14.8	350	259	10 (25)	350	229	15 (35)	81.9
ZAN	15.15	307	235	15 (25)	449	335	10 (25)	59.9
α-ZAL	15.45	433	309	20 (35)	433	295	20 (35)	26.1
ß-ZAL	15.68	433	295	15 (35)	307	73	10 (35)	82.2
ZON	15.95	462	151	10 (25)	462	333	10 (25)	76.9
α-ZOL	16.45	305	73	15 (25)	305	289	15 (20)	12.7
ß-ZOL	16.82	536	333	10 (35)	536	446	15 (20)	66.1

Q1: precursor ion; Q3: product ion; CE: collision energy; Dt: dwell time

For recovery studies, the samples obtained from the local market were spiked with the standard solution at the appropriate levels. Following this method, 10 mL sample were fortified with the working standard solution. The samples were then allowed to stand overnight until analysis. The final spiking concentration levels in the samples for recovery studies were 50, 100, and 200 µg/L. Precision studies were determined in fortified beer samples at the same spiking levels above mentioned and calculated as percentage of relative standard deviation (RSD, %). Both recovery and precision studies were conducted in triplicate in the same day (intra-day precision) and in four different days (inter-day precision). Limits of

detection (LODs) and quantitation (LOQs) were calculated as the concentrations for which signal-to-noise ratios were 3 and 10, respectively.

Student's t-test statistical analysis was performed for data evaluation; *p* values < 0.05 were considered significant.

3. Results and discussion

3.1 Analytical features of the proposed method

The here proposed procedure for the determination of fourteen mycotoxins in beer was validated in lager beer, the most numerous group of the collected samples (58 out of 154) as a representative of the whole beer samples.

The good performance of the method was confirmed by the validation data reported in the Table 2.

The analyses of blank samples showed that no interfering signals were presented at the retention time of the investigated analytes, assessing method specificity. Regression equations were obtained using eight standard concentrations on the abscissa and the area of the chromatogram peaks as vertical coordinates. Linear range was tested at eight concentration levels in triplicate from 0.1 to 500 μg/L. Relative standard deviations among the triplicate were below 5% at all calibration curve points. The determination coefficients (R^2) of all analytes were >0.995. Matrix effect was observed (from 52 to 79%), and thus matrix-matched calibration curves were used for quantification purposes.

Table2: Validation parameters of the GC-MS/MS method

Mycotoxin	LOD (µg/L)	LOQ (µg/L)	SSE (%)	Recovery (%)			Intra-day precision (RSD %; *n = 3*)			Inter-day precision (RSD%; *n = 3*)		
				50 µg/L	100 µg/L	200 µg/L	50 µg/L	100 µg/L	200 µg/L	50 µg/L	100 µg/L	200 µg/L
DON	0.05	0.10	61	76	82	79	9	7	8	13	10	9
3-ADON	2	4	63	82	71	76	6	10	9	10	7	13
FUS-X	8	16	53	98	95	81	8	5	10	8	12	14
DAS	4	8	66	77	83	85	6	9	5	12	9	7
NIV	0.5	1	79	78	75	84	12	8	6	15	11	14
NEO	2	4	73	82	84	78	8	5	10	10	6	12
HT-2	2	4	52	98	101	108	9	7	7	8	14	15
T-2	4	8	59	109	97	109	7	5	8	10	6	9
ZAN	8	16	58	68	69	72	9	10	12	12	13	15
α-ZAL	4	8	71	70	74	72	6	9	10	9	12	12
ß-ZAL	4	8	76	73	68	70	8	7	11	10	9	13
ZON	8	16	70	75	73	69	5	6	9	13	15	8
α-ZOL	2	4	78	83	80	75	6	4	5	8	9	7
ß-ZOL	4	8	62	72	75	71	8	7	9	10	8	13

SSE: signal suppression/enhancement calculated based on equation 1

Accuracy and precision data were provided by recovery tests conducted in triplicate using the fortified blank samples at three fortification levels (50, 100 and 200 µg/L) within laboratory reproducibility conditions. Considering all the concentration levels, mean recoveries (*n = 3*) obtained with matrix-matched calibration curves were in the range of 68 – 109% (Table 2) with RSD values between 4% and 12%. Global inter-day precision was estimated as RSD of 12 determinations and was between 6% and 15%. The sensitivity of the method was expressed in terms LODs and LOQs, which ranged between 0.05 - 8 µg/L and 0.1 - 16 µg/L, respectively (Table 2). Results showed the suitability of the developed method for the determination of trace amounts of the selected mycotoxins in beer samples.

3.2 Exposure data: occurrence of mycotoxins in beer samples

The above described analytical method was applied to one hundred and fifty-four beer samples collected from 13 countries yielded data of exposure assessment to consumers. Table 3 displays the concentration of the occurrence of mycotoxins quantified in this study.

An important incidence of DON (59.7%) was found in the total samples assayed. Moreover, HT-2 co-occurred in 9.1% of total samples. The overall average of all 154 beer samples was 17.2 µg/L for DON and 2.8 µg/L for HT-2. Taking into consideration only the contaminated beers above the LOQ, the average contents reached 28.9 µg/L for DON (range: 24.5 – 47.7 µg/L) and 30.9 µg/L for HT-2 (range: 24.2 – 38.2 µg/L).

Table 3: Occurrence of mycotoxins in the analyzed beer samples based on country of production

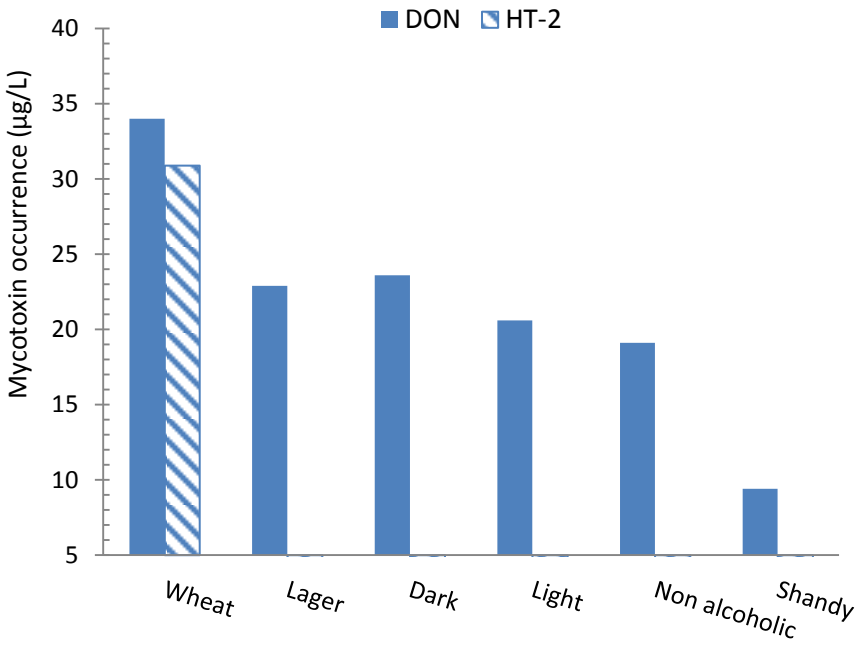
Country	*Deoxynivalenol*				*HT-2 toxin*			
	Incidence	Positive samples (%)	Mean (µg/L)	Range (µg/L)	Incidence	Positive samples (%)	Mean (µg/L)	Range (µg/L)
AR	1/5	20	28.2	28.2	n.d.	-	-	-
BE	7/7	100	30.5	27.1-40.3	1/7	14	31.3	31.3
CZ	2/6	33.3	35.1	29.3-42.5	n.d.	-	-	-
DE	20/24	83	32.3	24.5-43.1	9/24	37.5	33.6	29.3-38.2
DK	n.d.	-	-	-	n.d.	-	-	-
ES	45/60	75	27.0	25.2-47.7	2/60	3.3	27.0	25.1-30.4
FR	5/5	100	30.6	27.3-42.5	1/5	20	29.5	29.5
GB	1/6	16.7	26.7	26.7	1/6	16.7	24.2	24.2
IE	n.d.	-	-	-	n.d.	-	-	-
MX	5/5	100	26.2	25.1-27.3	n.d.	-	-	-
NL	1/7	14.3	28.9	28.9	n.d.	-	-	-
PT	3/8	37.5	31.4	30.3-32.8	n.d.	-	-	-
US	2/6	33.3	26.3	25.0-26.4	n.d.	-	-	-
Total (*n=114*)	92/154	59.7	28.9 [a] (*17.2*)	24.5 - 47.7 [a]	14/154	9.1	30.9 [a] (2.*8*)	24.2 - 38.2 [a]

[a] *data calculated based on positive samples only. Italic number: data calculated based on all samples*

The results of this survey were in agreement with previous European studies: Papadopoulou-Bouraoui, Vrabcheva, Valzacchi, Stroka & Anklam (2004) detected DON in 87% of analyzed beer samples *(n = 313*) at level of 4 – 56.7 µg/L. Kostelanska et al. (2009) quantified DON in 64% of samples (*n = 176*) in a concentration range of 1 – 35.9 µg/L whereas Bertuzzi et al. (2011) detected DON in 66% of samples *(n = 106*) at level of <0.5 – 18.6 µg/L. Recently, Kuzdraliński et al. (2013) quantified DON in 100% assayed beer samples (*n = 91*) at mean level of 20.66 µg/L and Varga et al. (2013) reported an average contamination of DON at 13.6 µg/L in the 77% of positive samples (*n = 374*). So far, a limited number of studies have reported HT-2 contamination in beer. The values here reported are also in good agreement with previous findings. For instance, Romero-González et al. (2009) detected HT-2 toxin in 26.7% of the analyzed beer samples *(n = 15*) but at levels of 1 µg/L. A low incidence of HT-2 toxin (6.1%) was detected by Rubert et al. (2013) in 49 beer samples but at contents similar to those here found (concentration range of 15.1 – 20 µg/L).

No significant statistical differences for a confidence interval of 95% in mycotoxin occurrence were observed between samples from different countries as previously described by Bertuzzi et al. (2011) and Kuzdraliński et al. (2013). Nonetheless, different mycotoxin occurrence was detected taking into consideration the different types of beers regardless of the country of production (Figure 1).

Figure 1: Mean levels of quantified mycotoxins in samples classified by type of beer



Wheat-based beers showed the highest mycotoxin incidence for both DON (76%) and HT-2 toxin (56%). In fact, the 14 out of 154 samples (9.1% of total beers) contaminated by HT-2 belong to this beer category (range total samples: <LOQ to 38.2 µg/L). In addition, wheat-based beers also showed the highest mean level of DON at 34.0 µg/L (range total samples: <LOQ to 47.7 µg/L). A similar trend was observed by Varga et al. (2013) who reported DON contamination in 78.3% of the 46 analyzed wheat beers at average content of 18.4 µg/L and maximum level of 49.6 µg/L. The high mycotoxin occurrence in this type of beers could be justified taken into account that the growth of *Fusarium graminearum* and

Fusarium culmorum, the major plant pathogens that are capable of producing trichothecenes in infected grains, are slightly more predominant in wheat than in barley and hence a greater mycotoxin contamination in wheat-based products is expected (Krstanović, Klapec, Velić & Milaković, 2005).

Concerning the lager beers, 42 out of 58 samples showed DON contamination at average content of 22.9 µg/L (range total samples: <LOQ – 42.0 µg/L). Similar results in terms of occurrence were obtained for dark beer samples (range total samples: <LOQ – 32.8 µg/L; mean content: 23.6 µg/L). Figure 2 shows a SRM chromatogram of a beer artificially contaminated by DON at 100 µg/L as well as SRM chromatogram of a wheat-based and barley-based beer naturally contaminated by DON at 47.7 µg/L and 24.5 µg/L, respectively.

The lowest averages of DON were found in light, non-alcoholic and shandy beers (20.6, 19.1 and 9.4 µg/L, respectively). These results could be probably related to the differences in the technological process involved, such as earlier stopped fermentation or the use of specific yeast, as highlighted in previous studies (Papadopoulou-Bouraoui et al. 2004; Kostelanska et al., 2009). Therefore, a longer fermentation process could possibly have contributed to the highest level of DON transfer from malt to beer being in agreement with the results here reported for wheat, pale and dark beers. In case of shandy, apart from the above mentioned it has also

to be considered the dilution of this type of beer with different ratios of lemonade.

Left-censored results (i.e. data below the analytical limits) were processed according to the dietary exposure assessment of chemicals in food recommendations (substitution method) that are the most commonly used and then evaluating how the exposure estimates change (IPCS, 2009). Two exposure scenarios were then defined: the lower bound scenario (LB) and the upper bound scenario (UB). The LB was obtained by assigning a zero value to those samples in which the analyte was non-detected or non-quantified and using these values to estimate dietary exposure. An UB dietary exposure was estimated by assigning the LOD to all samples with non-detected results and the LOQ to all samples with less than the LOQ but more than the LOD. It is widely considered that the LB scenario generally underestimates contamination and exposure levels and that the UB scenario overestimates them (EFSA, 2010). Hence, considering the LB and UB values of DON and HT-2 obtained from this survey, the latest mean beer consumption data in the European countries reported by FAO (FAO, 2011) and the default body weight for adults (70 kg) recommended by the Scientific Committee (EFSA, 2012), the daily average exposure was calculated.

Figure 2: SRM chromatogram of: beer artificially contaminated by DON at 100 µg/L (A), wheat-based beer naturally contaminated by DON at 47.7 µg/L (B) and barley-based beer naturally contaminated by DON at 24.5 µg/L (C).

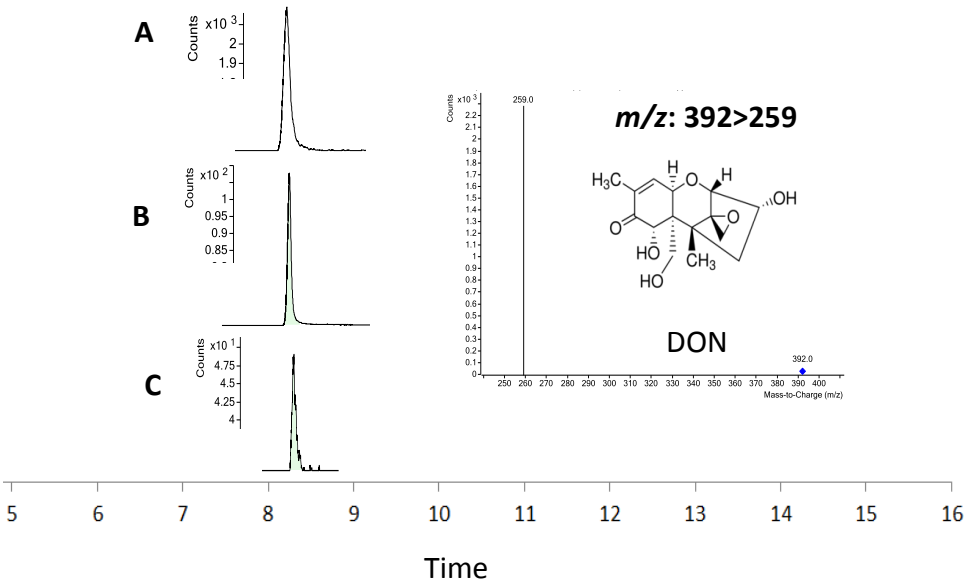


Table 4 shows the exposure estimates for all European countries based on the here reported mycotoxin occurrence. Generally, DON mean contribution to the tolerable intake in European population from beer is 5% (LB and UB) and HT-2 mean contribution ranged from 7% (LB) to 12% (UB). Consequently, the values obtained in this study demonstrate that the intake of DON though average beer consumption is not a matter of concern from a toxicological point of view, in agreement with previous studies (Harcz et al., 2007; Bertuzzi et al., 2011; Varga et al., 2013). Despite that, the intake of HT-2 from beer consumption in some countries should be

taken into account. In fact, a 10% of the HT-2 TDI was surpassed by 21.4 and 53.6% of European countries based on LB and UB, respectively. Irish population was the most exposed due to the highest mean beer consumption (0.39 L/day) amounting 10% of DON PMTDI and 15 - 25% of HT-2 TDI. Similar exposure estimates were here obtained for Czech and Austrian population (Table 4). Moreover, for high drinkers, beer could suppose an important source of exposure to mycotoxins. For instance, considering a daily consumption of two pints (1 L) and the average contents here reported, a significant exposure to mycotoxins in both scenarios were calculated (25% of the DON PMTDI and from 37% (LB) to 64% (UB) of the HT-2 TDI). Assuming even a worst case: consumption of 1 L of the most contaminated beer here analyzed (42 µg/L of DON and 33 µg/L of HT-2), a DON contribution of 60% of PMTDI was calculated whereas HT-2 could exceed the TDI by up to 5-fold.

Table 4: Daily average exposure (expressed as % tolerable daily intake) of Europeans through beer consumption

Country	Consumption [a] (kg/year)	% DON PMTDI		% HT-2 TDI	
		LB [b]	UB [c]	LB [b]	UB [c]
Austria	107	7	7	11	19
Belgium	88.7	6	6	9	15
Bulgaria	8.2	1	1	1	1
Croatia	83.9	6	6	9	15
Cyprus	2.5	0	0	0	0
Czech Republic	136.6	9	9	14	24
Denmark	66.7	5	5	7	12
Estonia	20.1	1	1	2	4
Finland	85.7	6	6	9	15

France	28.4	2	2	3	5
Germany	97.9	7	7	10	17
Greece	34.7	2	2	4	6
Hungary	69.1	5	5	7	12
Ireland	142.8	10	10	15	25
Italy	28.2	2	2	3	5
Latvia	1.5	0	0	0	0
Lithuania	13.5	1	1	1	2
Luxembourg	97.1	7	7	10	17
Malta	36.6	2	2	4	6
Netherlands	3	0	0	0	1
Poland	95.6	6	6	10	17
Portugal	50	3	3	5	9
Romania	1.4	0	0	0	0
Slovakia	73.4	5	5	8	13
Slovenia	81.2	5	5	8	14
Spain	75.3	5	5	8	13
Sweeden	54.5	4	4	6	9
United Kindom	79.1	5	5	8	14
Eastern Europe	*74*	*5*	*5*	*8*	*13*
Northern Europe	*79.2*	*5*	*5*	*8*	*14*
Southern Europe	*48.3*	*3*	*3*	*5*	*8*
Western Europe	*69.8*	*5*	*5*	*7*	*12*
European Union	*70.1*	*5*	*5*	*7*	*12*

[a] consumption data in the European countries reported by FAO; [b] LB: lower bound; [c] UB: upper bound

Note that beer is not the only foodstuff contributing to mycotoxin exposure. Cereals and cereal-based foods can also contain significant amounts of mycotoxins (Rodríguez-Carrasco, Moltó, Berrada & Mañes,

2014) and should be taken into consideration in exposure assessment studies as recommended by SCOOP Directive 93/5/EEC.

4. Conclusions

A suitable method based on a QuEChERS extraction using GC–MS/MS was developed and successfully validated for the detection and quantitation of mycotoxins in beer. The here proposed methodolody was able to determine simultaneously fourteen mycotoxins in beer proving a sensitive and robust technique. The occurrence of the studied analytes was evaluated in 154 beer samples from different countries of production. Two mycotoxins were found in a relatively high number of samples (59.7% for DON and 9.1% for HT-2). The overall average contents of 17.2 µg/L for DON and 2.8 µg/L for HT-2 contribute on average of 5% of the DON PMTDI and from 7% (LB) to 12% (UB) of HT-2 TDI for Europeans. Conclusively, a moderate consumption of beer do not raise any toxicological concern as regards exposure to mycotoxins. Nonetheless, for heavy drinkers beer consumption could imply an important source of mycotoxins.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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3.5. Determination of mycotoxins in bee pollen by gas chromatography – tandem mass spectrometry

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Determination of mycotoxins in bee pollen by gas chromatography-tandem mass spectrometry

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Keywords: Bee pollen, Mycotoxins, QuEChERS, GC-MS/MS, Triple quadrupole

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ABSTRACT

Bee pollen, promoted as a natural food supplement, is consumed increasingly by people to maintain a healthy diet. Depending on environmental conditions, pollen can also be an optimum medium for growth of molds such as *Fusarium* and *Penicillium*. A quick, easy, cheap, rapid, and safe (QuEChERS) extraction procedure followed by a gas chromatography-tandem mass spectrometry (GC-MS/MS) determination of eight selected *Fusarium* toxins in bee pollen was developed and optimized. Recovery studies at 20, 80, and 1000 µg/kg showed values between 73% and 95% with relative standard deviations (RSDs) of <15% for all studied mycotoxins. Limits of quantitation (LOQ) ranged from 1 to 4 µg/kg. The proposed method was applied to the analysis of 15 commercial samples. Two of 15 samples showed quantifiable values for neosolaniol and nivalenol.

INTRODUCTION

Shifts of structure of global food consumption affect directly eating habits, and people are increasingly looking for healthy and nutritious foods. These diets include natural products such as hive products including honey, bee pollen, and royal jelly.[1] Bee pollen contains, in perfect balance, all essential amino acids that humans require to achieve and maintain optimum vitality. Moreover, it is a storehouse of vitamins, minerals, fats and oils, carbohydrates, and other healthy compounds such as antioxidants.[2,3] For these reasons, its use in the human diet is very highly appreciated, even becoming recognized officially as a medicine by the German Federal Board of Health.[1] In this regard, several papers have appeared in the literature regarding its digestive, antioxidant, and immunostimulation activities.[4–6] These and other properties of bee pollen converted this commodity to a nutrient-rich health food for many centuries, and its benefits have been widely lauded. It is also important, however, to verify the presence of contaminants that are harmful to health.[7]

Mycotoxins are secondary metabolites produced by a wide variety of fungal species such as *Fusarium, Aspergillus, Alternaria, Claviceps*, and *Penicillium*.[8] The ability of molds to produce mycotoxins is influenced by environmental factors, the most important being temperature and relative humidity.[9] There is an increasing concern of mycotoxin contamination in foods and feeds because they can be found in a wide range of commodities

including cereals, spices, dried fruits, apple products, wine, and coffee. Human exposure occurs mainly by ingestion of mycotoxin-contaminated products and can lead to serious health problems, including immunosuppression and even carcinogenesis.[10,11] Maximum levels of mycotoxins in foodstuffs and feedstuffs have been established in many countries.[12] For instance, maximum limits for deoxynivalenol in food matrices such as processed cereal-based foods and baby foods for infants and young children have been set at 200 μg/kg. Nevertheless, there is a lack of current legislation with regard to maximum limits of mycotoxins in bee pollen.

Bee pollen is also an adequate substrate for mycotoxin growth when no prompt and adequate drying is performed by the beekeeper after collection by bees.[13,14] The quality of bee pollen is strongly dependent on its preservation.[4,7] Therefore, the water content of the product determines microbiological and organoleptic qualities and also its shelf life. The initial water content of the fresh pollen is 14−18 g/100 g, and pollens should be dried to reduce the moisture content to 6% to keep their nutritional value for a long time.[9,15,16]

Pollen can be infected by different toxigenic molds, which potentially results in the co-occurrence of several mycotoxins. Interactions between concomitantly occurring mycotoxins can be antagonistic, additive, or synergistic.[17,18] In this context, there is a clear need for fast and efficient analytical methods to support the feed and food industry in the

management of mycotoxin residues. Multiclass or multiresidue analytical methodologies are becoming the required tools to provide reliable and wider knowledge about the occurrence of mycotoxins. However, the complex sample matrix may contain components that can interfere with good sample analysis. No data for the determination of mycotoxins in bee pollen are found in the scientific literature, with the exception of a screening ELISA test reported for the sum of aflatoxins[9] and a simultaneous chromatographic determination of ochratoxin A and aflatoxins retained by an immunoaffinity column.[19]

For the determination of trichothecenes, nonfluorescent analytes, GC has largely been the method of choice, providing sensitive and accurate results after analyte derivatization, generally based on trimethylsilylation and fluoroacylation.[20] Analytical performance characteristics comparable with those of GC methods can be achieved by application of HPLC methods with pre- or postcolumn derivatization.[21] MS/MS is a highly reliable analyte tool and has become a routine technique in food analysis with high impact on the field of mycotoxin analysis, particularly in the development of multimycotoxin methods. GC-QqQ-MS/MS detection remains a powerful technique for the quantitative determination of lower levels of mycotoxins in complex matrices even in the era of liquid chromatography-tandem mass spectrometry.[22,23]

In this study a multiresidue method for the determination of eight trichothecenes, including type A and type B (deoxynivalenol, 3-acetyl

deoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, neosolaniol, HT-2, and T-2), in bee pollen using GC-MS/MS was performed. Mycotoxins were extracted from pollen samples using a QuEChERS-based extraction procedure carefully optimized for this food matrix. The method was in house validated and applied to 15 bee pollen samples commercialized in Spain to evaluate the occurrence of the studied mycotoxins.

MATERIALS AND METHODS

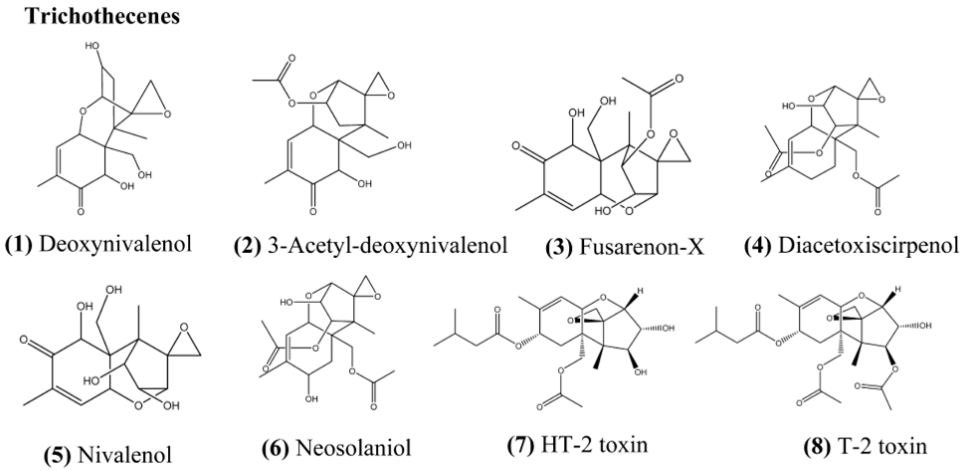
Chemical and Reagents

Solvents (acetonitrile, hexane, and methanol) were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate ($MgSO_4$) was obtained from Alfa Aesar GmbH & Co. (Karlsruhe, Germany); sodium chloride was purchased from Merck, and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, CA, USA). Bondesil primary-secondary amine (PSA) was acquired from Análisis Vínicos (Tomelloso, Spain). The derivatization reagent composed of BSA (N,O-bis-(trimethylsilyl)acetamide) + trimethylchlorosilane (TMCS) + N-trimethyl silylimidazole (TMSI) (3:2:3) was purchased from Supelco (Bellefonte, PA, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

Standards of the type A and type B trichothecenes, deoxynivalenol (1), 3-acetyldeoxynivalenol (2), fusarenon-X (3), diacetoxyscirpenol (4), nivalenol (5), neosolaniol (6), HT-2 (7), and T-2 (8) (Figure 1), were obtained

from Sigma-Aldrich (St. Louis, MO, USA). All stock solutions were prepared by dissolving 1.00 mg of the mycotoxin in 1.00 mL of pure methanol, obtaining a 1 mg/mL solution. The stock solutions were diluted with acetonitrile to obtain the appropriate multicompound working standard solutions (50 mg/L). All standards were kept at -20°C.

Figure 1: Structures of the trichothecenes studied.



GC-QqQ-MS/MS Conditions

One microliter of the extract of mycotoxins was injected in splitless mode at 250°C in programmable temperature vaporization (PTV) using an Agilent 7890A GC system coupled with an Agilent 7000A triple-quadrupole mass spectrometer with an inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, CA, USA). The mass spectrometer was operated in electron impact ionization (EI, 70 eV). The transfer line and source temperatures were 280 and 230°C, respectively.

The collision gas for MS/MS experiments was nitrogen, and helium was used as carrier gas at fixed pressure of 20.3 psi, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data were acquired and processed using Agilent MassHunter version B.04.00 software.

Analytes were separated on an HP-5MS 30 m × 0.25 mm × 0.25 µm capillary column. The oven temperature program was initially 80°C for 2 min, and the temperature was increased to 245°C at 80°C/min. After a 5 min hold time, the temperature was increased to 250°C at 5°C/min and finally to 27°C at 10°C/min and then held for 3 min.

Sampling

A total of 15 pollen samples (100 g) were randomly purchased in supermarkets located in the Valencia metropolitan area (Spain). All samples were homogenized using a laboratory mill and stored in a dark and a dry place in specific plastic food containers and analyzed within 3 days of sampling.

Sample Preparation

Extraction and Clean up. Five grams of milled bee pollen sample, weighed into a 50 mL centrifuge tube, was mixed with 10 mL of distilled water and sonicated for 15 min. To induce phase separation and mycotoxin partitioning, 7.5 mL of acetonitrile, 4 g of $MgSO_4$, and 1 g of NaCl were added to the tube, which was vortexed and centrifuged for 10 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase

extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$, 300 mg of C18, and 300 mg of PSA. The tube was vortexed for 30 s and centrifuged for 10 min at 4000 rpm. The extract (2 mL) was then evaporated to dryness under nitrogen flow for derivatization.

Derivatization. The dry extract was treated with 50 µL of BSA + TMCS + TMSI (3:2:3), and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 250 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to an autosampler vial for the chromatographic analysis.

Method Validation

The developed method was validated following the SANCO 12495/2011 document.[24] Linearity was first evaluated by a triplicate of standard calibration curves at seven concentration levels (5, 20, 80, 150, 500, 750, and 1000 µg/kg). Matrix-matched calibration curves were built by spiking blank sample extracts with selected mycotoxins at the same concentration levels as the standard calibration curves. The accuracy was verified by measuring the recoveries from spiked blank samples at three concentration levels (20, 80, and 1000 µg/kg), six replicates at each fortification level.

Precision (expressed as %RSD) of the method was determined by repeatability (intra-day precision) and reproducibility (inter-day precision).

Intra-day variation was evaluated in six determinations per concentration in a single day, whereas inter-day variation was tested on six different working days within 20 days. Sensitivity was evaluated by LOD and LOQ values. LOD was determined as the analyte concentration that produced a peak signal of 3 times the background noise from the chromatogram regarding SRM2, confirmation transition. LOQ was determined as the analyte concentration that produced a peak signal of 10 times the background noise from the chromatogram regarding SRM1, quantitation transition. Matrix effect is used to describe the analyte ionization efficiency. Recovery describes the efficiency of separating analyte from the sample. Process efficiency (PE) summarizes the efficiency of sample preparation (recovery) and analyte ionization (matrix effect). Therefore, process efficiency is suitable for assessing the overall performance of an analysis method. To assess PE the peak areas of pre-extraction addition extracts (A) with peak areas of calibration solutions prepared in solvent (B) were compared. Thus, the ratio (A/B × 100) was defined as the PE (%) as described Kruve et al.[25]

Two MS/MS transitions were acquired for each mycotoxin, giving four identification points with a defined SRM transitions ratio for the developed method as indicated in the requirements for mass spectrometry.[24,26] The most abundant SRM transition was used for quntitation (Q) and the second one for confirmation (q). In addition, the intensities ratio of the different transitions monitored was used as a confirmatory parameter.

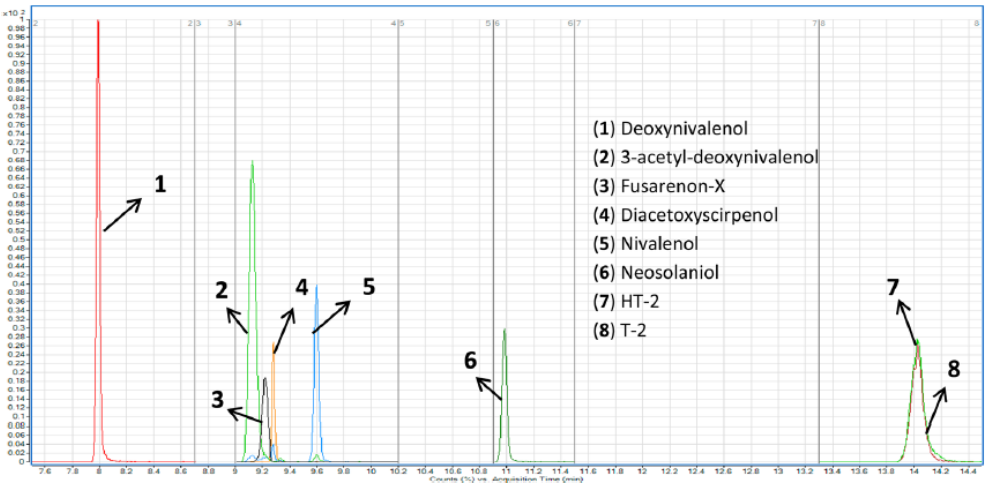
Table 1: Transitions reactions monitored by GC-ESI-MS/MS for the analysis of mycotoxins and peak area ratio with their limits of acceptance according to reference[a]

nº	Compound	t_R (min)	SRM transitions (m/z)	CE (V)	Dt (ms)	Ratio Q/q (RSD)
1	Deoxynivalenol	8.31	392>259	10	25	41.6 (3.2)
			407>197	10	25	
2	3-Acetyl deoxynivalenol	9.33	392>287	5	35	47.5 (12.3)
			467>147	10	25	
3	Fusarenon X	9.39	450>260	10	35	11.9 (7.0)
			450>245	20	35	
4	Diacetoxyscirpenol	9.45	350>229	15	35	56.9 (10.3)
			378>124	10	25	
5	Nivalenol	9.77	289>73	15	35	29.6 (2.7)
			379>73	15	35	
6	Neosolaniol	11.12	252>195	10	25	40.6 (4.)3
			252>167	15	35	
7	HT-2	13.73	347>157	10	25	86.7 (7.8)
			347>185	10	25	
8	T-2	13.74	350>259	10	25	81.9 (5.8)
			350>229	15	35	

[a] *t_R, retention time; SRM, selected reaction monitoring; CE, collision energy; Dt, dwell time; Q, quantitation transition; q, confirmation transition.*

The ion ratio was calculated as the quotient between Q/q areas. Relevant MS/MS data are reported in Table 1. A GC-MS/MS chromatogram obtained from a blank bee pollen sample spiked at 80 µg/kg is also provided in Figure 2.

Figure 2: GC-MS/MS chromatogram obtained from a blank bee pollen sample spiked at 80 µg/kg.



RESULTS AND DISCUSSION

The extraction of the selected mycotoxins in bee pollen was carried out according to the modified QuEChERS method previously developed for the determination of several mycotoxins in wheat semolina as a starting point.[23] In this previous study, the effect of the pH on extraction medium, the effect of solvent volume, and the influence of the cleanup step were carefully evaluated. Nonetheless, due to the complexity of the bee pollen as food matrix, some critical parameters such as volume of solvent and cleanup strategy required reappraisal.

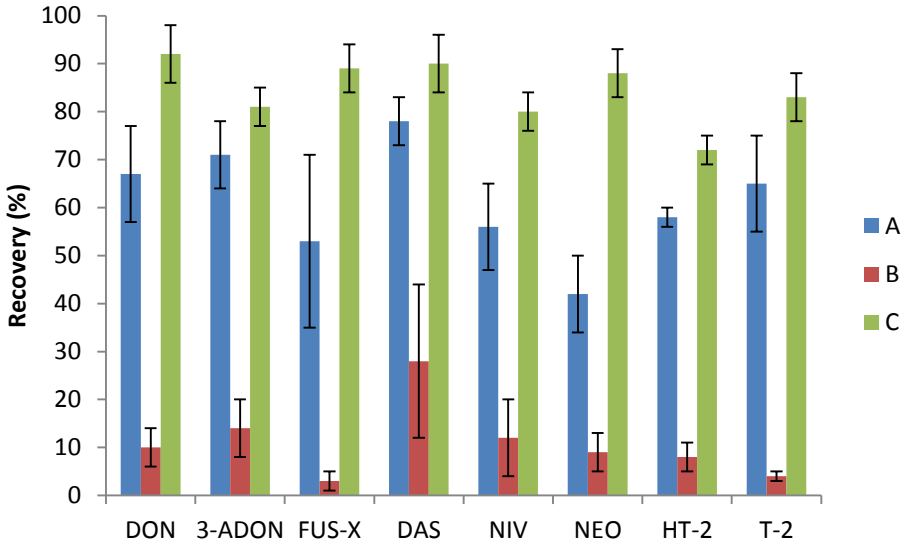
Optimization of the d-SPE

QuEChERS involves mainly acetonitrile extraction and extract purification using d-SPE. Sorbent type is an important parameter in the

QuEChERS method. $MgSO_4$ is employed to separate water from the organic solvent. C18 is the most popular hydrophobic silica based sorbent with high affinity for nonpolar compounds. On the other hand, PSA, originally employed in the QuEChERS analytical methodology developed by Anasstasiades et al.,[27] is commonly used to remove sugars and fatty and organic acids as well as some pigments. When PSA is used in combination with C18, additional lipids and sterols can be removed, offering the best performance in terms of cleanup efficiency, removing the greatest amount of interfering substances.[28] Therefore, different combinations of d-SPE were tested to check the best sorbent combinations regarding recovery results in spiked pollen samples at 80 µg/kg. Specifically, (A) 900 mg of $MgSO_4$ + 300 mg of C18, (B) 900 mg of $MgSO_4$ + 300 mg of C18 + 150 mg of activated carbon, and (C) 900 mg of $MgSO_4$ + 300 mg of C18 + 300 mg of PSA were employed to purify the extracts. The selection of the sorbents was carried out according to the QuEChERS application review.[29] The chromatograms of a spiked sample analyzed using $MgSO_4$ + C18 + PSA provided better cleanup than the other sorbents tried. Activated carbon retained apolar compounds; however, a thin fatty acid film was formed, leading to poor recoveries (<30%) (Figure 3). Similar results were previously reported for pesticide and veterinary drug extraction from honey and pollen.[30] Recoveries between 40 and 80% were obtained with a single use of C18 (Figure 3). The combination of PSA and C18 provided better cleanup than single-use of C18. With PSA, the solution after cleanup was colorless and transparent, whereas the color of the solution cleaned without PSA

was light yellow. Indeed, results showed that $MgSO_4$ in combination with PSA + C18 assured recoveries >70% for all of the mycotoxins studied (Figure 3).

Figure 3: Effect of different mixtures of d-SPE sorbents on cleanup of fortified bee pollen samples (error bars are ± SD*; n = 6*) at a level of 80 µg/kg of studied mycotoxins.



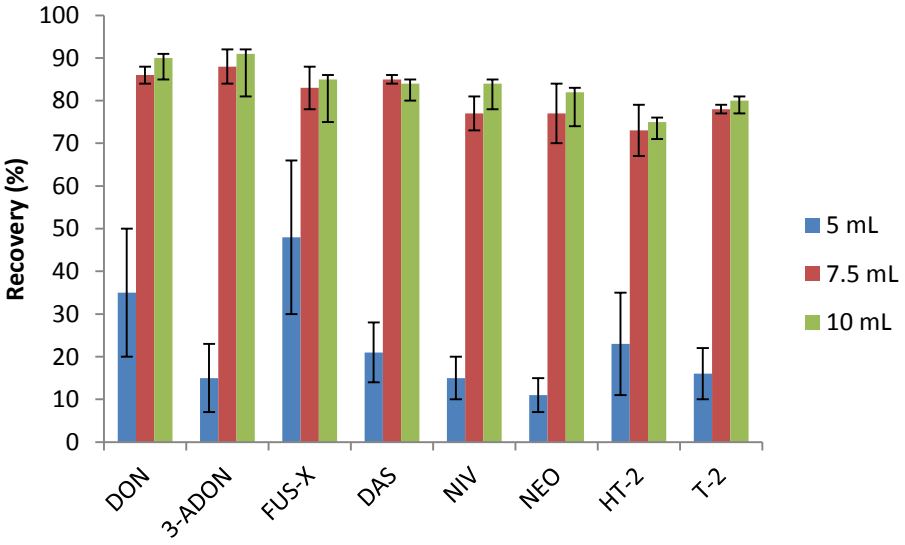
(A) 900 mg of $MgSO_4$ + 300 mg of C18; (B) 900 mg of $MgSO_4$ + 300 mg of C18 + 150 mg of activated carbon; (C) 900 mg of $MgSO_4$ + 300 mg of C18 + 300 mg of PSA

Optimization of the Volume of Solvent

The main objective of this step was to minimize the use of solvent employed ensuring acceptable recovery results. Three different volumes were assayed (5, 7.5, and 10 mL). Statistical analysis (Student's t test) of repeated measures (*n = 6*) was applied to analyze the results. No significant

statistical differences for a confidence interval of 95% were found in terms of recovery results when 7.5 and 10 mL of acetonitrile were employed, obtaining with both volumes tested recoveries up to 70% in all analytes evaluated (Figure 4).

Figure 4: Effect of different acetonitrile volumes on extraction of fortified bee pollen samples (error bars are ± SD; *n = 6*) at a level of 80 µg/kg of studied mycotoxins.



However, 5 mL of solvent was not enough to create an upper layer after centrifugation to be submitted to d-SPE. Finally, 7.5 mL of acetonitrile was chosen due to its being less time-consuming when the subsequent extract was dried.

Analytical Performance

The linear ranges of determination were from LOQ to 1000 µg/L with coefficients of determination >0.990. LODs were from 0.3 to 1.2 µg/kg, and LOQs obtained were in a range from 1 to 4 µg/kg (Table 2). Matrix-matched calibration standards were used to compensate matrix effects. The recoveries obtained ranged from 78 to 95% for the 20 µg/kg spiking level, from 73 to 88% for the 80 µg/kg spiking level, and from 83 to 90% for the 1000 µg/kg spiking level. In all spiking levels the values of intra-day precision (*n = 6*) were <15%, whereas those for inter-day precision were <20% (Table 2). Following the EU guideline[24] (recovery of 70 − 110%, RSD ≤20%), the proposed method was found to be accurate, with satisfactory recoveries at three fortification levels. Process efficiency is the overall performance characteristic of the method. Percent PE values near 100% generally indicate that both the percentage of matrix effect and the percentage of recovery are near 100%. Table 2 shows the PE results obtained for the selected mycotoxins in bee pollen samples analyzed. For the majority of compounds, PE values of <100% were obtained. Ion suppression may be due to matrix effects and compound losses during the sample preparation process. A few compounds showed PE > 100%, which was probably due to matrix effects resulting in ionization enhancement.

Table 2: Performance characteristics of the proposed method for determining mycotoxins in bee pollen

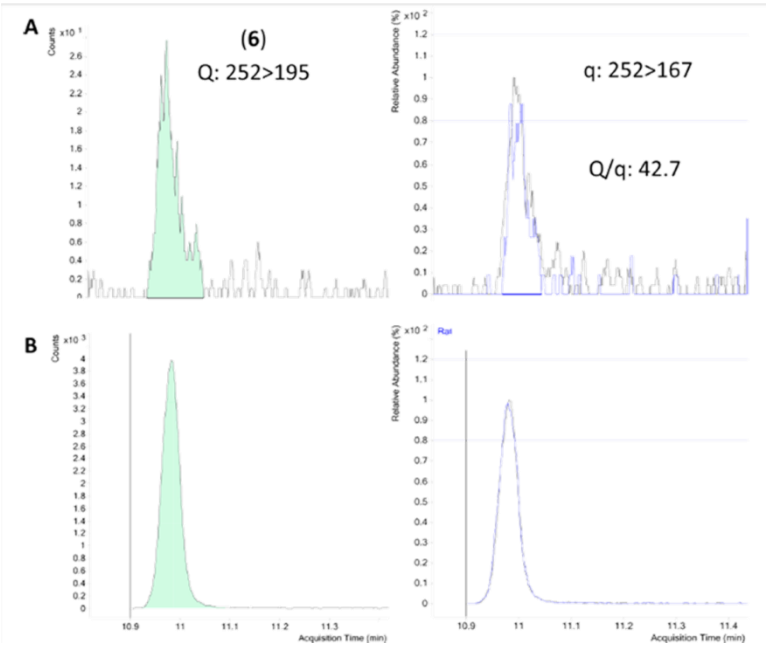
nº	Compound	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	coefficient of determination (R^2)	Recovery (RSD) (%)			PE[a] (%)
					20 µg kg^{-1}	80 µg kg^{-1}	1000 µg kg^{-1}	
1	DON	0.3	1	0.998	78 (7)	86 (2)	83 (7)	68
2	3-ADON	0.3	1	0.992	81 (8)	88 (11)	88 (10)	70
3	FUS-X	1.2	4	0.990	93 (11)	83 (14)	87 (13)	63
4	DAS	1.2	4	0.991	79 (5)	85 (1)	83 (7)	86
5	NIV	0.3	1	0.997	87 (9)	77 (4)	83 (10)	30
6	NEO	0.7	2	0.995	87 (12)	77 (7)	90 (15)	141
7	HT-2	0.3	1	0.999	95 (10)	73 (6)	87 (7)	115
8	T-2	1.2	4	0.991	92 (12)	78 (1)	86 (4)	122

[a] *PE, process efficiency.*

Analysis of Samples

Very little literature is reported regarding mycotoxin-producing fungi in bee pollen and their capability to produce mycotoxins under some circumstances. A survey carried out by González et al.[13] highlighted the occurrence of fungi in bee pollen and reported the isolation of *Penicilium spp., Aspergillus spp*., *and Fusarium spp*., which are producers of ochratoxin A, aflatoxins, and trichothecenes. Nevertheless, neither aflatoxins nor ochratoxin A was detected in the 20 bee pollen samples analyzed by Garcia-Villanova et al.[19]

Figure 5: GC-MS/MS chromatograms for (A) blank bee pollen sample spiked at LOQ (2 µg/kg) of neosolaniol and (B) bee pollen sample naturally contaminated with neosolaniol at 29 µg/kg.



In the present work, 15 bee pollen samples were analyzed with the previously described methodology. Two out of 15 bee pollen samples presented mycotoxin contamination. Neosolaniol (6) was detected in both naturally contaminated samples at 30 ± 5 and 22 ± 3 μg/kg (mean ± SD; *n = 3*), respectively. Nivalenol (5) was also found in these samples with values close to the limit of quantitation (1 μg/kg). Figure 5 shows the GC-MS/MS chromatogram of one naturally contaminated bee pollen sample with neosolaniol as well as a GC-MS/MS chromatogram of blank bee pollen sample spiked with neosolaniol at the LOQ value (2 μg/kg).

None of the other studied mycotoxins were detected. GC-QqQ-MS/MS proposed in this paper allows the single QuEChERS extraction and simultaneous determination of eight *Fusarium* mycotoxins in bee pollen with a chromatographic run of 14 min. The method performance fulfilled the EU guideline standardized in the SANCO/12495/2011 document, offering reliable results in terms of sensitivity, mean recovery, precision, and limit of quantitation. The results reported in the present work show for the first time the presence and co-occurrence of *Fusarium* toxins in two bee pollen samples collected in Spain.

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Notes

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ABBREVIATIONS USED

GC-MS/MS, gas chromatography–tandem mass spectrometry; QqQ, triple quadrupole; PE, process efficieny; d-SPE, dispersive solid phase extraction; SRM, selected reaction monitoring; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantitation.

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3.6. Exposure estimates to *Fusarium* mycotoxins through cereals intake

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Exposure estimates to *Fusarium* mycotoxins through cereals intake

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Abstract

Mycotoxins are harmful substances produced by fungi in several commodities with a widespread presence in foodstuffs. Human exposure to mycotoxins occurs mainly by contaminated food. The quantitation of mycotoxins in cereal-based food, highly consumed by different age population, is of concern. In this survey, 159 cereal-based samples classified as wheat, maize and rice-based, have been evaluated for the occurrence of patulin, deoxynivalenol, 3-acetyl-deoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, neosolaniol, HT-2, T-2 and zearalenone by gas chromatography–tandem mass spectrometry. Intakes were calculated for average consumers among adults, children and infants and compared with the tolerable daily intakes (TDI). Data obtained were used to estimate the potential exposure levels. 65.4% of the samples were contaminated with at least one mycotoxin and 15.7% of the analyzed samples showed co-occurrence of mycotoxin. The dietary exposure to HT-2 and T-2 toxins was estimated as 0.010 and 0.086 μg kg^{-1} bw d^{-1}, amounting to 10% and 86% of the TDI, for adults and infants respectively. These results back up the necessity to take a vigilant attitude in order to minimize human intake of mycotoxins.

1. Introduction

Mycotoxins are secondary metabolites produced by a wide variety of fungal species leading nutritional losses and representing a significant hazard to the food chain (Mankeviciene et al., 2011). In fact, the Food and Agricultural Organization (FAO) has estimated that one-quarter of the world's food crop is contaminated, to some extent, with mycotoxins (JECFA, 2001). The molds ability to produce mycotoxins is greatly influenced by environmental factors, being the most important temperature, relative humidity, insect damage, drought and inadequate storage conditions (Miraglia et al., 2009; Prandini et al., 2009). The *Fusarium* toxins such as the trichothecenes and zearalenone, occur mainly in cereals grown in more moderate climates. While these contaminants may be of lower toxicity than aflatoxins, ochratoxin A, etc., their occurrence in food commodities that are eaten more widely by consumers means that levels must also be rigorously controlled in food and feed. Thus, the occurrence of mycotoxins in agricultural commodities has been recognized as a potential hazard for the human and animal health.

Among the thousands of species of fungi, only about 100 belonging to genera *Fusarium, Aspergillus, Penicillium, Alternaria, Claviceps*, etc. are known to produce mycotoxins. Trichothecenes and zearalonene constitute one of the largest groups of mycotoxins produced by *Fusarium* in cereal grains. Most *Fusarium* species produce either type A or type B trichothecenes. Type A trichothecenes includes HT-2 and T-2 toxins,

diacetoxyscirpenol (DAS) and neosolaniol (NEO). Type B trichothecenes is mainly represented by deoxynivalenol (DON) and by its derivates 3-acetyl-deoxynivalenol (3-ADON) and 15-acetyl-deoxynivalenol (15-ADON). Other type B trichothecenes are fusarenon-X (FUS-X) and nivalenol (NIV). Immunosuppressive and immunomodulatory effects were described as trichothecenes toxicological effect. Zearalenone (ZON) has high oestrogenic activity and can be an important etiologic agent of intoxication in young children (Zinedine et al., 2007; Metzler et al., 2010). Patulin (PAT) is produced by different species of *Penicillium, Aspergillus*, and *Byssochlamys* and it is related to neurotoxic, immunotoxic and gastrointestinal effects in animals.

The human risk to mycotoxins exposure is related to consumption of plant-based foods like cereal grains or animal origin as kidney, liver, milk and eggs (Erdogan, 2004; Atanda et al., 2007). Cereal foods are important in infants and young children diet and they are among the first solid food eaten. Wheat, rice and maize are the most consumed cereals worldwide; the latest data reported by FAO estimated an annual global mean consumption of 66, 53.3 and 17.1 kg/per capita for wheat, rice and maize, respectively. Additionally, the Spanish population is one of the largest consumers of wheat in the world, with an average consumption of 86.10 kg/per capita (FAO, 2009).

Mycotoxins are relatively stable to cooking and processing, thus food preparation procedures do not guarantee their removing. Monitoring

studies are required in order to obtain reliable information about the real exposure of human population to mycotoxins. In this sense, in the European Union, efforts to assess human exposure to mycotoxins have been undertaken within SCOOP (Co-operation on Questions relating to Food) projects. The latest data were reported in the SCOOP task 3.2.10 and compiles about 35000 results received about occurrence of thricothecenes in various food and food raw materials from 12 countries (Schothorst and van Egmond, 2004).

Nowadays, increased efforts have been carried out in order to improve analytical methods not only for the detection of very low concentrations of mycotoxins in foodstuffs but also for the simultaneous determination of different types of mycotoxins (Shephard, 2008; Koeppen et al., 2010; Meneely et al., 2011; Capriotti et al., 2012). In this way, the European Union Reference Laboratory (EU-RL) for mycotoxins aims to facilitate the implementation of European legislation related to monitoring of mycotoxins in food of plant origin and animal feed. The Joint Research Centre Institute for Reference Materials and Measurements and the EU-RL for mycotoxins develop analytical approaches and validate them in-house (JRC Technical Notes, 2011). In the same way, the Association of Official Analytical Chemist (AOAC 985.18, 2002; 986.18, 2002 International) reported official methods for mycotoxins determination, for instance Official Method 986.18 for deoxynivalenol determination in wheat or Official Method 985.18 for zearalenone determination in corn.

Taking into account the methods of analysis for the official control of the levels of contaminants in foodstuffs, an analytical strategy to determine ten mycotoxins using QuEChERS-based extraction and gas chromatography-tandem mass spectrometry (GC–MS/MS) with triple quadrupole (QqQ) mass analyzer have been recently optimized and validated in our laboratory in cereal products and bee pollen (Rodríguez-Carrasco et al., 2012, 2013).

To ensure food safety, the European Commission (EC) has set maximum limits for some mycotoxins in foodstuffs (EC No. 1881/2006; EC No. 1126/2007) (Table 1). Maximum levels of DON permitted in Europe are 750 μg kg^{-1} in pasta, cereals intended for human consumption and cereal flour and 200 μg kg^{-1} in processed cereal-based foods and baby foods for infants and young children. The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) established provisional maximum tolerable daily intakes (PMTDI) for DON and its acetylated derivates (3-ADON and 15- ADON) of 1 μg kg^{-1} bw, whereas for NIV and the sum of T-2 and HT-2 toxins the SCF proposed a temporary tolerable daily intake (t-TDI) of 0.7 and 0.06 μg kg^{-1} bw, respectively (SCF, 2002). Recently, the Scientific Committee on Food (SCF) concluded that a full TDI of 0.1 μg kg^{-1} bw for the sum of T-2 toxin and HT-2 toxin can be now established based on recent data (SCF, 2011a).

EC set also a maximum level of ZON of 75 μg kg^{-1} in cereals intended for direct human consumption and cereal flour, 100 μg kg^{-1} in maize intended for direct human consumption and maize flour and

20 µg kg^{-1} in processed cereal-based foods and baby foods for infants and young children. In 2000 the SCF established a t-TDI of 0.2 µg kg^{-1} bw for ZON. However, in 2011 the SFC concluded that a TDI of 0.25 µg kg^{-1} bw can be established based on recent data in the most sensitive animal species (SCF, 2011b).

Table 1: Summary of maximum levels specified for deoxynivalenol and zearalenone in foodstuffs

Toxin	Food product	Maximum level (µg kg^{-1})
DON	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 2.4.7, 2.4.8 and 2.4.9	750
	Pasta (dry)	750
	Processed cereal-based foods and baby foods for infants and young children	200
ZON	Cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, with the exception of foodstuffs listed in 2.5.6, 2.5.7, 2.5.8, 2.5.9 and 2.5.10	75
	Maize intended for direct human consumption, maize-based snacks and maize-based breakfast cereals	100
	Processed cereal-based foods (excluding maize-based foods) and baby foods for infants and young children	20
	Processed maize-based foods for infants and young children	20

Concerning PAT, Codex Alimentarius have recommended PAT concentration of <50 μg kg^{-1} for apple products intended for human consumption and the European Union (EU) established a limit of 10 μg kg^{-1} for patulin in baby food and infant formulae. On the other hand a PMTDI of 0.4 μg kg^{-1} bw was established by JEFCA (JECFA, 1995).

The occurrence of PAT, DON, 3-ADON, DAS, NIV, FUS-X, NEO, T-2, HT-2 and ZON was evaluated in this study in cereal-based products at first stage for screening purposes followed by a risk assessment approach for these toxins in infants, children and adults.

2. Materials and methods

2.1. Sampling

One hundred and fifty-nine cereal-based samples purchased in 2012 from different shops located in Valencia (Spain) were evaluated. The samples were classified as wheat-based (*n = 119*), rice-based (*n = 23*), and maize-based (*n = 17*). Additionally, they were categorized as store brands (*n = 79*) and name brands (*n = 80*). A subdivision of the collected samples depending on agricultural practice was also performed. Thereby, 70 out of 79 store brand samples were classified as conventional while the remaining nine were organic ones. Similarly, 56 out of 80 name brand samples were conventionally produced while the 24 remaining were organic ones. Almost entirely samples collected were categorized as minute pasta (such as alphabet, little threads, melon seeds, little stars, couscous, little shells and semolina) which required less than 8 min for cooking. The other samples

were ready-to-eat cereal-based foods. All of them were packaged in plastic food containers (100 g) and stored under cool conditions (4°C) until analysis.

2.2. Analytical method

A total of ten mycotoxins, including PAT, DON, 3-ADON, FUS-X, DAS, NIV, NEO, HT-2, T-2 and ZON, were determined in cereal-based samples following a QuEChERS-based extraction and GC–MS/MS analysis after derivatization step. Briefly, 5 g of milled sample were added to 25 mL of distilled water and were sonicated for 15 min. The main extraction involved the addition of 7.5 mL of acetonitrile, 4 g of $MgSO_4$ and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18-E (50 µm, 65 A) and centrifuged for 1 min at 1500 rpm. Finally the extract was evaporated to dryness under nitrogen flow. The dry extract was treated with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 250 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the hexane was washed with 1 mL of phosphate buffer (60 mM, pH 7). Finally, the hexane layer was injected in splitless mode at 250°C in programmable temperature vaporization (PTV) using an Agilent 7890A GC system coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies,

Palo Alto, CA). The mass spectrometer was operated in electron impact ionization (EI, 70 eV). The transfer line and source temperatures were 280°C and 230°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as carrier gas at fixed pressure of 20.3 psi, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent MassHunter version B.04.00 software.

Analytes were separated on a HP-5MS 30 m x 0.25 mm x 0.25 µm capillary column. The oven temperature program was initially 80°C for 2 min, and the temperature was increased to 245°C at 80°C min^{-1}. After a 5 min hold time, the temperature was increased to 250°C at 5°C min^{-1} and finally to 270°C at 10°C min^{-1} and then held for 3 min (Rodríguez-Carrasco et al., 2012).

2.3. Consumption data

The population groups considered in this study were: infants (0–3 years), children (5–12 years) and adults (18–65 years). Wheat consumption data was derived from different Spanish surveys published in the Spanish Food Safety and Nutrition Agency (AESAN, 2012). Rice-based and maize-based foods consumption data were taken from the latest Food Balance Sheets data reported by FAO regarding Spanish population (FAO, 2009).

2.4. Calculation of mycotoxins intakes

For the exposure assessment based on a deterministic approach, the probable daily intake (PDI) were obtained by an integration of mycotoxin analysis data obtained from the samples analyzed combined with the food consumption assumption of different population groups with a body weight of 10, 25 and 70 kg for infants, children and adults, respectively (Fernandes et al., 2011; Marin et al., 2011; Törnkvist et al., 2011). The PDI of each mycotoxin was calculated as indicated in the following equation:

$$PDI_m = {(C_m \; x \; K)}/{_{bw}} \qquad (1)$$

where PDI_m is the probable daily intake (µg kg^{-1} bw d^{-1}) for each mycotoxin m; C_m is the mean content of a mycotoxin (µg kg^{-1}); K is the average consumption of the commodity (g d^{-1}) and bw is the body weight used in each population group. Once calculated the PDI_m, the health risk characterization of each mycotoxin (% of relevant TDI) was performed by dividing the PDI_m with the tolerable daily intake TDI_m) (µg kg^{-1} bw d^{-1}) as indicated in Eq. (2) (Nakanishi et al., 2003). For NEO, DAS and FUS-X, TDI data is not regulated and the hypothetic value of 0.1 µg kg^{-1} bw d^{-1} was used.

$$\%\, TDI = (PDI_m / TDI_m)\; x\; 100 \qquad (2)$$

%TDI was useful to evaluate consumer's exposure in those samples contaminated only with one mycotoxin. An approximation of exposure assessment for samples multicontaminated was also carried out. The concentrations of the mycotoxins found in sample multicontaminated were summed determining the range of contamination and thus, $\sum C_{min}$ and $\sum C_{max}$ were obtained for the samples analyzed. Then a combined health risk characterization ($\sum_{n=1}^{i} \%TDI$) was proposed as follows:

$$\sum_{n=1}^{i} \%TDI_{min} = \sum_{n=1}^{i} (C_{m,min}\, x\, K)/TDI_m$$

$$\sum_{n=1}^{i} \%TDI_{max} = \sum_{n=1}^{i} (C_{m,max}\, x\, K)/TDI_m \quad (3)$$

Values of $\sum_{n=1}^{i} \%TDI$ higher than a hundred could predict a possible health risk scenario.

3. Results and discussion

3.1. Presence of mycotoxins in samples

The analytical method was in-house validated with respect to selectivity, linearity, LOD, LOQ, precision and accuracy according to SANCO 12495/2011 document (SANCO, 2011). Matrix-matched calibrations were used for quantitation. Linear range studied was from LOQ to 100 times LOQ. A good linearity (R^2 > 0.990) within the tested range was obtained in all food matrices evaluated. For the recovery analysis, one wheat, rice and

maize-based sample, previously analyzed to assure the absence of studied mycotoxins, were used in the validation procedure. Recoveries of fortified cereals ranged between 76–108% and 77–114% at 20 and 80 μg kg^{-1} respectively, in the three food matrices evaluated. Intra-day precision of <9% were achieved for both spiked levels while an inter-day precision lower than 14% was obtained. LOD was defined as the concentration with a signal-to-noise ratio (S/N) of 3:1 whereas LOQ was defined as the concentration with a signal-to-noise ratio (S/N) of 10:1. Ranges from 0.6 to 5 μg kg^{-1} and from 1.25 to 10 μg kg^{-1} were obtained for LOD and LOQ, respectively.

Occurrence of mycotoxins in wheat-based cereal samples is shown in Table 2. The most frequently mycotoxins found were DON, HT-2 and NIV with an overall incidence of 79.8%, 16.8% and 13.4%, respectively. Conventionally and organically produced cereals showed similar occurrence of mycotoxins. These results are in agreement with other *Fusarium* mycotoxins occurrence studies. For instance, Edwards (2009) and Ok et al. (2011), did not find statistically significant differences in the trichothecenes content in cereals obtained from different agricultural practices analyzed. Nevertheless, in the survey reported by Edwards (2009), organic samples had a significantly lower concentration of HT-2 and T-2. Over the last years, many consumers perceived healthier organic foods and preferred those to conventional ones.

Table 2: Incidence and levels of mycotoxins wheat-based products analyzed.

Wheat (*n = 119*)	Agricultural practice	Parameter	Mycotoxin									
			PAT	DON	3-ADON	FUS-X	DAS	NIV	NEO	HT-2	T-2	ZON
Store brands (n = 62)	Conventional (n = 57)	% Positive samples	-	75.4	1.7	1.7	-	15.8	-	10.5	-	-
		Mean (µg kg^{-1})	n.d.	19.5	5.3	10.8	n.d.	13.9	n.d.	16.4	n.d.	n.d.
		Maximum (µg kg^{-1})		43.2	5.3	10.8		28.3		22.9		
	Organic (n = 5)	% Positive samples	-	80	20	-	-	40	-	20	-	-
		Mean (µg kg^{-1})	n.d.	26.2	3.8	n.d.	n.d.	39.5	n.d.	7.1	n.d.	n.d.
		Maximum (µg kg^{-1})		83.2	3.8			53.6		7.1		
Name brands (n = 57)	Conventional (n = 42)	% Positive samples	-	83.3	-	-	-	9.5	2.4	19.0	-	-
		Mean (µg kg^{-1})	n.d.	10.5	n.d.	n.d.	n.d.	17.6	15.5	8.9	n.d.	n.d.
		Maximum (µg kg^{-1})		16.8				43.1	15.5	23.5		
	Organic (n = 15)	% Positive samples	-	86.7	13.3	-	-	26.7	-	33.3	6.7	-
		Mean (µg kg^{-1})	n.d.	12.1	4.8	n.d.	n.d.	21.6	n.d.	15.6	13.7	n.d.
		Maximum (µg kg^{-1})		25.4	5.1			35.8		28.2	13.7	
Overall incidence (%)			-	**79.8**	**3.4**	**0.8**	**-**	**13.4**	**0.8**	**16.8**	**0.8**	-

Some authors reported that reduced use of fungicides may lead to a greater contamination by mycotoxins in organic food (Juan et al., 2008). However, contradictory results have been found in scientific literature (Ariño et al., 2007; Suproniene et al., 2010; Bernhoft et al., 2012).

Statistical analysis (Student's t-test) was applied to analyze the results obtained from store brands (*n = 62*) and name brands (*n = 57*). No significant differences were found.

As far as rice-based cereals are concerned, a total of 23 samples were analyzed. The eighteen conventionally produced samples did not show quantifiable values of the mycotoxins studied whereas 3 out of 5 organically produced samples showed contamination with DON and NEO at average contents of 5.0 and 15.5 μg kg^{-1}, respectively. Note that organic samples were also brown rice. Relating to maize-based cereals, 17 samples were analyzed showing mycotoxins contamination in both agricultural practices. 2 out of 9 conventionally produced showed DON contamination with an average content of 4.1 μg kg^{-1}, ZON was also found at 10 μg kg^{-1} and HT- 2 at 6.4 μg kg^{-1}. DON was also found in 4 out of 8 organic maize-based samples with a mean content of 11.2 μg kg^{-1} and NIV was quantitate in 2 organic samples (Table 3).

In brief, none of the samples assayed contained toxins at non-permitted level according to the EU legislation. Despite that, it has to be highlighted that 65.4% of the samples showed mycotoxin contamination by at least one mycotoxin. Results indicated that wheat-based samples

showed the highest mycotoxin contamination. DON was the most frequently detected mycotoxin in the three food matrices studied followed by HT-2 toxin and NIV. No significant differences were found neither in the type of brand nor in the agricultural practice employed.

Thus, the overall contamination data in the three commodities studied were used to calculate the risk assessment approach.

One of the critical point in dietary exposure assessment comes from data below the limit of detection (LOD) or quantitation (LOQ) also known as left-censored data, determined by the performance of the analytical methods. The most commonly used method in food risk assessment is to substitute the non-detects by LOD, LOD/2 or LOD/$\sqrt{2}$. A considerable percentage of samples analyzed in this study were below LOQ. The value of ½ LOD was assigned to all non-detected samples as commonly used in exposure assessment of populations by mycotoxins (Joint FAO/WHO, 2009).

3.2. Exposure assessment

Table 4 summarizes the risk characterization of mycotoxins evaluated in different population groups based on probable daily intake following the Eq. (1). The dietary exposure to DON through the consumption of the three commodities evaluated was about 77.3 x 10^{-3}, 63.3 x 10^{-3} and 9.1 x 10^{-3} μg kg^{-1} bw d^{-1} amounting to 8%, 6% and 1% of the TDI for infants, children and adults, respectively.

Table 3: Incidence and levels of mycotoxins in rice- and maize-based products analyzed

Commodity	Brand type	Agricultural practice	Parameter	Mycotoxin									
				PAT	DON	3-ADON	FUS-X	DAS	NIV	NEO	HT-2	T-2	ZON
Rice (n = 23)	Store brands (n = 10)	Organic (n = 2)	% Positive samples	-	50	-	-	-	-	50	-	-	-
			Mean (µg kg^{-1})	n.d.	5.5	n.d.	n.d.	n.d.	n.d.	15.5	n.d.	n.d.	n.d.
			Maximum (µg kg^{-1})		5.5					15.5			
		Conventional (n = 8)	% Positive samples	-	-	-	-	-	-	-	-	-	-
			Mean (µg kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			Maximum (µg kg^{-1})										
	Name brands (n = 13)	Conventional (n = 10)	% Positive samples	-	-	-	-	-	-	-	-	-	-
			Mean (µg kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			Maximum (µg kg^{-1})										
		Organic (n = 3)	% Positive samples	-	66.7	-	-	-	-	-	-	-	-
			Mean (µg kg^{-1})	n.d.	4.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
			Maximum (µg kg^{-1})		4.8								
	Overall incidence (%)			-	13.0	-	-	-	-	4.3	-	-	-

Maize (n = 17)	Store brands (n = 7)	Organic (n = 2)	% Positive samples	-	50	-	-	-	100	-	-	-	-
			Mean (µg kg^{-1})	n.d.	12.6	n.d.	n.d.	n.d.	4.9	n.d.	n.d.	n.d.	n.d.
			Maximum (µg kg^{-1})		12.6				5.4				
		Conventional (n = 5)	% Positive samples	-	40	-	-	-	-	-	-	-	20
			Mean (µg kg^{-1})	n.d.	4.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	10.0
			Maximum (µg kg^{-1})		4.7								10.0
	Name brands (n = 10)	Organic (n = 6)	% Positive samples	-	50	-	-	-	-	-	25	-	-
			Mean (µg kg^{-1})	n.d.	10.8	n.d.	n.d.	n.d.	n.d.	n.d.	6.4	n.d.	n.d.
			Maximum (µg kg^{-1})		22.1						6.4		
		Conventional (n = 4)	% Positive samples	-	-	-	-	-	-	-	25	-	-
			Mean (µg kg^{-1})	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	6.4	n.d.	n.d.
			Maximum (µg kg^{-1})								6.4		
	Overall incidence (%)			-	35.3	-	-	-	11.8	-	5.9	-	5.9

The other mycotoxins studied followed a very similar trend as concerned risk characterization. It has to be highlighted the percent of relevant TDI obtained for the sum of HT-2 and T-2 toxins, amounting to 86%, 79% and 10% of the TDI for infants, children and adults, respectively. A higher percent of relevant TDI was obtained for the sum of HT-2 and T-2 than for DON because TDI for the sum of HT-2 and T-2 is 10 times lower than the TDI of DON (0.1 μg kg^{-1} bw d^{-1}).

These results suggest that the current exposure levels of these toxins might not pose a health risk for the average consumer, although mycotoxins intake by certain populations consuming high amounts of cereal-based products, such as infants, children or vegetarians, could exceed the safety limits.

With regard to co-occurrence of mycotoxin in the samples analyzed, Table 5 shows the lowest ($\sum C_{min}$) and the highest ($\sum C_{max}$) sum of concentrations of mycotoxins found in the same sample.

The combined risk characterization of those multi-contaminated samples was calculated for infants, children and adults following the Eq. (3). Results showed up slightly higher levels of exposure if they are compared with one mycotoxin data.

Table 4: Risk characterization of mycotoxins studied in different population groups based on probable daily intake.

Toxins	Mean ($\mu g\ kg^{-1}$)	Consumption ($g\ day^{-1}$)			Probable Daily Intake (PDI) ($x\ 10^{-3}\ \mu g\ kg^{-1}\ bw\ day^{-1}$)			TDI ($\mu g\ kg^{-1}\ bw\ day^{-1}$)	Risk characterization (% TDI)		
		Infants	Children	Adults	Infants	Children	Adults		Infants	Children	Adults
PAT	n.d				*8.23*	*7.94*	*0.90*	0.4	*2.06*	*1.99*	*0.22*
DON + 3-ADON	17.4		Wheat		57.25	55.26	6.24	1.0	5.72	5.53	0.62
FUS-X	10.8				35.53	34.30	3.87	0.1	35.53	34.30	3.87
DAS	n.d	32.9	79.4	25.1	*4.11*	*3.97*	*0.45*	0.1	*4.11*	*3.97*	*0.45*
NIV	19.2				63.17	60.98	6.88	0.7	9.02	8.71	0.98
NEO	n.d				*4.11*	*3.97*	*0.45*	0.1	*4.11*	*3.97*	*0.45*
HT-2 + T-2	24.1				79.29	76.54	8.64	0.1	79.29	76.54	8.64
ZON	n.d				*8.23*	*7.94*	*0.90*	0.25	*3.29*	*3.18*	*0.36*

PAT	n.d	Rice	*7.88*	*3.15*	*1.13*	0.4	*1.97*	*0.79*	*0.28*
DON + 3-ADON	5		15.75	6.30	2.25	1.0	1.58	0.63	0.22
FUS-X	n.d		*3.94*	*1.58*	*0.56*	0.1	*3.94*	*1.58*	*0.56*
DAS	n.d	31.5	*3.94*	*1.58*	*0.56*	0.1	*3.94*	*1.58*	*0.56*
NIV	n.d		*2.27*	*0.91*	*0.32*	0.7	*0.32*	*0.13*	*0.05*
NEO	15.5		48.83	19.53	6.98	0.1	48.83	19.53	6.98
HT-2 + T-2	n.d		*3.94*	*1.58*	*0.56*	0.1	*3.94*	*1.58*	*0.56*
ZON	n.d		*7.88*	*3.15*	*1.13*	0.25	*3.15*	*1.26*	*0.45*
PAT	n.d		*1.03*	*0.41*	*0.15*	0.4	*0.26*	*0.10*	*0.04*
DON + 3-ADON	10.5	Maize	4.31	1.72	0.62	1.0	0.43	0.17	0.06
FUS-X	n.d		*0.51*	*0.21*	*0.07*	0.1	*0.51*	*0.21*	*0.07*
DAS	n.d	4.1	*0.51*	*0.21*	*0.07*	0.1	*0.51*	*0.21*	*0.07*
NIV	4.9		2.01	0.80	0.29	0.7	0.29	0.11	0.04
NEO	n.d		*0.51*	*0.21*	*0.07*	0.1	*0.51*	*0.21*	*0.07*
HT-2 + T-2	6.4		2.62	1.05	0.37	0.1	2.62	1.05	0.37
ZON	n.d		*1.03*	*0.41*	*0.15*	0.25	*0.41*	*0.16*	*0.06*

Italic data show the calculated value in non-detected samples assuming ½ LOD. LODs (µg kg^{-1}): PAT (5), DON (0.6), 3-ADON (0.6), FUS-X (2.5), DAS (2.5), NIV (1.2), NEO (2.5), HT-2 (2.5), T-2 (2.5), ZON (5).

Despite that, intake of mycotoxins values were also lower than TDIs except for those samples contaminated simultaneously by four and five mycotoxins which showed slightly higher values of one hundred in the combined risk characterization for infants (118.1%) due to their lower body weight and their higher cereal-based consumption. However, those samples above 100% represented only 1.9% of the total samples analyzed (3 out of 159 cereal-based samples).

Other studies have already estimated the mycotoxins intake, for instance, in the French Total Study Diet, estimated total intakes of DON of 45 x 10^{-3} and 28 x 10^{-3} µg kg^{-1} bw d^{-1} from total diet were reported for children and adults, respectively (Leblanc et al., 2005). The estimated DON intake related to pasta consumption (20 x 10^{-3} µg kg^{-1} bw d^{-1} for children and 8 x 10^{-3} µg kg^{-1} bw d^{-1} for adults), was similar than those reported in the present study (55 x 10^{-3} and 6 x 10^{-3} µg kg^{-1} bw d^{-1} for children and adults, respectively). Results reported in the survey carried out by Cano-Sancho et al. (2011) from processed cereal-based foods (*n = 928*) for children and adults were also in accordance with those exposed before.

Concerning the mean adults exposure to NIV, 7.5 x 10^{-3} µg kg^{-1} bw d^{-1} was obtained in this study, as sum of the PDI from the three commodities evaluated, while a PDI of 8 x 10^{-3} µg kg^{-1} bw d^{-1} was published in the French study from the same commodities.

Table 5: Co-occurrence mycotoxins data based on the sum of the concentrations found in the same sample and combined risk characterization ($\sum_{n=1}^{i} \%\mathrm{TDI}$) in different population groups.

Co-occurrence	number of samples (%)	$\sum C_{min}$ (µg kg^{-1})	$\sum C_{max}$ (µg kg^{-1})	$\sum_{n=1}^{i} \%TDI_{min}$			$\sum_{n=1}^{i} \%TDI_{max}$		
				Infants	Children	Adults	Infants	Children	Adults
2 mycotoxins									
DON-HT-2	8 (5)	12.3	61.5	24.5	9.8	3.4	44.8	17.9	6.4
DON-NIV	4 (2.5)	13.4	56.5	5.5	2.2	0.8	22.9	9.2	3.2
3 mycotoxins									
DON-3-ADON-NIV	1 (0.6)	51.4	51.4	50.4	20.2	7.2	50.4	20.2	7.2
DON-HT-2-NIV	8 (5)	26.4	72.5	35.6	14.2	5.1	50.7	20.3	7.3
DON-HT-2-FUS-X	1 (0.6)	28.2	28.2	61.0	24.4	8.7	61.0	24.4	8.7
4 mycotoxins									
DON-3-ADON-HT-2-NIV	2 (1.3)	80.1	90.9	80.5	32.2	11.6	118.1	47.3	16.9
5 mycotoxins									
DON-3-ADON-HT-2-T-2-NIV	1 (0.6)	77.7	77.7	118.3	47.3	16.9	118.3	47.3	16.9

Regarding T-2 and HT-2 toxins, median estimates exposure reported by Cano-Sancho et al. (2012) ranged from 36 x 10^{-3} to 77 x 10^{-3} μg kg^{-1} bw d^{-1} in children. Similarly, Schothorst et al. (2005) calculated 40 x 10^{-3} μg kg^{-1} bw d^{-1} as the combined mean exposure to T-2 and HT-2 toxins for infants from diet of a complete day (*n = 74*). These results are in accordance with the values obtained in this study as the sum of the PDI from the three food matrices evaluated being 86 x 10^{-3} and 79 x 10^{-3} μg kg^{-1} bw d^{-1}, for infants and children respectively. The average intake of T-2 and HT-2 estimated by Schothorst and van Egmond (2004) for European regional diet was 17 x 10^{-3} μg kg^{-1} bw d^{-1}, very similar to 10 x 10^{-3} μg kg^{-1} bw d^{-1} found in this study. The combined health risk characterization proposed here is an approximation of exposure assessment in multicontaminated samples. In this sense, the Scientific Steering Committee of the Norwegian Scientific Committee for Food Safety have published a risk assessment in cereal grains multicontaminated by several mycotoxins (VKM, 2013); but literature of health risk scenarios of multicontamination by mycotoxin is scarce.

4. Conclusions

The occurrence of PAT, DON, 3-ADON, DAS, NIV, FUS-X, NEO, T-2, HT-2 and ZON has been evaluated in 159 cereal-based samples commonly consumed in Spain mainly by infants and children. Our findings show that the levels of contamination of mycotoxins in cereal-derived products were lower than the permitted levels by EU for safe consumption. As regards

occurrence of mycotoxins, no significant differences were found neither in the type of brand nor in the agricultural practice employed. However, a considerable number of contaminated samples at least with one mycotoxin (65.4%) were found. Additionally, 15.7% of the positive samples showed mycotoxin co-occurrence. As for the exposure assessment calculated, it was found that average consumer intakes were below TDI values but certain populations consuming high amounts of cereals and cereal-based products could exceed the safety limits of some mycotoxins

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3.7. Development of a GC–MS/MS strategy to determine 15 mycotoxins and metabolites in human urine

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Development of a GC–MS/MS strategy to determine 15 mycotoxins and metabolites in human urine

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Abstract

The widespread mycotoxins contamination of food commodities has made the monitoring of their levels essential. To overcome the disadvantages of the indirect approach by food analysis, detection of mycotoxin as biomarkers in urine provides a useful and specific data for exposure assessment to these food contaminants. In this work, a sensitive, rapid and accurate method based on gas chromatography-tandem mass spectrometry procedure to determine 15 mycotoxins and metabolites in human urine was optimized and validated taking into consideration the guidelines specified in Commission Decision 2002/657/EC and 401/2006/EC. A salting-out assisted acetonitrile-based extraction was used for sample preparation. The extraction recoveries were in a range of 72 – 109 %, with intra-day relative standard deviation and inter-day relative standard deviation lower than 10% and 13%, respectively for all mycotoxins at 50, 100 and 200 µg/L spiking levels. The limits of quantitation ranged from 0.25 to 8 µg/L. Matrix effect was evaluated and matrix-matched calibration was used for quantitation. The proposed procedure was applied to 10 urine samples collected from children. Mycotoxins were quantified in 30 % of samples.

1. Introduction

Mycotoxins are an heterogeneous group of secondary metabolites of filamentous fungi mainly belonging to *Apergillus, Penicillium, Alternaria* and *Fusarium spp*. Naturally occurring mycotoxins are of public concern due to their association with a wide range of adverse health effects [1]. In fact, mycotoxins were ranked as the most important chronic dietary risk factors, ahead of synthetic contaminants, plant toxins, and pesticide residues [2]. The diversity of mycotoxins leads to a wide range of acute and chronic toxic effects in animals and humans such as skin irritation, feeding refusal, nausea, vomiting, diarrhea, anemia, hemorrhage, immune suppression, etc. [3].

Most mycotoxins are chemically stable; they survive storage and processing, and could even remain in cooked food as previously reported in literature [4]. Thus, to protect consumers from these health risks, many countries have adopted food regulations to limit exposure to mycotoxins [5, 6]. Many mycotoxins are metabolized and efficiently excreted. Since high levels of mycotoxins were detected in widely consumed cereals [7], quantifiable amounts of these toxins are expected in urine and it is also possible to find toxin derivatives that result from its biotransformation [8-9]. Thus, in order to understand the possible links between mycotoxins and human disease, it is necessary to measure the exposure of a population to the multiple toxins.

In this line, urine is a very convenient sample for screening because large amounts can be easily and non-invasively collected [10]. Consequently, the development and validation of methods for multi-mycotoxin determination is necessary to assess the levels and frequencies of human mycotoxins exposure [11, 12].

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) has become the most powerful tools for determining multiresidues in several matrices [13]. The use of MS/MS in combination with gas chromatography (GC) or liquid chromatography (LC) is gaining ground providing better sensitivity and confirmation realiability. In fact, the important requirements set by the Commission Decision 2002/657/EC [14] as regards criteria and procedures for the validation of analytical methods is satisfied by MS/MS. In this sense, high dynamic range and good performance reached in selected reaction monitoring (SRM) mode make the triple quadrupole (QqQ) one of the most widely employed analyzer [15]. Similarly, appropriate sample preparation protocol involving extraction, purification and concentration of the extract is crucial to obtain the desired sensitivity [16]. Sample preparation techniques have been reported in literature for measuring mycotoxins in urine, such as liquid-liquid extraction [17], solid-phase extraction [18] or immunoaffinity columns purification [19]. Salting-out assisted liquid/liquid extraction (SALLE) is also an alternative sample preparation technique based on the salting-out effect to separate water-miscible organic solvent such as acetonitrile [20].

Up to date, a few multi-mycotoxin methods for determining mycotoxin and their metabolites in human urine have been described in literature [17, 18, 21, 22]. In response, a simple optimized analytical procedure for the simultaneous determination of 15 mycotoxins and metabolites, namely de-epoxydeoxynivalenol, deoxynivalenol, 3-acetyl deoxynivalenol, fusarenon-X, diacetoxyscirpenol, nivalenol, neosolaniol, HT-2, T-2, zearalanone, α-zearalanol, ß-zearalanol, zearalenone, α-zearalenol, ß-zearalenol in human urine is described herein. This method merges the advantages of a solvent extraction at high ionic strength followed by dispersive solid phase extraction with the sensitivity of gas chromatography tandem mass spectrometry (GC-MS/MS) technique. Selected compounds have been determined in SRM mode, using a state of the art triple quadrupole analyzer reaching a chromatographic separation by 17 min total run. To the best knowledge of the authors, the paper here presented is the first application of GC–QqQ–MS/MS to evaluate multiple mycotoxins in human urine. In addition, the optimized validated analytical procedure was used to evaluate the occurrence of target mycotoxins and metabolites in 10 urine samples.

2. Material and Methods

2.1. Standards

Mycotoxin standards and metabolites namely de-epoxy deoxynivalenol (DOM-1), deoxynivalenol (DON), 3-acetyldeoxy nivalenol (3-ADON), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), nivalenol (NIV),

neosolaniol (NEO), HT-2, T-2, zearalanone (ZAN), α-zearalanol (α-ZAL), ß-zearalanol (ß-ZAL), zearalenone (ZON), α-zearalenol (α-ZOL), ß-zearalenol (ß-ZOL) were obtained from Sigma-Aldrich (St. Louis, USA). Individual stock solutions of all analytes were prepared at the same concentration (1000 mg/L) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (50 mg/L). All standards were stored in darkness and kept at -20°C until the GC-MS/MS analysis.

2.2. Chemical, reagents and other material

The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilylimidazole) (3:2:3) was purchased from Supelco (Bellefonte, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain). Picric acid (moistened with water, ≥98%) and creatinine (Crea) standard were supplied by Sigma-Aldrich (St. Louis, USA) whereas sodium hydroxide was acquired from BDH Prolabo – VWR International (Barcelona, Spain).

All solvents, acetonitrile, hexane and methanol (HPLC grade), were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate (thin powder) was obtained from Alfa Aesar GmbH & Co (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, USA).

2.3. Apparatus

For simultaneous determination of all mycotoxins, a GC-MS/MS method was developed for their separation and detection. A GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, USA) were used for MS/MS analysis. Chromatographic separation was achieved on a HP-5MS 30 m × 0.25 mm, 0.25 µm capillary column.

Aliquots of 1µL of sample extracts were injected into the gas chromatograph in splitless mode at 250°C in programmable temperature vaporization (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially set at 80°C, and the temperature was increased to 245°C at 60°C/min. After a 3 min hold time, the temperature was increased to 260°C at 3°C/min and finally to 270°C at 10°C/min and then held for 10 min. All analytes eluted within 17 min, reaching the requirement for a high throughout determination.

Quantitation data were acquired at SRM mode and the mass spectrometer operated in electrospray ionization (EI) mode. The transfer line and source temperatures were 280°C and 230°C, respectively. The EI energy used was 70 eV as in that region the maximum abundance was observed. The collision energies varied from 5-20 eV, depending on the precursor and product ions. The analysis was performed with a filament-multiplier delay of 3.50 min. The collision gas for MS/MS experiments was

nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). The dwell times also varied from 5-35 eV. Data was acquired and processed using the Agilent Masshunter version B.04.00 software.

2.4. Sample Preparation

All urine samples were first centrifuged at 4000 rpm for 5 min. A 10 mL portion of the centrifuged urine was then used for analysis. Five mL of acetonitrile were added to the urine samples followed by the addition of 4 g of $MgSO_4$ and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18 and centrifuged for 1 min at 1500 rpm. Finally the extract was evaporated to dryness under nitrogen flow.

The dry extract was added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 200 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the diluted derivatized sample was added with 1 mL of phosphate buffer (60 mM, pH 7) and the upper layer (hexane phase) was transferred to an autosampler vial for the chromatographic analysis.

2.5 Sampling

Sampling was carried out in a primary school located in Valencia, Spain. Ten urine samples from children (from 8 to 11 years old) were

randomly collected during June 2013. A written and approved informed consent was obtained from the progenitors of all volunteers. The informed consent for the mycotoxin evaluation in urine was in accordance to the Helsinki Declaration on ethical principles for medical research involving human subjects.

All urine samples were obtained in urine recipients and frozen within 6h after collection. The samples with undetectable levels of mycotoxins were used for spiking and recovery studies.

2.6 Method validation

Commission Decision 2002/657/EC [14] and 401/2006/EC [23] were used as guidelines for the validation studies. All the parameters were evaluated by spiking blank samples. Samples were spiked and left to equilibrate over night before the analysis. For identification purposes, retention times of mycotoxins in standards and samples were compared at tolerance of ± 0.5%. Moreover, in accordance with the 2002/657/EC Decision, the relative ion intensity of analytes studied in the standard solution and the spiked samples at the concentration levels used for the calibration curve were compared.

Method performance characteristics such as linearity, limits of detection (LOD), limits of quantitation (LOQ), matrix effect, extraction recovery, repeatability and reproducibility were evaluated for all tested mycotoxins.

2.7. Creatinine analysis

Creatinine urinary levels were determined based on a spectrophotometric method slightly modified [17]. In summary, 3.5 mM picric acid was reacted with 1000 mM NaOH to form alkaline picrate. This solution was stored in the dark in an amber glass recipient. Alkaline picrate (1 mL) was reacted with 1 mL of diluted urine (1/10, v/v, in ultrapure water). The optical density was measured at 500 nm after 30 min using a Shimadzu mini 1240 spectrophotometer. Mycotoxin urinary concentrations were correlated to the creatinine content of a sample expressed as µg/g creatinine.

3. Results and Discussion

3.1. Optimization of the GC-MS/MS conditions

The concentrations of the mycotoxins and/or metabolites in urine samples often occur in low µg/L, hence it is important to optimize the GC-MS/MS method to reach the required levels. Due to regulatory requirements for confirmatory methods, MS/MS instrumentation was selected in order to fulfill the rule of identification points. Each compound was identified and several characteristic fragment ions were obtained as described below. In the present manuscript, GC-MS/MS optimization of 6 metabolites namely DOM-1, ZAN, α-ZAL, ß-ZAL, α-ZOL and ß-ZOL were described for the first time. The optimization of MS/MS method consisted of (i) acquisition of respective MS spectra in full scan mode (m/z 50 – 650 mass range); (ii) selection and fragmentation of appropriate precursor ions;

(iii) product ion scans at different collision energies (CEs) 5, 10, 15 and 20 eV to obtain the best product ion transition signal and different dwell times (Dts) 5, 15, 25 and 35 ms to provide a good peak shape; and (iv) fine tuning of CE and Dt in SRM mode. Figure 1 shows the optimization of collision energies and dwell times carried out in the studied metabolites. Once the final choices were made, the SRM conditions were tested in mixed sample extracts spiked or not with the mycotoxins under study in order to evaluate the sensitivity and selectivity of the method. An example of the chromatogram of urine spiked with the mixture of all target mycotoxins at 25 µg/L is shown in Figure 2.

Retention time in standards and samples must agree, so restrictive tolerances were set. Excellent repeatability in terms of retention times (RSD < 0.5%) was observed and thus fulfilling the tolerance of ± 0.5% considered for quantitation purpose as specified in Commission Decision 2002/657/EC. Table 1 shows the optimized GC-MS/MS parameters for all target mycotoxins.

Table 1: Experimental conditions of the optimized GC-MS/MS method for the selected mycotoxins and metabolites

Compound	t_R (min)	SRM transitions (m/z)	CE (eV)	Dwell time (ms)	Ratio Q/q (RSD)
DOM-1	7.63	181>73	15	25	41.6 (3.2)
		391>271	15	35	
DON	8.60	392>259	10	25	41.6 (3.2)
		407>197	10	25	

3-ADON	9.68	392>287	5	35	47.5 (12.3)
		467>147	10	25	
FUS-X	9.73	450>260	10	35	11.9 (7.0)
		450>245	20	35	
DAS	9.85	350>229	15	35	56.9 (10.3)
		378>124	10	25	
NIV	10.15	289>73	15	35	29.6 (2.7)
		379>73	15	35	
NEO	11.68	252>195	10	25	40.6 (4.3)
		252>167	15	35	
HT-2	14.73	347>157	10	25	86.7 (7.8)
		347>185	10	25	
T-2	14.80	350>259	10	25	81.9 (5.8)
		350>229	15	35	
ZAN	15.15	307>235	15	25	59.9 (7.2)
		449>335	10	25	
α-ZAL	15.45	433>309	20	35	26.1 (4.9)
		433>295	20	35	
ß-ZAL	15.68	433>295	15	35	82.2 (9.7)
		307>73	10	35	
ZON	15.95	462>151	10	25	76.9 (3.9)
		462>333	10	25	
α-ZOL	16.45	305>73	15	25	12.7 (10.7)
		305>289	15	20	
ß-ZOL	16.82	536>333	10	35	66.1 (8.4)
		536>446	15	20	

3.2. Method performance

The suitability of the quantitation method for urinary mycotoxin levels was evaluated by a validation study. Table 2 shows the figures of

merit of the proposed method, namely, linearity, extraction recovery, repeatability, reproducibility, LODs and LOQs and matrix effect (as signal suppression/enhancement, SSE).

Figure 1: Optimization of collision energy (CE) and dwell time for the quantitation transition.

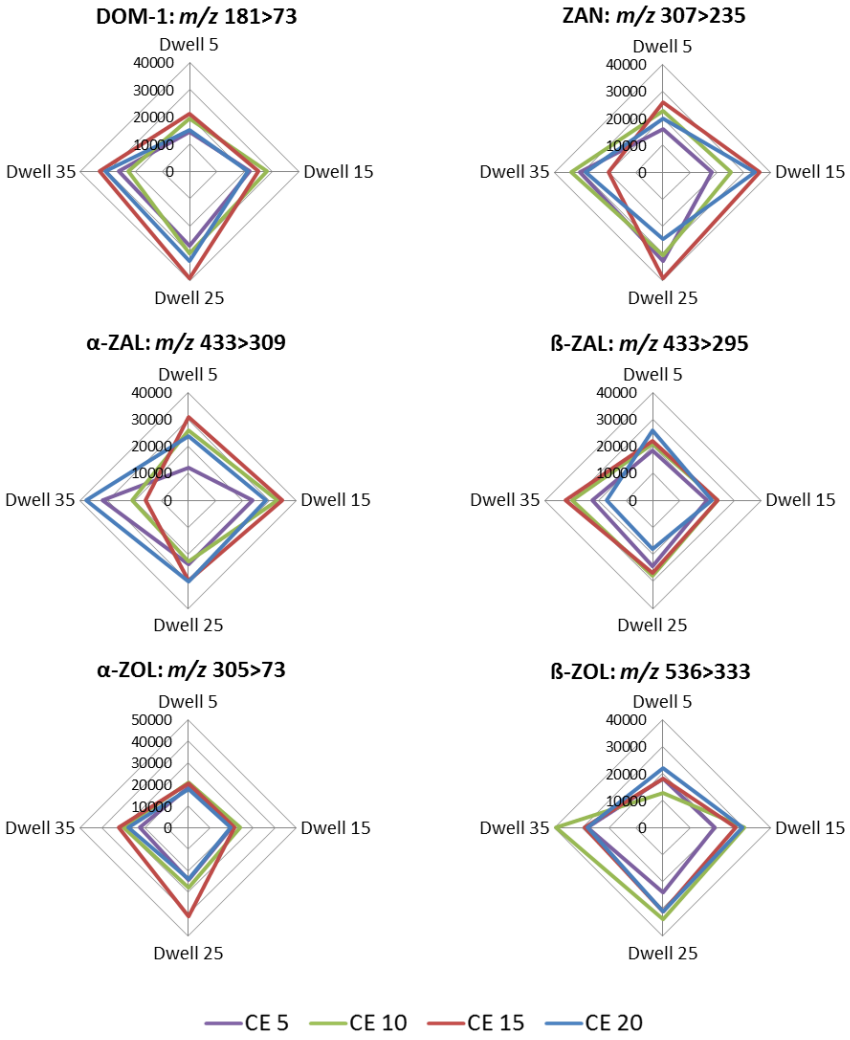
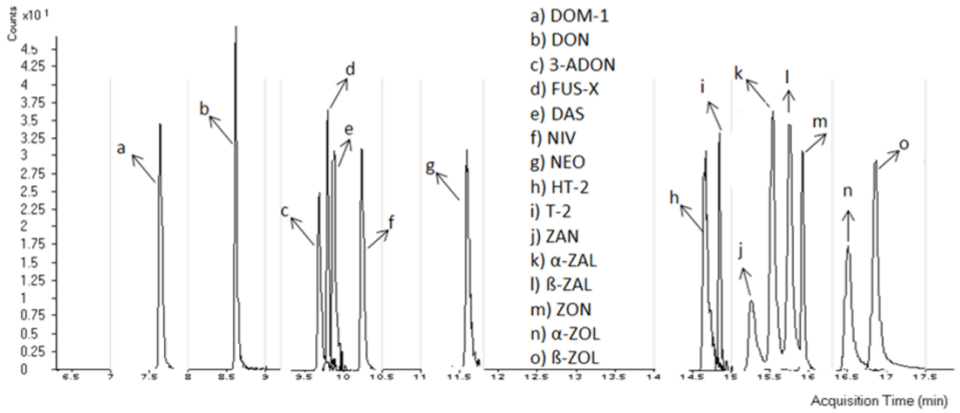


Figure 2: SRM chromatogram of blank urine sample spiked with a mixture of the studied mycotoxins at 25 µg/L.



The linearity of the MS/MS method was established by eight calibration points within a concentration range from LOQ to 250 µg/L. Each calibration point was obtained as the mean of three injections. Correlation between the response and the amount of analytes was verified by plotting signal intensity against analyte concentrations. Good linearity was achieved in all cases with regression coefficients higher than 0.990. Calibration curves were checked at the end of the analysis to assess the response drift of the method.

The specificity and selectivity of the method relies on the chromatographic retention time of each analyte and on the SRM transition used. Selectivity and specificity were assessed by recognizing both quantitation (Q) and confirmation (q) transitions of each mycotoxin at same concentration levels as used for the construction of the calibration

curve. Additionally the ion ratio, defined as the ratio between both transitions (Q/q), of the real samples had to be in agreement with the ion ratio of the matrix-matched calibration curve to confirm a finding. The average ion ratios are listed in Table 1.

The sensitivity of the method was assessed by the limits of detection and quantitation. The LODs and LOQs were calculated as the lowest matrix-matched calibration providing signal-to-noise ratios greater than 3 and 10, respectively, at both Q and q transitions and matching the intensity ratio observed for the particular compound in the standard solution. LOD and LOQ obtained (from 0.12 to 4 µg/L and from 0.25 to 8 µg/L, respectively) showed the suitability of the developed method for the determination of trace amounts of the selected mycotoxins in urine samples.

A blank urine sample extract, from a urine sample previously analyzed to confirm the absence of target mycotoxins, was analyzed (*n = 5*) to study signals obtained from the matrix and to evaluate possible sample interferences. The good specificity of the technique makes possible to have no signal at all in the blank urine for any of the mycotoxins.

Co-eluting matrix components can negatively influence the accuracy and quantitative methods through ion suppression or enhancement in the ion source, thus the effects of a possible matrix mismatch were assessed. The matrix effect for each analyte is defined as the percentage of the matrix-matched calibration slope (A) divided by the

slope of the standard calibration in solvent (B). The ratio ($A/B \times 100$) is defined as the absolute matrix effect. A value of 100% indicates that there is no absolute matrix effect whereas values higher than a hundred indicates signal enhancement and values below 100% indicates signal suppression. Significant signal suppression (SSE) were observed (from 6 to 36%) between the slopes of the calibration lines meaning that matrix effect is present and thus quantitation should be conducted with matrix-matched calibration standards in order to have reliable and accurate results.

Recovery and precision, expressed as percentage relative standard deviation (% RSD), were determined by analyzing urine samples at three different concentrations, 50, 100 and 200 μg/L. Blank sample extracts were employed in method's trueness and precision. Intra-day precision data were obtained from three analyses performed on one day; inter-day data were tested on three different working days within 20 days. Precision studies showed that the method was repeatable (RSD <10%) and reproducible (RSD <13%). Satisfactory results in terms of recoveries (*n = 9*) were found. The fifteen studied analytes showed recoveries results inside the range 72 – 109%. The results obtained are in agreement with the recoveries accepted by the Commission Regulation (EC) No. 401/2006.

The key performance characteristics documented through the above outlined validation protocol also met the criteria established in SANCO document No. 12495/2011 [24].

Table 2: Overview of the extraction recovery, repeatability and reproducibility (Rec (RSD), %), limits of detection (LOD) and quantitation (LOQ) and signal suppression/enhancement (SSE) for the studied analytes.

Proposed biomarker	Correlation coefficient (*r*)	Rec (intra-day RSD, %)			Rec (inter-day RSD, %)			LOD (µg/L)	LOQ (µg/L)	SSE (%)
		Low level (50 µg/L)	Medium level (100 µg/L)	High level (200 µg/L)	Low level (50 µg/L)	Medium level (100 µg/L)	High level (200 µg/L)			
DOM-1	0.990	84 (2)	92 (3)	90 (5)	87 (6)	81 (8)	91 (4)	0.25	0.50	20
DON	0.996	96 (4)	89 (6)	93 (2)	93 (8)	97 (10)	88 (9)	0.12	0.25	23
3-ADON	0.992	92 (5)	96 (1)	84 (9)	102 (6)	94 (4)	96 (11)	0.25	0.50	27
FUS-X	0.992	95 (3)	89 (4)	84 (6)	83 (6)	94 (13)	90 (6)	2	4	12
DAS	0.998	89 (4)	94 (3)	83 (2)	94 (10)	98 (11)	95 (8)	1	2	35
NIV	0.996	87 (3)	82 (7)	95 (2)	90 (7)	94 (7)	85 (4)	0.50	1	6
NEO	0.999	93 (5)	98 (7)	106 (7)	98 (5)	106 (3)	109 (11)	0.25	0.50	36
HT-2	0.999	96 (4)	105 (5)	102 (4)	102 (10)	93 (11)	92 (8)	1	2	28
T-2	0.998	102 (6)	91 (1)	89 (1)	96 (8)	104 (9)	93 (6)	0.50	1	8
ZAN	0.993	72 (7)	75 (6)	79 (6)	77 (10)	79 (11)	80 (8)	4	8	36
α-ZAL	0.998	79 (5)	83 (9)	74 (10)	78 (6)	82 (12)	77 (8)	4	8	28
ß-ZAL	0.997	77 (8)	74 (6)	89 (9)	75 (12)	77 (9)	83 (12)	4	8	33
ZON	0.991	81 (5)	87 (3)	96 (5)	83 (12)	89 (5)	79 (8)	3	6	23
α-ZOL	0.995	88 (2)	81 (8)	93 (8)	98 (6)	80 (12)	84 (4)	1	2	19
ß-ZOL	0.991	80 (6)	78 (7)	83 (5)	78 (9)	84 (8)	79 (9)	2	4	14

Validation results here reported are in agreement with other analytical methodologies previously reported for the determination of several mycotoxins in human urine (Table 3). For some mycotoxins, an improvement of the validation parameters such as recovery range, intra- and inter-day precision and limits of detection and quantitation were obtained. Thus, according to the obtained results, the developed method seems robust and suitable for its purpose, and can be an alternative method for the determination of mycotoxins and their metabolites in human urine.

Table 3: DON performance characteristics of five LC-MS/MS-based multibiomarker methods in human urine compared with the here reported GC-MS/MS methodology.

Reference	[17]	[20]	[21]	[27]	[28]	Present study
Recovery (%)	92	65	88	107	77	96
$RSD_{r\ (\%)}$	16	20	6	3	n.i.	4
$RSD_{R\ (\%)}$	19	22	8	5	n.i.	8
LOD (µg/L)	2.85	0.5	4	10	0.45	0.12
LOQ (µg/L)	5.7	1.7	13	35	1.51	0.25
Extraction and clean up	LLE-SPE	SALLE	-[a]	IAC	SPE-IAC	SALLE-dSPE
No. of detected mycotoxins	18	12	14	11	7	15
Total elution time (min)	15	15	15	9	n.i.	17

RSD_r: intra-day relative standard deviation; RSD_R: inter-day relative standard deviation;
[a] Dilute and shoot; n.i.: not indicated

3.3. Application to real samples

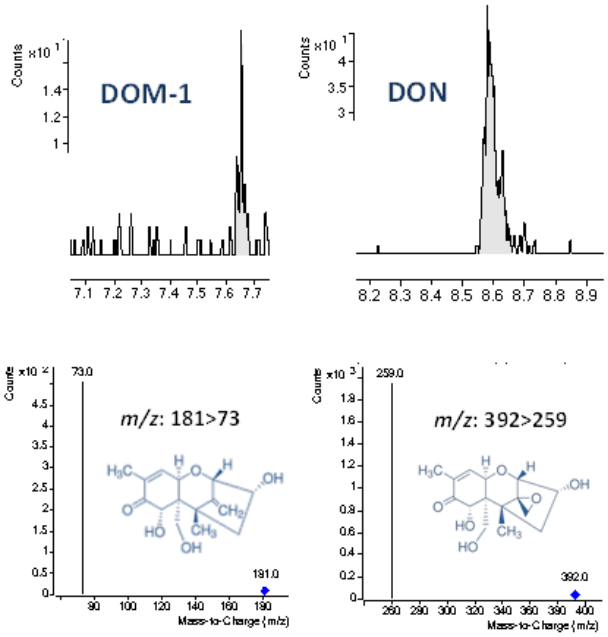
The optimized and validated analytical procedure was used to evaluate the occurrence of the target analytes in 10 children urine samples. Table 4 summarizes the results obtained.

Table 4: Summary of the analytes found in the analyzed urine samples.

Samples	Parameter	Biomarker	
		DOM-1	DON
Urine	Incidence	1/10	3/10
(*n = 10*)	% positive samples	10	30
	Mean positive (µg/g Crea)	1.3	7.4
	Maximum (µg/g Crea)	1.3	21.1

Of the 15 mycotoxins and metabolites detectable by the here proposed method, only two analytes, namely DOM-1 and DON, were quantified in the urine samples assayed. 3 out of 10 urine samples were found positive at least with one mycotoxin. Results showed that DOM-1 was detected in one urine sample at 1.3 µg/g creatinine, whereas DON was quantified in 3 out of 10 urine samples at average concentrations of 7.4 µg/g creatinine. GC-MS/MS chromatograms of one naturally contaminated urine sample in which co-occurred DOM-1 and DON at 1.3 and 21.1 µg/g creatinine, respectively are shown in Figure 3.

Figure 3: GC-MS/MS chromatograms of a child urine sample naturally contaminated with DOM-1 and DON at 1.3 and 21.1 µg/g creatinine, respectively.



3.4. Extrapolation of the DON urinary data

Urinary contamination data can be employed to estimate an exposure assessment approach to contaminants. As an example, urinary DON and DOM-1 levels ($\sum^{\mathrm{DON_{eq\ (\mu g)}}}\!/_{\mathrm{g\ Crea}}$) here reported from one child urine sample were used to assess DON exposure and compared with the established DON provisional maximum tolerable daily intake (PMTDI) set at 1 µg/kg bw [25]. On the basis of an assumed of 1.5 L daily urine volume for

children (V_{urine}), an average body weight of 35 kg and an estimated DON excretion rate (Exc_r) of 72% [26], the amount of DON probable daily intake (DON PDI) were roughly calculated as indicated in the following equation:

$$\text{DON PDI} = \frac{\left[\sum \frac{\text{DON}_{\text{eq (µg)}}}{\text{g Crea}}\right] \cdot \text{CI} \cdot \text{V}_{\text{urine(L)}} \cdot \frac{1}{\text{Exc}_{\text{r (\%)}}}}{\text{body weight (kg)}}$$

where CI is the creatinine index (obtained from 2.7 Section) expressed as g Crea/L urine.

Taken into account the above considerations, a DON probable daily intake of 1.39 µg/kg bw was calculated. Then, a risk characterization, expressed as % PMTDI, was obtained by comparing the DON PDI to DON PMTDI. DON daily intake represented 139% of the established PMTDI which exceeded the tolerable limits suggested by the Scientific Committee on Food [25]. That could indicate a possible health risk scenario. Thus, further studies should focus on bio-monitoring of mycotoxin contamination to properly understand the extent of exposure and to propose intervention strategies to reduce potentially associated health risks.

4. Conclusions

The present study was conducted to produce a sensitive, rapid and accurate method to determine 15 mycotoxins and metabolites in human urine. As urine sampling is non-invasive technique, mycotoxin analysis in this matrix is a promising alternative as exposure biomarker. The determination of the analytes in a GC-MS/MS system working in SRM

mode allowed an accurate determination of even (ultra)trace levels of mycotoxins due to the triple quadrupole MS analyzer. It was, together with the use of an easy and cheap extraction procedure and the benefits from its application, the most important features of this analytical procedure. The method performance fulfilled the EU guideline standardized in the Commission Decision 2002/657/EC and 401/2006/EC. The recoveries of all target analytes were within the acceptable range of 72 – 109% and precision studies conducted at three spiking levels were ≤ 13%. Under the optimized conditions the LOQs were in the range of 0.25 – 8 μg/L. Thus, the analytical strategy optimized in this study represents a reliable tool for rapid quantitation of mycotoxins and their metabolites in urine. The here proposed method was used to evaluate the occurrence of the selected mycotoxins in 10 children urine samples. Occurrence of mycotoxins were detected in 30% of samples.

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3.8. Exposure assessment approach through mycotoxin/creatinine ratio evaluation in urine by GC–MS/MS

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Exposure assessment approach through mycotoxin/creatinine ratio evaluation in urine by GC–MS/MS

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Abstract

In this pilot survey human urine samples were analyzed for presence of 15 mycotoxins and some of their metabolites using a novel urinary multi-mycotoxin GC–MS/MS method following salting–out liquid–liquid extraction. Fifty–four urine samples from children and adults residents in Valencia were analyzed for presence of urinary mycotoxin and expressed by gram of creatinine. Three out of 15 mycotoxins were detected namely, HT-2 toxin, nivalenol and deoxynivalenol (DON). 37 samples showed quantifiable values of mycotoxins. Co–occurrence of these contaminants was also observed in 20.4% of assayed samples. DON was the most frequently detected mycotoxin (68.5%) with mean levels of 23.3 μg/g creatinine (range: 2.8 – 69.1 μg/g creatinine). The levels of urinary DON were used to carry out an exposure assessment approach. 8.1% of total subjects were estimated to exceed the DON provisional maximum tolerable daily intake (PMTDI) (1 μg/kg b.w.). Two out of 9 exposed children exceeded the DON PMTDI thus, making them the most exposed group based on the urinary results.

1. Introduction

Mycotoxins are secondary metabolites produced by toxigenic fungi belonging to various genera such as *Apergillus, Penicillium, Alternaria* and *Fusarium spp*. Mycotoxin can contaminate crop plants and fruits before and after harvesting. In this way, the Food and Agricultural Organization (FAO) reported that over one-quarter of global food and feed crop output are affected by mycotoxins (JECFA, 2001). They are known to have potential adverse effects in humans and animals such as liver cancer, kidney damage, gastrointestinal disturbances, reproductive disorders or suppression of the immune system (Marin et al., 2013). Consequently, many countries have adopted food regulations to limit mycotoxins exposure and to protect consumers from their health risks (EC No 1881/2006; EC No 1126/2007).

Data on the occurrence of the principal mycotoxins on foods and beverages are increasing due to the availability and use of modern and sensitive LC–MS/MS and GC–MS/MS methodologies suitable for simultaneous determination of mycotoxins (Capriotti et al., 2012; Rodríguez-Carrasco et al., 2012). Several studies reported an important occurrence data of some major mycotoxins in the Mediterranean area (Oueslati et al., 2012; Soleimany et al., 2012; Juan et al., 2013; Rodríguez-Carrasco et al., 2014). For instance, the second French total diet study reported a dietary exposure to DON of 0.379 µg/kg bw/day (Sirot et al., 2013) and the latest DON probable daily intake data reported in the SCOOP

task 3.2.10 derived from food analysis in Europe was 0.34 µg/kg bw/day (SCOOP, Directive 93/5/EEC).

Exposure to mycotoxins can also originate from the ingestion of their masked forms (mycotoxins covalently or non-covalently bound to matrix component) that can be released in the gastrointestinal part into parent and bioavailable mycotoxin (Berthiller et al., 2011).

The mycotoxins exposure assessment has been traditionally evaluated based on their occurrence in food products combined by consumption data. The probable daily intake (PDI) of mycotoxins is expressed in µg/kg body weight (bw) and it is compared with the tolerable daily intake (TDI). However, only a certain fraction of the compound reaches the blood stream and will be able to exert toxic effects (Maul et al., 2012).

As a consequence, monitoring of mycotoxins in urine as biomarkers will lead to a more realistic scenario in order to assess the exposure by an easy and non-invasive sampling (Ahn et al., 2010). In addition, it is also possible to detect mycotoxin metabolites in urine samples with different toxic potential as α-zearalanol, ß-zearalanol, ß-zearalenol and α-zearalenol, this last one more estrogenic than zearalenone. DON is also reported as excreted in its unmetabolized form via the urine (Haschek and Voss, 2013). Thus, analytical methodology must be sensitive enough to detect the low levels of mycotoxins and some of their metabolites found.

To date, only a few analytical methods for a simultaneous determination of mycotoxin and their metabolites in human urine have been reported (de Andrés et al., 2008; Ahn et al., 2010; Warth et al., 2012a). For instance, aflatoxin metabolites detected in human urine were related dose dependently to aflatoxin intake (Polychronaki et al., 2008) and urinary deoxynivalenol (free DON + DON metabolites) have also been associated with DON ingestion (Turner et al., 2010a). Urinary occurrence of some mycotoxins, such as DON, has been lately investigated in European countries such as Spain (*n = 27*; 33.3% incidence, levels < LOQ, LOQ = 35 µg/L) (Rubert et al., 2011), Portugal (*n = 13*; 69% incidence, mean levels = 16.3 µg/L) (Cunha and Fernandes, 2012), Italy (*n = 10*; 70% incidence, mean levels = 3.67 µg/L) (Solfrizzo et al., 2011) or United Kindom (*n = 34*; 100% incidence, mean levels = 17.8 µg/L) (Turner et al., 2011).

These indicators could reveal the human mycotoxin exposure level in a more accurate way than food analyses (Wild and Gong, 2010). Furthermore, additive or synergistic interactions of co-occurring mycotoxins should be taken into account as reported by Grenier and Oswald (2011) who reviewed more than 100 studies on mycotoxin interactions. Therefore, continuous monitoring of the extent of mycotoxin contamination is indispensable.

Considering the above described situation, this survey serves as a pilot study to examine the utility of this novel multi-mycotoxin assay by using GC–MS/MS to determine levels of urinary de-epoxydeoxynivalenol

(DOM-1), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), nivalenol (NIV), neosolaniol (NEO), HT-2, T-2, zearalanone (ZAN), α-zearalanol (α-ZAL), ß-zearalanol (ß-ZAL), zearalenone (ZON), α-zearalenol (α-ZOL), ß-zearalenol (ß-ZOL) in 54 urine samples. The amounts of mycotoxins ingested were estimated through urinary mycotoxin/creatinine levels, total urine volume and estimated mycotoxin excretion kinetics previously described in literature (Turner et al., 2010b). Therefore, the aim of this study was to assess the exposure of a group of children and adults inhabitants of the city of Valencia (Spain) to the *Fusarium* toxins above mentioned, by means of its detection in urine as well as by the detection of their metabolites.

2. Materials and methods

2.1. Sampling

Candidates with good physical and psychological health and willingness to provide a written informed consent to participate in the study were kindly welcomed. Exclusion criteria such as any important systemic or psychiatric disorders incompatible with the study were considered. The participants were not subjected to any diet restriction before and during the sampling. Furthermore, all samples were anonymous but participants indicated their weight and gender, male (M) or female (F), whereas urine from children (C) was labeled differently. An approved informed consent was obtained from all volunteers. In case of urine collected from children, the informed consent was signed by their

progenitors. This project was approved by the University of Valencia Institutional human research Committee and the study purposes and procedures were justified and accepted for this study. In total 54 urine samples were collected during April and July 2013. Samples were acquired from a group of 22 male, 16 female and 16 children. Samples were classified by gender and age. Three age groups were considered namely children (from 8 to 14 years old), young adults (from 18 to 28 years old) and adults (>28 years old). All urine samples were collected into sterile plastic vessels. The samples were immediately stored in a freezer (-20°C) until analysis. Samples with undetectable levels of mycotoxins were used for spiking and recovery studies.

2.2. Materials

Mycotoxin standards and metabolites DOM-1, DON, 3-ADON, FUS-X, DAS, NIV, NEO, HT-2, T-2, ZAN, α-ZAL, ß-ZAL, ZON, α-ZOL, ß-ZOL were obtained from Sigma–Aldrich (St. Louis, USA). The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl)acetamide) + TMCS (tri methylchlorosilane) + TMSI (N-trimethylsilylimidazole) (3:2:3) was purchased from Supelco (Bellefonte, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

All solvents, acetonitrile, hexane and methanol (HPLC grade), were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate (thin powder) was obtained from Alfa Aesar GmbH &

Co (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, USA). Picric acid (moistened with water, ≥98%) and creatinine (Crea) standard were supplied by Sigma–Aldrich (St. Louis, USA) whereas sodium hydroxide was acquired from BDH Prolabo – VWR International (Barcelona, Spain).

2.3. Standard preparation

Individual stock solutions of all analytes were prepared at the same concentration (1000 mg/L) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (50 mg/L). All standards were stored in darkness and kept at -20°C until the GC–MS/MS analysis.

2.4. Sample preparation

All urine samples were first centrifuged at 4000 rpm for 5 min. A 10 mL portion of the centrifuged urine was then used for the analysis. 5 mL of acetonitrile were added to the urine samples followed by the addition of 4 g of $MgSO_4$ and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18 and centrifuged for 1 min at 1500 rpm. Finally the extract was evaporated to dryness under nitrogen flow.

The dry extract was added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatized

sample was diluted to 200 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the diluted derivatized sample was added with 1 mL of phosphate buffer (60 mM, pH 7) and the upper layer (hexane phase) was transferred to an autosampler vial for the chromatographic analysis.

2.5. Analytical methods

A GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, USA) were used for MS/MS analysis. Chromatographic separation was achieved on a HP-5MS (5% Phenyl 95% dimethylpolysiloxane phase) 30 m x 0.25 mm x 0.25 µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode at 250°C in programmable temperature vaporization (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially 80°C, and the temperature was increased to 245°C at 60°C/min. After a 3 min hold time, the temperature was increased to 260°C progressively at 3°C/min and finally to 270°C at 10°C/min and then held for 10 min. Chromatographic analysis time was performed in 17 min, which reached the requirement for a high throughput determination. The mass spectrometer operated in electron impact ionization (EI, 70 eV). The source and transfer line temperatures were 230°C and 280°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos

Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent Masshunter version B.04.00 software. Optimized MS/MS parameters and both quantitation and confirmation transitions of the studied analytes are shown in Table 1.

Table 1: GC-MS/MS parameters for the analytes under study.

Analyte	Retention time (min)	Quantitation transition, Q m/z (CE, eV)	Dt (ms)	Confirmation transition, q m/z (CE, eV)	Dt (ms)	Ion Ratio (%)
DOM-1	7.63	181 > 73 (15)	25	391 > 271 (15)	35	41.6
DON	8.60	392 > 259 (10)	25	407 > 197 (10)	25	41.6
3-ADON	9.68	392 > 287 (5)	35	467 > 147 (10)	25	47.5
FUS-X	9.73	450 > 260 (10)	35	450 > 245 (20)	35	11.9
DAS	9.85	350 > 229 (15)	35	378 > 124 (10)	25	56.9
NIV	10.15	289 > 73 (15)	35	379 > 73 (15)	35	29.6
NEO	11.68	252 > 195 (10)	25	252 > 167 (15)	35	40.6
HT-2	14.73	347 > 157 (10)	25	347 > 185 (10)	25	86.7
T-2	14.80	350 > 259 (10)	25	350 > 229 (15)	35	81.9
ZAN	15.15	307 > 235 (15)	25	449 > 335 (10)	25	59.9
α-ZAL	15.45	433 > 309 (20)	35	433 > 295 (20)	35	26.1
ß-ZAL	15.68	433 > 295 (15)	35	307 > 73 (10)	35	82.2
ZON	15.95	462 > 151 (10)	25	462 > 333 (10)	25	76.9
α-ZOL	16.45	305 > 73 (15)	25	305 > 289 (15)	20	12.7
ß-ZOL	16.82	536 > 333 (10)	35	536 > 446 (15)	20	66.1

CE: collision energy; Dt: dwell time

2.6. Method performance

Validation of the analytical method was based on the following criteria: selectivity, linearity, limit of detection (LOD), limit of quantitation (LOQ), precision and accuracy according to SANCO (2011) document. Two

different types of calibration curve were studied: matrix-matched calibration and standard calibration. Eight concentration levels for independent determination for three parallel sets between LOQ and 250 µg/L were employed for linearity evaluation. Matrix-matched calibration curves were built by spiking blank sample extracts with selected mycotoxins at the same concentration levels than standard calibration curves. Linear calibration graphs were constructed by least-squares regression of concentration versus relative peak area of the calibration standards. Quantitation was performed using matrix-matched calibration. The LODs and LOQs were estimated as the lowest matrix-matched calibration level providing signal-to-noise ratios greater than 3 and 10, respectively, at both quantitation and confirmation transitions and matching the intensity ratio established in the standard solution.

For recovery purpose, 10 mL urine blank sample were spiked before determination procedure by the addition of a mixture of standard mycotoxins at 50, 100 and 200 µg/L of each compound. Spiking samples were left to stand overnight. They were then prepared according to the sample preparation method described above. Recovery studies were performed in triplicate in the same day as well as in three different days. Recoveries of spiked samples were calculated from equation below:

$$Rec\ (\%) = {C_{calculated}}/{C_{spiked}}\ x\ 100$$

where $C_{calculated}$ and C_{spiked} are estimated and spiked concentrations.

The repeatability (intra-day precision) and reproducibility (inter-day precision), expressed as the relative standard deviation (RSD), were also calculated. The spiking levels used for precision studies were 50, 100 and 200 µg/L.

2.7. Creatinine analysis

Creatinine urinary levels were determined based on a spectrophotometric method slightly modified (Njumbe Ediage et al., 2012). In summary, 3.5 mM picric acid was reacted with 1000 mM NaOH to form alkaline picrate. This solution was stored in the dark in an amber glass recipient. Alkaline picrate (1 mL) was reacted with 1 mL of diluted urine (1/10, v/v, in ultrapure water). The optical density was measured at 500 nm after 30 min using a Shimadzu mini 1240 spectrophotometer. Mycotoxin urinary concentrations were correlated to the creatinine content of a sample expressed as µg/g Crea.

3. Results and discussion

3.1. Analytical performance

Results of the method performance are shown in Table 2. The obtained results showed signal suppression for all analytes when compared to a pure analytical standard and thus matrix-matched calibration was used to compensate matrix effect for quantitation purpose. A good linearity ($r > 0.990$) for all studied mycotoxins within the tested range was obtained.

Table 2: Results of linearity (coefficient of correlation), limits of detection and quantitation, matrix effect (signal suppression effect in %), trueness (recovery in %), repeatability and intermediate precision (*n = 3*, RSD in %) in spiked urine samples.

Analyte	Linearity (r)	LOD (µg/L)	LOQ (µg/L)	Matrix effect (% SSE)	Spiking level (µg/L)	Intra-assay		Inter-assay	
						Recovery (%)	RSD (%)	Recovery (%)	RSD (%)
DOM-1	0.990	0.25	0.50	20	50	84	2	87	6
					100	92	3	81	8
					200	90	5	91	4
DON	0.996	0.12	0.25	23	50	96	4	93	8
					100	89	6	97	10
					200	93	2	88	9
3-ADON	0.992	0.25	0.50	27	50	92	5	102	6
					100	96	1	94	4
					200	84	9	96	11
FUS-X	0.992	2	4	12	50	95	3	83	6
					100	89	4	94	13
					200	84	6	90	6
DAS	0.998	1	2	35	50	89	4	94	10
					100	94	3	98	11
					200	83	2	95	8
NIV	0.996	0.50	1	6	50	87	3	90	7
					100	82	7	94	7
					200	95	2	85	4
NEO	0.999	0.25	0.50	36	50	93	5	98	5

					100	98	7	106	3
					200	106	7	109	11
					50	96	4	102	10
HT-2	0.999	1	2	28	100	105	5	93	11
					200	102	4	92	8
					50	102	6	96	8
T-2	0.998	0.50	1	8	100	91	1	104	9
					200	89	1	93	6
					50	72	7	77	10
ZAN	0.993	4	8	36	100	75	6	79	11
					200	79	6	80	8
					50	79	5	78	6
α-ZAL	0.998	4	8	28	100	83	9	82	12
					200	74	10	77	8
					50	77	8	75	12
ß-ZAL	0.997	4	8	33	100	74	6	77	9
					200	89	9	83	12
					50	81	5	83	12
ZON	0.991	3	6	23	100	87	3	89	5
					200	96	5	79	8
					50	88	2	98	6
α-ZOL	0.995	1	2	19	100	81	8	80	12
					200	93	8	84	4
					50	80	6	78	9
ß-ZOL	0.991	2	4	14	100	78	7	84	8
					200	83	5	79	9

Recoveries of fortified urine ranged between 72% and 109% at the three assayed concentrations (50, 100 and 200 µg/L). Intra-day precision and inter-day precision were lower than 10% and 13%, respectively at the three assayed spiking levels. LODs from 0.12 to 4 µg/L and LOQs from 0.25 to 8 µg/L for all studied mycotoxins were obtained. The method was sensitive and reliable enough to detect and determine very low levels of the target mycotoxins in human urine samples.

3.2. Results of pilot survey

Table 3 reports the mycotoxins incidence and levels of urine analyzed samples (*n = 54*) as regards age ranges and gender with the exception of children where no gender difference was carried out. Three mycotoxins, namely DON and its metabolite DOM-1, HT-2 and NIV, were quantified in the urine assayed samples. 68.5% of the analyzed samples showed, at least, one mycotoxin. An overall incidence of HT-2 and NIV were measured in 7.4% and 13% of the investigated ones, respectively.

DON and its metabolite DOM-1 were found in 68.5% and 3.7% of the assayed samples, respectively. Results by population groups showed that 56.2% urine samples from children, 75.0% urine samples from young adults and 72.7% urine samples from adults were DON contaminated. Despite its high occurrence, the DON average urinary concentrations were 14.8, 27.8 and 32.9 µg/g Crea for adults, children and young adults, respectively. No significant differences concerning urinary mycotoxins levels between female and male were found.

Table 3: Mycotoxins incidence and levels of analyzed urine samples.

Population group	Parameter	Biomarker			
		DON	DOM-1	NIV	HT-2
Children	Incidence	9/16	1/16	n.d	1/16
(*n* = 16)	% samples (+)	56.2	6.2		6.2
	Mean (µg/g Crea)	27.8	1.3		12.6
	Maximum (µg/g Crea)	84.5	1.3		12.6
Young adults	***Female (n = 6)***				
(*n* = 16)	Incidence	5/6	n.d	2/6	n.d
	% samples (+)	83.3		33.3	
	Mean (µg/g Crea)	41.4		12.7	
	Maximum (µg/g Crea)	69.1		15.2	
	Male (n = 10)				
	Incidence	7/10	1/10	1/10	n.d
	% samples (+)	70.0	10.0	10.0	
	Mean (µg/g Crea)	13.7	4.2	14.4	
	Maximum (µg/g Crea)	34.6	4.2	14.4	
	Overall				
	Incidence	12/16	1/16	3/16	n.d
	% samples (+)	75.0	6.2	18.7	
	Mean (µg/g Crea)	32.9	4.2	13.3	
	Maximum (µg/g Crea)	69.1	4.2	15.2	
Adults	***Female (n = 10)***				
(*n* = 22)	Incidence	7/10	n.d	2/10	2/10
	% samples (+)	70.0		20.0	20.0
	Mean (µg/g Crea)	26.9		16.9	14.5
	Maximum (µg/g Crea)	49.6		17.3	15.8
	Male (n = 12)				
	Incidence	9/12	n.d	2/12	1/12
	% samples (+)	75.0		16.7	8.3
	Mean (µg/g Crea)	15.7		16.5	13.9
	Maximum (µg/g Crea)	59.9		17.6	13.9
	Overall				

	Incidence	16/22	n.d	4/22	3/22
	% samples (+)	72.7		18.2	13.6
	Mean (µg/g Crea)	14.8		16.7	14.3
	Maximum (µg/g Crea)	56.9		17.6	15.8
Overall incidence(%) (*n* = 54)		68.5	3.7	13.0	7.4

The mean urinary levels found in this work were comparable with those reported before in literature. In particular, a study conducted in 13 volunteers from Portugal showed a DON average concentration of 16.3 µg/L in urine (range: 1.9–26.2 µg/L) (Cunha and Fernandes, 2012) whereas a mean concentration of 15.3 µg/L (range: 1.6–30.4 µg/L) is here reported. A similar range was observed by Piekkola et al. (2012) who quantified urinary DON in 63 out of 93 Egyptian pregnant women at mean value of 2.8 µg/g Crea and 2 out of 69 were also DOM-1 positive (0.10 and 0.12 µg/g Crea). Low levels of DOM-1 (range: 0.2–2.8 ng/mL) were also found in 34% of the male farmers (*n = 76*) in Normandy, France, whereas DON was detected in 75/76 urine samples (range: 0.5–28.8 ng/mL) as elucidated Turner et al. (2010a). Recently, DON was detected in 87% urine samples (*n = 53*) at mean values of 11.3 µg/g Crea in a study conducted by Shephard et al. (2013) in Transkei, region of South Africa and in Italy Solfrizzo et al. (2014) quantified DON in 96% of human urine samples (*n = 52*) at mean concentration of 11.89 ng/mL.

3.3. Co-existence of mycotoxins in human urine

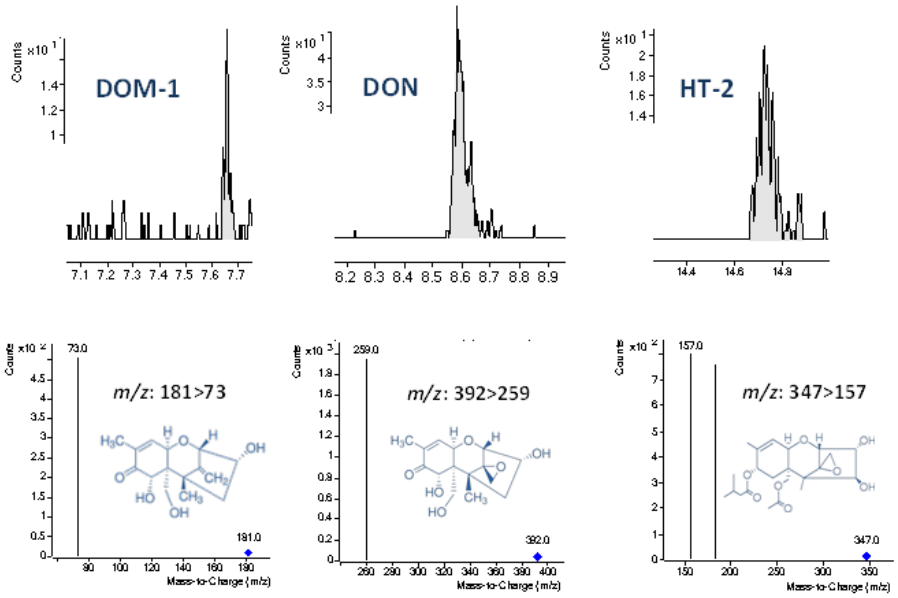
There is an increasing concern about the health hazard derived from exposure of mycotoxin mixtures in humans and animals as a consequence of synergistic and additives effects. Lately, preliminary evidence using *in vitro* models suggests that simultaneous exposure to mycotoxins at low dose showed loss of cell viability whereas single mycotoxin exposure at the same dose were not cytotoxic (Wan et al., 2013). Nonetheless, the nature of combined effect or the relative potencies of the mycotoxins are still not well described.

As regards the samples assayed in this pilot study, 11 out of 54 samples contained more than one mycotoxin (Table 4). Four of the mixtures included DON and HT-2 and 7 had DON and NIV occurring simultaneously. GC–MS/MS chromatograms of naturally contaminated urine sample by HT-2 and DON as well DOM-1 at 12.6, 20.1 and 1.3 μg/g Crea, respectively are shown in Fig. 1.

Table 4: Co-occurrence of mycotoxins in the assayed urine samples.

Co-occurred mycotoxins	Frecuency (%)	Σ mycotoxin content in the highest contaminated sample
DON and HT-2	4/54 (7.4)	27 μ DON g/g Crea + 18.8 μg HT-2/g Crea= 42.8 μg/g Crea
DON and NIV	7/54 (13.0)	34.6 μg DON/g Crea + 17.6 μg NIV/g Crea= 52.2 μg/g Crea

Figure 1: GC-MS/MS chromatograms of a child urine sample naturally contaminated by DOM-1, DON and HT-2 at 1.3, 20.1 and 12.6 µg/g creatinine, respectively.



3.4. Estimated exposure approach through urinary DON levels

Exposure assessment in relation to regulatory recommendations was subsequently conducted based on the assumption that 72% of the dietary intake of DON was excreted in the urine of both children and adults, and daily volumes of urine were 1 L and 1.5 L for children and adults, respectively (Turner et al., 2010b). The DON PDI for each participant was calculated based on the total individual urinary DON (DON + DOM-1 levels) normalized for creatinine here reported taken into account the

creatinine index obtained from Section 2.7 and the body weight provided in the inform consent from each participant.

A DON exposure assessment in young children has been recently published and the average percentage of DON excreted in the urine from children was 74% (Srey et al., 2014). This is in very good agreement with the 72% estimated excretion in UK in adults reported by Turner et al. (2010b), suggesting there is no significant difference between children and adults.

Table 5 presents DON estimated exposure results through the urine samples. Matching the results with the DON PMTDI value, which is set at 1 μg/kg bw, it comes out that the resulting exposures represent a range between 6% and 107% PMTDI. 8.1% of total exposed subjects were estimated to exceed the PMTDI limits suggested by the Scientific Committee on Food (SCF, 2002). In addition, 51.3% of exposed individuals amounted from 50% to 99% of the DON PMTDI. As regards children, 2 out of 9 exposed children exceeded the safety levels. Children are considered susceptible group due to their lower body weight and higher metabolic rate. Comparable results reported by Srey et al. (2014) also highlighted the mycotoxin exposure levels above safety limits in children. One out of 28 adults also exceeded the safety levels based on the exposure estimated throughout the mycotoxin urinary data. A higher exposure to DON was reported by Solfrizzo et al. (2014) who reported that 40% of studied population (*n = 52*) exceeded the DON PMTDI. A similar range was

observed by Warth et al. (2012b) who recently reported exposures data from 38% to 220% DON PMTDI in the Austrian pilot survey carried out in 27 volunteers.

Table 5: Estimation of the DON exposure (expressed as % PMTDI) through urinary levels obtained.

Individuals	[µg DON/L]	[µg DON/g Crea]	% PMTDI
Child 1	15.1	64.2	77
Child 2	19.9	21.1	107
Child 3	24.7	43.9	102
Child 4	22.1	16.2	82
Child 5	23.1	24.1	69
Child 6	18.5	14.8	60
Child 7	13.6	14.9	66
Child 8	6.4	11.9	35
Child 9	14.8	18.9	78
Woman 1	17.3	25.7	55
Woman 2	15.5	25.4	52
Woman 3	26.2	69.1	94
Woman 4	16.8	19.7	52
Woman 5	8.5	21.0	31
Woman 6	17.4	40.9	59
Woman 7	14.7	52.1	49
Woman 8	15.8	49.6	70
Woman 9	6.3	19.8	22
Woman 10	21.8	28.9	72
Woman 11	30.4	40.3	106
Woman 12	1.6	2.8	6
Man 1	10.4	7.0	32
Man 2	12.4	8.0	37
Man 3	5.8	5.1	17
Man 4	8.7	4.8	26
Man 5	23.6	19.3	65

Man 6	7.2	5.0	19
Man 7	11.3	9.0	36
Man 8	5.3	4.2	15
Man 9	27.4	18.1	77
Man 10	12.9	7.2	38
Man 11	11.6	9.7	34
Man 12	3.5	3.3	10
Man 13	18.8	17.4	54
Man 14	21.9	27.0	77
Man 15	17.3	34.6	60
Man 16	17.8	56.9	61

It has to be highlighted that results here reported are based on a DON excretion rate assumption and thus, inter-individual variations, derived from different metabolism activities, should be taken into account. Moreover, the excretion rate may also vary in the same subject.

4. Conclusions

Analysis of mycotoxins in human urine is another important data source for exposure assessment as well as is an easy and non-invasive sampling. In this pilot study, fifty-four human urine samples were analyzed using a gas chromatography–tandem mass spectrometry procedure to evaluate the presence of 15 mycotoxins and metabolites. Occurrence of at least one mycotoxin namely DON, NIV and HT-2 were detected in 68.5% of the assayed samples. Mycotoxin urinary concentrations were related to creatinine rates and used to estimate mycotoxin exposure. The range of urinary levels indicated an exposure similar to previously reported

estimates. Co-occurrence of analytes was also present in 20.4% of samples. The estimated DON probable intake in the present study could imply a health risk, as in the 8.1% cases the DON provisional maximum tolerable daily intake was exceeded. Moreover a range between 50% and 99% PMTDI were estimated in 51.3% of individuals. To date, the potential health effects associated with such chronic exposure to multiple mycotoxins remain unexplored and should be studied further.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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3.9. Preliminary estimation of deoxynivalenol excretion through a 24h pilot study

Toxins, 7 (2015) 705-718

Preliminary estimation of deoxynivalenol excretion through a 24h pilot study

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Keywords: Deoxynivalenol, Duplicate diet study, GC-MS/MS, Urine,

Risk characterization

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Abstract

A duplicate diet study was designed to explore the occurence of 15 *Fusarium* mycotoxins in the 24h-diet consumed by one volunteer as well as the levels of mycotoxins in his 24h-collected urine. The employed methodology involved solvent extraction at high ionic strength followed by dispersive solid phase extraction and gas chromatography determination coupled to mass spectrometry in tandem. Satisfactory results in method performance were achieved. The method's accuracy was in a range of 68 – 108%, with intra-day relative standard deviation and inter-day relative standard deviation lower than 12% and 15%, respectively. The limits of quantitation ranged from 0.1 – 8 μg/Kg. Matrix effect was evaluated and matrix-matched calibrations were used for quantitation. Only deoxynivalenol (DON) was quantified in both food and urine samples. A total DON daily intake was amounted to 49.2 ± 5.6 μg whereas DON daily excretion of 35.2 ± 4.3 μg was determined. DON daily intake represented 68.3% of the established DON provisional maximum tolerable daily intake (PMTDI). Valuable preliminary information was obtained as regards DON excretion and needs to be confirmed in large-scale monitoring studies.

1. Introduction

Cereals are the most important source of food for both direct human consumption and livestock production. In fact, the latest published data by the Food and Agricultural Organization (FAO) reported an annually cereal global consumption (excluding beer) of 146.7 kg/capita [1]. Mycotoxins are secondary fungi metabolites produced in several commodities that could exert toxic effects on animals and humans [2] and mycotoxin contamination of cereals is also frequently reported as public health threat [3, 4]. Acute exposures to mycotoxins are related to gastrointestinal manifestations such as diarrhea, vomiting and melena, while chronic exposures and the most worrisome one are related to degenerative effects on the immune, neural and reproductive systems. Chronic exposure to some mycotoxins are also related to cancer induction [5]. Mycotoxins have also been classified as priority food contaminants by the Global Environment Monitoring System-Food Contamination Monitoring Assessment Programme (GEMS/Food) of the WHO [6]. Moreover, mycotoxins were the main hazards in the European Union with 425 border rejection notifications as highlighted its annual report for 2012, according to the Rapid Alert System for Food and Feed [7].

Among the wide number of mycotoxins, zearalonene and trichothecenes (type A mainly represented by T-2 and HT-2 toxins, and type B by DON) constitute one of the largest groups of mycotoxins produced by *Fusarium* in cereal grains [8, 9]. Mycotoxin production in

agricultural crops can occur at various stages in the food chain like pre-harvest, harvest, drying and storage. In addition, mycotoxins tend to persist during the transformation and processing of contaminated plants and are also still reported in cooked and sterilized food [10].

The European Commision (EC) has set maximum limits for some mycotoxins in foodstuffs [11, 12] and the Joint FAO/WHO Expert Committee on Food Additives (JEFCA) established maximum tolerable daily intakes to ensure food safety [13]. According to the guidelines published by the World Health Organization (WHO), the basic approaches that can be used to determine the intake of a food contaminant include: total diet studies, duplicate diet studies, and selective studies of individual foods, which combine food consumption patterns and contamination level [14]. Duplicate diet studies may be a good alternative to total diet studies, especially when there are important economical limitations to perform a suitable total diet studies. In addition, duplicate diet studies are particularly interesting to consider not only basic cooking methods, but real cooking, something essential when evaluating the dietary intake in specific individuals, countries or regions [15]. A step forward in the individual exposure assessment could be provided by biomarkers measured in biological fluids. The identification of mycotoxins and their main metabolized products in urine could therefore serve as such biomarkers and could facilitate effective exposure assessment [16, 17].

Knowledge on mycotoxins in vivo metabolism in humans has been rarely investigated and it is fundamental to carry out some studies which serve as an approach to assess the exposure. In this line some experiments were designed to provide tentative information about the human in vivo metabolism of major Fusarium mycotoxins. For instance, Mirocha et al., [18] studied the zearalenone metabolite pattern in 24h urine after ingestion of 100 mg zearalenone at once by one volunteer. Similarly, Warth et al., [19] carried out a study to investigate the human deoxynivalenol and zearalenone in vivo metabolism through the analysis of urine samples obtained from one volunteer following a naturally contaminated diet containing 138 µg DON and 10 µg ZON over a period of four days. Thus, this preliminary work was designed as the previously ones to provide basic information about the human in vivo metabolism and serves for the purpose to develop a method which might principally suited as a screening tool. The dietary intake of 15 mycotoxins was studied in a single individual using the duplicate diet approach. All food products as prepared, served and consumed were analyzed. The occurrence of mycotoxins and metabolites was also evaluated in the 24h urine collection and expressed by µg/g of creatinine. The aims of the study were to investigate the daily mycotoxin intake through complete cooked meals, to estimate the mycotoxin urinary excretion, and to carry out a risk characterization approach for the participant.

2. Results and Discussion

2.1. Method performance

Regression equations were obtained using eight standard concentrations on the abscissa and the area of the chromatogram peaks as vertical coordinates. Linear range was tested at eight concentration levels in triplicate from LOQ to 100 µg/kg. Relative standard deviations among the triplicate were below 5% at all calibration curve points. The determination coefficients (R^2) of all analytes were > 0.995. Matrix effect was observed (from 83 to 91%), and thus matrix-matched calibration curves were used for quantification purposes. Apparent recovery for each mycotoxin was determined in composite, beer and urine samples spiked at low and high level (Table 1). Recoveries values obtained, from 68 to 108%, were in agreement with the range set in legislation [20]. Corrections based on recovery percentages were not performed. A precision study was performed by determining the repeatability (intra-day precision) (*n = 6*) and reproducibility (inter-day precision) (*n = 4*), and was fulfilled in conformity with the described criteria in current legislation. Intra-day and inter-day precision were lower than 12 and 15%, respectively in the assayed matrices. The sensitivity of the method was expressed in terms of limits of detection (LOD) and limits of quantitation (LOQ). LOD and LOQ values were calculated from spiked samples chromatograms based on a signal-to-noise ratio of 3:1 and 10:1, respectively (Table 1). LODs varied in the following ranges of 0.6 – 5 µg/kg, 0.05 – 8 µg/kg and 0.1 – 4 µg/kg for

composite, beer and urine respectively. LOQs varied in the following ranges of 1.2 – 10 µg/kg, 0.1 – 16 µg/kg and 0.2 – 8 µg/kg, for composite, beer and urine respectively, which guaranteed quantitation at low ppb-level.

2.2. Deoxynivalenol reduction during cooking

Mycotoxin analyses in pasta (spaghetti) and whole-wheat pasta (little stars) were carried out prior the cooking step and then cooked after the drying process. Analyses were performed in triplicate. The purpose was to evaluate the percentage of mycotoxin reduction during food preparation procedure. Most of the exposure assessment approach to contaminants has been carried out based on uncooked food, and thus assuming some uncertainty in the reported data. In this work, not only cooked meals but also the regular cooking practices and the serving size were taking into account. The aim was not to serve as a representative data of percentage of reduction for wheat-based products but to minimize the uncertainty of the obtained data in order to allow a closer exposure assessment approach

Table 1: Method performance for fifteen mycotoxins and metabolites in composite diet, beer and urine.

Analyte	Composite diet				Beer				Urine			
	REC ± RSD (%)		LOD (µg/kg)	LOQ (µg/kg)	REC ± RSD (%)		LOD (µg/kg)	LOQ (µg/kg)	REC ± RSD (%)		LOD (µg/kg)	LOQ (µg/kg)
	Low level[a]	High level[c]			Low level[b]	High level[c]			Low level[a]	High level[c]		
DOM-1	87 ± 7	93 ± 5	0.6	1.2	73 ± 6	77 ± 8	0.1	0.2	84 ± 2	86 ± 4	0.2	0.5
DON	89 ± 5	91 ± 6	0.6	1.2	75 ± 9	83 ± 9	0.05	0.1	96 ± 4	94 ± 8	0.1	0.2
3-ADON	95 ± 4	90 ± 7	0.6	1.2	82 ± 6	80 ± 5	2	4	92 ± 5	94 ± 5	0.2	0.5
FUS-X	84 ± 5	89 ± 3	2.5	5	98 ± 8	93 ± 9	8	16	95 ± 3	90 ± 6	2	4
DAS	103 ± 3	99 ± 6	2.5	5	78 ± 6	82 ± 5	4	8	89 ± 4	84 ± 8	1	2
NIV	79 ± 6	82 ± 5	1.2	2.5	77 ± 12	81 ± 9	0.5	1	87 ± 3	93 ± 6	0.5	1
NEO	97 ± 8	92 ± 5	2.5	5	83 ± 8	88 ± 6	2	4	93 ± 5	94 ± 5	0.2	0.5
HT-2	93 ± 7	89 ± 8	1.2	2.5	97 ± 9	93 ± 4	2	4	96 ± 4	91 ± 8	1	2
T-2	84 ± 9	90 ± 6	2.5	5	108 ± 7	97 ± 8	4	8	102 ± 6	94 ± 9	0.5	1
ZAN	85 ± 5	90 ± 4	5	10	68 ± 9	73 ± 9	8	16	72 ± 7	74 ± 8	4	8

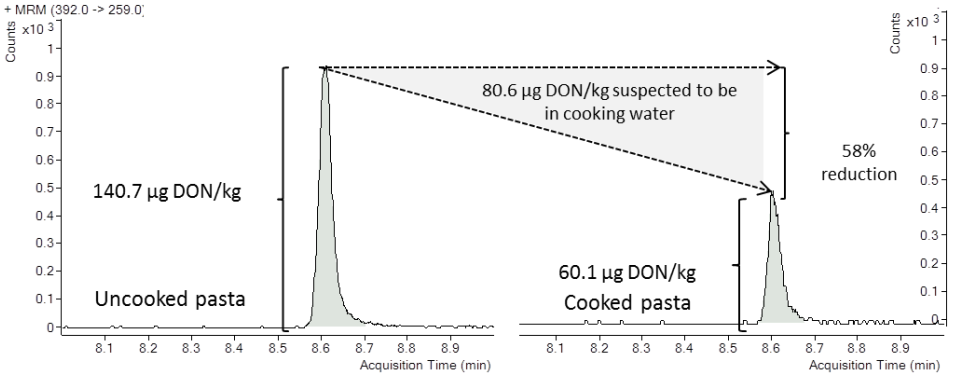
α-ZAL	72 ± 8	79 ± 8	5	10	70 ± 6	78 ± 7	4	8	79 ± 5	82 ± 5	4	8
ß-ZAL	79 ± 6	77 ± 6	5	10	73 ± 8	79 ± 8	4	8	77 ± 8	74 ± 6	4	8
ZON	87 ± 8	84 ± 7	2.5	5	71 ± 5	78 ± 6	8	16	81 ± 5	84 ± 7	3	6
α-ZOL	83 ± 9	80 ± 7	2.5	5	78 ± 6	83 ± 4	2	4	88 ± 2	93 ± 5	1	2
ß-ZOL	77 ± 6	78± 9	2.5	5	74 ± 8	73 ± 8	4	8	80 ± 6	84 ± 9	2	4

[a]Spiking level: 10 µg/kg; [b]Spiking level: 20 µg/kg; [c]Spiking level: 100 µg/kg

A reduction of 13 and 58% was obtained for whole-wheat pasta and pasta, respectively after culinary treatment (Figure 1).

That difference could be attributed to the distinct serving. While the cooking water is removed in spaghetti serving, little stars are consumed as a soup and thus, the amount of mycotoxin intake in little stars was higher than in spaghetti. The percentage of reduction obtained in this work were comparable than those reported Visconti et al., [10] in a larger study showing average DON reduction levels of 40%. Moreover, they indicated that the amount of DON retained by cooked pasta consistently decreased by increasing the pasta/water ratio during cooking.

Figure 1. SRM chromatograms of the naturally DON contaminated pasta before and after culinary treatment at 140.7 µg/kg and 60.1 µg/kg, respectively, on a dry weight basis, and percentage of mycotoxin reduction.



2.3. Deoxynivalenol content in food

All analyzed meals were wheat-based related. Within the 15 mycotoxins targeted in the proposed procedure only DON was detected in the analyzed food commodities. This high DON prevalence was highlighted before in cereal and their derivative products by several authors [5] as well as in urine samples [21]. Quantified DON (*n = 3*) in the different food items are presented in Table 2. The food items presenting the highest concentrations of DON were whole-wheat pasta and toasts with average values of 272.4 and 190.6 µg/kg, respectively. Despite beer was the matrix with the lowest DON content, it was the food item with the highest significant contribution to DON intake (28.6 µg) because of the consumption data (0.83L). A total DON daily intake throughout the consumption of the followed diet was estimated in 49.2 ± 5.6 µg. Mean

DON contents of the studied food matrices were in line with results reported by other authors [22-25]. The estimated levels of mycotoxins in the composite are also presented in Table 2. DON was quantified at mean level of 120.5 ± 7.3 µg/Kg. A mean DON intake of 19.9 µg was obtained throughout the solid food items consumed. The composite's result was in line with the 20.5 µg of DON obtained from the sum of each food item. Thus, composite could be presented as an alternative tool to reduce sampling and analysis cost. However, the resulting information concerning contaminants in each selected food item will be missed.

2.4. DON content in urine

Quantifiable amounts of mycotoxins, and its toxin derivatives that result from its biotransformation, are expected to be found in urine. For instance, DON can be metabolized within the intestinal lumen by gut microbiota, generating the less toxic de-epoxy metabolite known as DOM-1. Further metabolism of DON and ZON to a less toxin metabolite involves the addition of glucuronic acid, catalyzed by UDP-glucuronyltransferase [26, 27]. As regards mycotoxin conjugation, the uncertainness exists, since it has been related individual difference in the enzymatic system. On the other hand, Turner et al., [28, 29] suggested that un-conjugated DON can also persist and it can be excreted in urine. In this line, this work was focused on the investigation of un-metabolized DON as a preliminar step since it needs to be extended in the future to understand

the relation between the mycotoxin intake and mycotoxin levels in urine, both metabolized and un-metabolized fractions.

Table 2. Overview of contamination level, consumption data and mycotoxin intake contribution of the food items consumed.

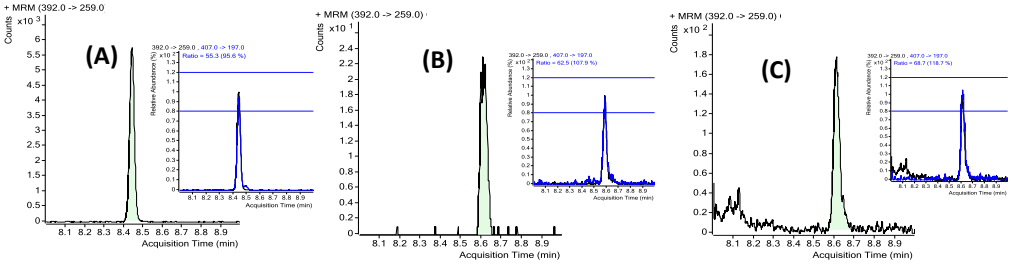
Time of consumption	Food	Consumption (g/day)	Mean DON ± SD (µg/kg)	Mean DON intake (µg)
8 am	Toast	45	190.6 ± 2.3	8.7
11 am	Breadsticks	30	49.7 ± 4.6	1.5
2 pm	Pasta	67[a]	58.2 ± 2.7	3.9
7 pm	Wheat beer	500	36.4 ± 1.8	18.1
8 pm	Beer	330	32.1 ± 6.2	10.5
10 pm	Whole-wheat pasta	24[a]	272.4 ± 5.9	6.5
				Σ DON Intake: 49.2 µg
	Composite[b]	166	120 ± 7.3	19.9 µg

[a]on a dry weight basis; [b]no beverages included

Analyses in urine carried out by other authors have revealed the occurrence of DON in a high incidence of samples. For instance, Gratz et al., [30] reported an incidence of DON in all analyzed samples (*n = 54*). Similarly, DON incidences in urine samples of 33.3% (*n = 27*) and 67.6% (*n = 34*) were reported by Rubert et al., [31] and Turner et al., [21], respectively. In this work, a total of 1.87 L urine was collected as 24h urine volume and was in the normal excretion range according to sex and age

[32]. DON was quantified at 17.5 ± 2.7 µg/g creatinine (*n = 3*) (equivalent to 18.8 ± 3.5 µg/L). No other mycotoxin was found in the urine sample being according with the results reported by Rubert et al., [31] and Warth et al. [33] who did not find neither any other trichothecenes nor zearalenone in the analyzed urine samples. GC-MS/MS chromatograms of naturally DON contaminated composite, beer and urine are shown in Figure 2. Urinary free DON levels of 18.8 ± 3.5 µg/L (equivalent to 35.2 ± 4.3 µg DON) was calculated in this study. Data found in literature were very similar. For instance, DON average contents of 20.4 µg /L were reported in an Austrian survey [27] (*n = 27*; incidence of 22%) and a range from 0.5 to 28.8 µg/L were reported in a French study (*n = 76*; incidence of 98.7%) [26].

Figure 2. (a) SRM chromatograms of the naturally contaminated composite; (b) wheat-beer and (c) urine by deoxynivalenol at 122.6 µg/kg, 37.2 µg/kg and 20.8 µg/L, respectively.



2.5. Exposure estimates

On the basis of the calculated data, DON daily intake was compared with the established PMTDI of 1 µg/kg bw [13]. The dietary

exposure level to DON estimated in the present study was 0.683 µg/kg bw day, lower than the value set by JECFA. Comparable values as regards dietary exposure to DON were reported in the second French total diet study [34] (mean: 0.379 µg/kg bw day) as well as in a duplicate diet study carried out in Dutch young children (mean: 0.66 µg/kg bw day) [35]. These results are also within the latest DON probable daily intake data (0.34 µg/kg bw day) reported in the SCOOP task 3.2.10 derived from food analysis in Europe [36] and that reported by FAO/WHO (1.4 µg/kg bw day) [37].

3. Experimental Section

3.1. Materials

Mycotoxin standards and metabolites namely de-epoxy deoxynivalenol (DOM-1), DON, 3-acetyldeoxynivalenol (3-ADON), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), nivalenol (NIV), neosolaniol (NEO), HT-2, T-2, zearalanone (ZAN), α-zearalanol (α-ZAL), ß-zearalanol (ß-ZAL), zearalenone (ZON), α-zearalenol (α-ZOL), ß-zearalenol (ß-ZOL) were obtained from Sigma-Aldrich (St. Louis, USA). The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl)acetamide) + TMCS (trimethyl chlorosilane) + TMSI (N-trimethylsilylimidazole) (3:2:3) was purchased from Supelco (Bellefonte, USA). Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

Certified reference material BRM 003004 (artificially contaminated wheat, DON 1062 ± 110 µg/kg) was purchased from Biopure Referenzsubstanzen GmBH (Tulln, Austria).

All solvents, acetonitrile, hexane and methanol (HPLC grade), were purchased from Merck KGaA (Darmstadt, Germany). Anhydrous magnesium sulfate (thin powder) was obtained from Alfa Aesar GmbH & Co (Karlsruhe, Germany); sodium chloride was purchased from Merck and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, USA). Picric acid (moistened with water, ≥98%) and creatinine standard were purchased from Sigma-Aldrich (St. Louis, USA) whereas sodium hydroxide was acquired from BDH Prolabo – VWR International (Barcelona, Spain).

3.2. Standard Preparation

Individual stock solutions of all analytes were prepared at the same concentration (1000 mg/L) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (50 mg/L). All standards were stored in darkness and kept at -20°C until the GC-MS/MS analysis. Calibration function of both neat solvent standards and spiked samples were established by plotting peak areas versus analyte concentrations in the measured solutions and performing linear regression. Linear range was tested from 0.1 – 100 µg/kg by spiking at eight concentration levels. In order to reveal the presence of matrix effect, matrix-matched calibration prepared by spiking extracts of blank samples with mycotoxins at similar

concentrations than the calibration built in neat solvent without any matrix were compared. The slopes of the resulting linear calibration functions were compared and the signal suppression/enhancement due to matrix effects (ME) was determined as follows:

$$\boldsymbol{ME}\ (\%) = \frac{\boldsymbol{Slope}_{\boldsymbol{matrix-matched\ calibration}}}{\boldsymbol{Slope}_{\boldsymbol{standard\ in\ solvent}}}\ \boldsymbol{x}\ \mathbf{100}$$

3.3. Study design and sampling

In this 24h preliminary study, a farinaceous-based diet was designed and conducted by a 26 years old, healthy male volunteer. Duplicate meals as prepared, served and consumed, based on a "duplicate plate" method were provided for subsequent individual analysis in sterile plastic food containers and kept with ice packs in a cooler until they were returned to the laboratory. The selection of food items analyzed in the present study was based on two criteria: first, the food must be identified by GEMS/Food as potential sources of mycotoxins and second, their level of consumption must exceed 1 g/person day. Four complete meals were consumed during the day of the study as well as two beers in the afternoon. The food groups items selected were bread, wholegrain cereals, pasta and wheat or barley-based beer. Food items such as pasta (spaghetti) and whole-wheat pasta (little stars) were cooked for 7 min in boiling water. A pasta/water ratio of 1:5 (w/v) was respected as recommended the

preparation mode established in the food packaging labels. Salt and spices were also added as in regular cooking practices.

Upon arrival at the laboratory all meals were weighted and dried in an oven at 100 ± 4°C to reach constant weight for subsequent mycotoxin analysis. The dried products obtained were thoroughly grounded and homogenized using a laboratory mill and kept at 4°C under dark and dry conditions into a specific plastic food containers. Note that once dried and milled, all dried products were wheat flour-related foods. For the cooked pasta-based products, both dry cooked form and the dry uncooked ones were subjected to mycotoxin analysis to evaluate the percentage of mycotoxin reduction during the cooking.

Urine sample was collected as 24h urine throughout the day of the study due to it was demonstrated that the main part of absorbed *Fusarium* toxins showed a rapid elimination within 24h after ingestion [38]. Urine collected was stored at -20°C until analysis. The 24h period lasted from 8 am to 8 am on the next day to include the first morning urine. A written and approved informed consent was obtained from the volunteer. This project was approved by the University of Valencia Institutional human research Committee and the study purposes and procedures were justified and accepted for this study.

3.4. Composite diet sample

The composite diet was intended to be representative of 24h duplicate diet collected and included all food items consumed over the

monitoring period, without beverages. The dried food items were briefly homogenized, carefully mixed, and finally combined keeping the diet proportions.

3.5. Sample preparation

Composite and individual meals were analyzed as described in detail elsewhere [39] in order to know the contribution of each food item. In brief, 5 g of homogenized sample was added to 25 mL distilled water and 7.5 mL of acetonitrile followed by the addition of 4 g of $MgSO_4$ and 1 g of NaCl prior to be shaken vigorously and centrifuged for 3 min at 4000 rpm. Then the upper layer was submitted to a dispersive solid phase extraction (d-SPE) with a mixture of 900 mg of $MgSO_4$ and 300 mg of C18 and centrifuged for 1 min at 1500 rpm. After centrifugation the liquid extract was separated from the solid salts and finally the extract was evaporated to dryness under nitrogen flow.

Beer samples were first completely degased by sonication for 15 min prior the analysis. A 10 mL portion was then used for the analysis. 5 mL of acetonitrile were added to the sample followed by the addition of the mixture of salts ($MgSO_4$ and NaCl) and then submitted to a d-SPE as previously described.

Urine sample was first centrifuged at 4000 rpm for 5 min. A 10 mL portion of the centrifuged urine was then used for the subsequent analysis as indicated above.

All dry extracts were added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the samples were left for 30 min at room temperature. The derivatized samples were diluted to 200 µL with hexane and mixed thoroughly on a vortex for 30 s. Then the diluted derivatized samples were added with 1 mL of phosphate buffer (60 mM, pH 7) shaken and the upper layers (hexane phases) were transferred to autosampler vials for the chromatographic analysis.

For quality control, certified reference material BRM 003004 (artificially contaminated wheat, DON 1062 ± 110 µg/kg) was used and included. Certified reference material was used as provided without further grinding. It was stored under the same conditions, extracted and determined with the same protocol as the analyzed samples. Each sample was analyzed in triplicate and measured in separate batches.

3.6. GC-MS/MS method

A GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, USA) were used for MS/MS analysis. Chromatographic separation was achieved on a HP-5MS 30 m × 0.25 mm × 0.25 µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode (equivalent to 25 mg of dried food matrix) at 250°C in programmable temperature vaporization (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially 80°C, and the

temperature was increased to 245°C at 60°C /min. After a 3 min hold time, the temperature was increased to 260°C at 3°C /min and finally to 270°C at 10°C /min and then held for 10 min. Chromatographic analysis time was performed in 17 min, which reached the requirement for a high throughout determination [39].

The mass spectrometer operated in electron impact ionization (EI, 70 eV). The source and transfer line temperatures were 230 and 280°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent Masshunter version B.04.00 software [39].

3.7. Calculation of DON daily intake

A deterministic approach was applied for the calculation of DON dietary exposure. The volunteer filled a 1-day food consumption record and was asked to provide his body weight (bw: 72 kg). DON daily intake, expressed as µg DON/kg bw, was calculated by combining food consumption (g foodstuff/kg bw day) with DON food contamination (µg DON/g foodstuff) data. The contribution of each food to the average dietary exposure was also calculated.

3.8. Creatinine analysis

Creatinine urinary levels were determined based on a spectrophotometric method slightly modified [40]. In summary, 3.5 mM

picric acid was reacted with 1000 mM NaOH to form alkaline picrate. This solution was stored in the dark in an amber glass recipient. Alkaline picrate (1 mL) was reacted with 1 mL of diluted urine (1/10, v/v, in ultrapure water). The optical density was measured at 500 nm after 30 min using a Shimadzu mini 1240 spectrophotometer. Mycotoxin urinary concentrations were correlated to the creatinine content of a sample expressed as μg/g creatinine.

4. Conclusions

A total DON daily intake derived from the 24h duplicate diet study amounted to 49.2 ± 5.6 μg whereas 35.2 ± 4.3 μg of DON were quantified in the urine collected in the same. DON incidence in urine of the participant confirms his exposure to DON and evidence the usefulness of DON and its metabolites in urine as biomarker of exposure to such contaminants. The values of DON PDI estimated herein with the urinary biomarker approach matched quite well the intake derived from food analysis. DON data was further correlated to the established DON PMTDI value in order to obtain a risk characterization approach. DON daily intake represented a 68.3% of the established PMTDI. The obtained data from this preliminary study is subjected to intra- and inter-day variations. Therefore, this experiment needs to be extended to a larger group of individuals to investigate these variations and to elucidate the relation between ingested mycotoxins and excreted ones and their corresponding metabolites in humans. In this

sense, the *in vitro* digestibility/metabolic models are very useful to complete the full-scale metabolism studies.

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Conflicts of Interest

The authors declare no conflict of interest.

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3.10. Mycotoxin multi-biomarker method in human urine based on salting-out liquid-liquid extraction and gas chromatography-tandem mass spectrometry

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Mycotoxin multi-biomarker method in human urine based on salting-out liquid-liquid extraction and gas chromatography-tandem mass spectrometry

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Dispersive liquid-liquid microextraction, Urine, GC-MS/MS

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Abstract

Simple, highly efficient and rapid sample preparation procedures namely dispersive liquid-liquid microextraction (DLLME) and salting-out liquid-liquid extraction (SALLE) for the analysis of 10 *Fusarium* mycotoxins and metabolites in human urine were compared. Various parameters affecting extraction efficiency such as type and volume of extraction and disperser solvents, salt addition and extraction time were evaluated. Under the optimum extraction conditions, SALLE-based procedure showed better results than DLLME. Hence, a multi-biomarker method based on SALLE followed by gas chromatography-tandem mass spectrometry determination (GC-QqQ-MS/MS) was proposed. Satisfactory results in terms of validation were achieved. The method resulted in low limits of detection and quantitation (LOQ) within the range of 0.12 - 4 µg/L and 0.25 - 8 µg/L, respectively. The method accuracy and precision was evaluated at three spiking levels (8, 25 and 100 µg/L) and the recoveries were in a range from 70 – 120% with relative standard deviations lower than 15%. Matrix effect was evaluated and matrix-matched calibrations were used for quantitation purpose. The methodology developed was successfully applied to determine mycotoxins human urine samples (*n = 12*) and total deoxynivalenol (free + conjugated) was found in 83% samples at average concentration of 31.6 µg/L.

1. Introduction

Mycotoxins are considered toxic substances affecting as much as one-quarter of global food and feed crop output [1]. The main classes of *Fusarium* mycotoxins with respect to production and animal health are trichothecenes such as deoxynivalenol (DON) and T-2 toxin, fumonisins and zearalenone (ZON). In animals, DON toxicological effects mainly concern the immune system and the gastrointestinal tract whereas prolonged dietary ZON exposure include carcinogenicity, genotoxicity, reproductive toxicity, endocrine effects and immunotoxicity [2]. Despite that, IARC carncinogenic evaluation of DON and ZON concluded that they are not classifiable regarding their carcinogenicity to humans (Group 3) [3].

Exposure to mycotoxins has been traditionally assessed based on occurrence data combined with consumption data [4]. However, only a certain fraction of the compound reaches the blood stream and will be able to exert toxic effects, and thus biomarkers have been proposed as suitable targets to assess the exposure to mycotoxins [5]. Factors such as food consumption, exposure routes, diet composition, food preparation techniques, metabolism and excretion are taken into account when assessing the exposure *via* biomarkers. The parent toxins themselves and/or major phase I and phase II metabolites (e.g. glucuronide conjugates) are the typical biomarkers of exposure which are measured in biological fluids such as urine or plasma/serum [6]. Urine is often the

preferred sample for screening because large amounts can be easily and non-invasively collected.

In pigs and probably in humans, ZON is rapidly absorbed after oral administration and can be metabolized in intestinal cells and further degraded into α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL [7]. DON can be metabolized within the intestinal lumen by gut microbiota, generating the less toxic de-epoxy metabolite known as DOM-1. Further metabolism of DON and ZON to more hydrophilic metabolites involves addition of glucuronic acid, catalyzed by UDP-glucuronyltransferase [8]. Glucuronidation enhances water solubility facilitating the excretion by urine. The β-glucuronidase pretreatment allows the combined measure of urinary unmetabolized or free mycotoxin and the glucuronide ones in a single step [9]. This combined measure is suggested as a putative exposure biomarker. Consequently, the development of rapid multi-biomarker methods to assess human exposure to mycotoxins are highly desirable [10].

Sample preparation procedures such as liquid-liquid extraction (LLE) [11], solid-phase extraction (SPE) [12], immunoaffinity columns (IAC) [13] or QuEChERS [14] have been reported in literature for determining mycotoxins in urine yielding in general satisfactory results. More recently, dispersive liquid-liquid microextraction (DLLME) [15] as well as salting out liquid-liquid extraction (SALLE) [16] have also been reported as a good

alternative to conventional sample preparation techniques offering simplicity, cost- and time-efficient, and overall increase throughput.

DLLME is a miniaturized liquid extraction developed by Rezaee et al., (2006) [17]. An appropriate mixture of extraction and disperer solvent is rapidly injected into the aqueous sample containing analytes and a cloudy dispersion of droplets of the extraction solvent is produced. Droplet formation enhances the effective surface contact area between the organic and the aqueous phase, and the extraction equilibrium is reached rapidly. The SALLE method combines water and a water-miscible organic solvent by adding an electrolyte. It involves reducing the solubility of a non-electrolyte in a water-miscible phase, and thus separating it, followed by simultaneous extraction of the target solute into the separated miscible organic phase [18].

In this study, DLLME and SALLE were developed and compared for the urinary determination of DON, ZON and their major metabolites namely 3-ADON, 15-ADON, DOM-1 and α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL, respectively. Critical parameters affecting extraction efficiency were carefully evaluated. Coupled to an optimized GC-QqQ-MS/MS method, the proposed procedure was validated and successfully applied to measure the concentrations of free and conjugated mycotoxins in human urine after enzymatic treatment.

2. Material and methods

2.1. Chemicals and reagents

Mycotoxin standards and metabolites DON, $^{13}C_{15}$-DON, DOM-1, 3-ADON, 15-ADON, ZON, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL, were obtained from Sigma–Aldrich (St. Louis, USA). The derivatization reagent composed of BSA (N,O-bis(tri methylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilyilimidazole) (3:2:3) was purchased from Supelco (Bellefonte, USA).

Sodium dihydrogen phosphate and disodium hydrogen phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain). All solvents, acetonitrile, hexane, methanol, chloroform, ethyl acetate and dichloromethane were purchased from Merck KGaA (Darmstadt, Germany). Acetic acid (glacial) was of analytical grade obtained from Panreac (Barcelona, Spain). Sodium chloride and ammonium acetate were all analytical grade and obtained from Merck KGaA (Darmstadt, Germany) and C18-E (50 µm, 65 A) was purchased from Phenomenex (Torrance, USA). Picric acid (moistened with water, P98%) and creatinine (Crea) standard were supplied by Sigma–Aldrich (St. Louis, USA) whereas sodium hydroxide was acquired from BDH Prolabo – VWR International (Barcelona, Spain).

β-Glucuronidase Type H-1 from Helix pomatia (≥3,000,000 U/g solid glucuronidase and ≥ 10,000 U/g solid sulfatase) was purchased from Sigma–Aldrich (St. Louis, USA).

Individual stock solutions of all analytes were prepared at the same concentration (20 mg/L) in methanol. The stock solutions were diluted with acetonitrile in order to obtain the appropriate multi-compounds working standard solutions (2 mg/L). All standards were stored in darkness and kept at -20°C until the GC–MS/MS analysis.

2.2. GC-MS/MS analysis

A GC system Agilent 7890A coupled with an Agilent 7000A triple quadrupole mass spectrometer with inert electron-impact ion source and an Agilent 7693 autosampler (Agilent Technologies, Palo Alto, USA) were used for MS/MS analysis. Chromatographic separation was achieved on a HP-5MS (5% Phenyl 95% dimethylpolysiloxane phase) 30 m x 0.25 mm x 0.25 µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode at 250°C in programmable temperature vaporization (PTV) inlet employing helium as carrier gas at fixed pressure of 20.3 psi. The oven temperature program was initially 80°C, and the temperature was increased to 245°C at 60°C/min. After a 3 min hold time, the temperature was increased to 260°C progressively at 3°C/min and finally to 270°C at 10°C/min and then held for 10 min. Chromatographic analysis time was performed in 17 min, which reached the requirement for a high throughput determination. The mass spectrometer operated in electron impact ionization (EI, 70 eV). The source and transfer line temperatures were 230°C and 280°C, respectively. The collision gas for MS/MS experiments was nitrogen, and the helium was

used as quenching gas, both at 99.999% purity supplied by Carburos Metálicos S.L. (Barcelona, Spain). Data was acquired and processed using the Agilent Masshunter version B.04.00 software. Optimized MS/MS parameters and both quantitation and confirmation transitions of the studied analytes are shown in Table 1. MATLAB R2013a (Mathworks Corporation, USA) was employed to develop the response surface.

Table 1: MS/MS parameters of the studied mycotoxins and metabolites

MS/MS	DON	$^{13}C_{15}$-DON	DOM-1	3-ADON	15-ADON
MRM (Q)	392>259	437>300	181>73	392>287	392>217
CE (V)	*10*	*15*	*15*	*5*	*10*
Dt (ms)	*25*	*25*	*25*	*35*	*25*
MRM (q)	407>197	437>284	391>271	467>147	392>184
CE (V)	*10*	*15*	*15*	*10*	*15*
Dt (ms)	*25*	*35*	*35*	*25*	*35*
Ion ratio	42 ± 3	45 ± 5	10 ± 3	48 ± 12	43 ± 9

MS/MS	ZON	α-ZOL	β-ZOL	ZAN	α-ZAL	β-ZAL
MRM (Q)	462>151	305>73	536>333	307>235	433>309	307>292
CE (V)	*10*	*15*	*10*	*15*	*20*	*15*
Dt (ms)	*25*	*25*	*35*	*25*	*35*	*35*
MRM (q)	462>333	305>289	536>446	449>335	433>295	307>277
CE (V)	*10*	*15*	*15*	*10*	*20*	*10*
Dt (ms)	*25*	*20*	*20*	*25*	*35*	*35*
Ion ratio	77 ± 4	13 ± 4	66 ± 8	60 ± 7	26 ± 5	80 ± 12

Q: Quantification transition; q: confirmation transition; Ion ratio: Q/q ± RSD

The script was run in Windows 2010 on a personal computer. Student's *t*-teest statistical analysis was performed for data evaluation; *p* values < 0.05 were considered significant.

2.3. Sample collection

Candidates with good physical and psychological health and willingness to provide a written informed consent to participate in the study were kindly welcomed. The participants were not subjected to any diet restriction before and during the sampling. Furthermore, all samples were anonymous but participants indicated their weight and gender, male (M) or female (F). This project was approved by the University of Valencia Institutional human research Committee and the study purposes and procedures were justified and accepted for this study. In total 12 urine samples were collected during November 2014. Samples were acquired from a group of 6 males and 6 females. All urine samples were collected into sterile plastic vessels. The samples were immediately stored in a freezer (-20°C) until analysis. Samples with undetectable levels of mycotoxins were used for spiking and recovery studies.

2.4. Sample preparation

2.4.1. Enzymatic hydrolysis

All urine samples were first centrifuged at 4000 rpm for 5 min at 4°C. Then a 1 mL aliquot was placed into a 2mL Eppendorf tube and spiked with 50 µL of $^{13}C_{15}$-DON at 2 µg/mL (to achieve 100 µg/L). One mL of urine

was added with 250 µL ammonium acetate buffer (1 M, pH 5.0) containing 20000 U of β-glucuronidase/mL. The hydrolysis was allowed to proceed for 18 h at 37°C with continuous shaking at 550 rpm [12].

2.4.2. SALLE procedure

Digested samples were centrifuged at 10000 rpm for 10 min at 4°C and the upper layer was placed into a 15 mL screw cap test tube with conical bottom. Then 1 mL of acetonitrile and a mixture of 0.3 g sodium chloride and 30 mg of C18 sorbent were added. The solution was vortexed for 30s and centrifuged at 4000 rpm for 3 min at 4°C. Finally, the upper layer was collected and evaporated to dryness under gentle nitrogen flow.

The extraction of free mycotoxins was carried out as described here with the exception of enzymatic hydrolysis.

2.4.3. DLLME procedure

One mL of centrifuged urine was placed into a 15 mL screw cap test tube with conical bottom. The mixture of 1 mL of acetonitrile and 100 µL of $CHCl_3$ was added quickly into the tube and the mixture emulsified, forming cloudy solution. The mixture was vortexed for 1 min and centrifuged at 4000 rpm for 5 min at 4°C to sediment the $CHCl_3$. The sedimented phase was removed using a 1 mL syringe, transferred to a vial and evaporated to dryness under a gentle stream of nitrogen.

2.5. Derivatization

The dry extract was added with 50 µL of BSA + TMCS + TMSI (3:2:3) and the sample was left for 30 min at room temperature. The derivatized sample was diluted to 200 µL with hexane and mixed thoroughly on a vortex for 30s. Then the diluted derivatized sample was added with 1 mL of phosphate buffer (60 mM, pH 7) and the upper layer (hexane phase) was transferred to an autosampler vial for the GC-MS/MS analysis [19].

3. Results and discussion

In residue analysis methods sample preparation is a critical step, especially when the concentration of the studied analytes is at the µg/L levels. Therefore two sample preparation procedures (DLLME and SALLE) were tested and compared to extract mycotoxins and metabolites from human urine samples. All experiments were performed in triplicate.

3.1. Optimization of DLLME

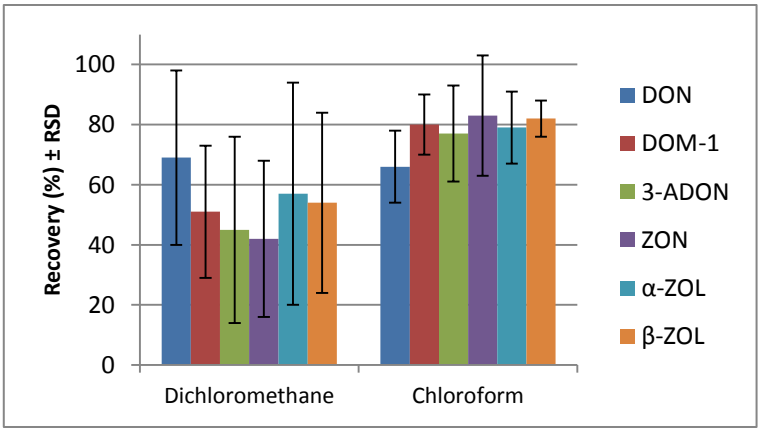
In order to find the appropriate conditions for DLLME, different experimental parameters were studied and optimized such as effect of extraction solvent and its volume, effect of disperser solvent and its volume, effect of salt addition and time of extraction.

3.1.1. Influence of the extraction solvent: type and volume

The ability of several higher density solvents with different polarities namely dichloromethane (CH_2Cl_2, density 1.32 g/mL) and

chloroform ($CHCl_3$, density 1.49 g/mL), to extract the studied mycotoxins was compared. For considering the influence of the solvent type on the extraction efficiency, a fixed volume of acetonitrile (1 mL) and 100 µL of extraction solvents were used. Figure 1 shows that chloroform was the most efficient solvent based on the accuracy and precision data.

Figure 1: Effect of the extraction solvent type on the extraction efficiency. Extraction conditions: concentration of analytes, 100 µg/L; volume of disperser solvent (acetonitrile), 1 mL; volume of dichloromethane and chloroform, 100 µL; no salt addition.



For considering the influence of the solvent volume on the extraction efficiency, different volumes of chloroform (from 60 to 120 µL with gaps of 20 µL) were evaluated. Lower volumes enhance the enrichment factor of the DLLME process, although reducing the volume of the sedimented phase. Figure 2 describes the MATLAB-based optimization procedure graphically. When chloroform was increased from 60 to 100 µL,

the recoveries of the mycotoxins were increased from 48 – 59% to 76 – 86%. Nonetheless, extraction efficiency was decreased by using the highest volume of chloroform. Thus, 100 µL of chloroform was selected as the optimum volume.

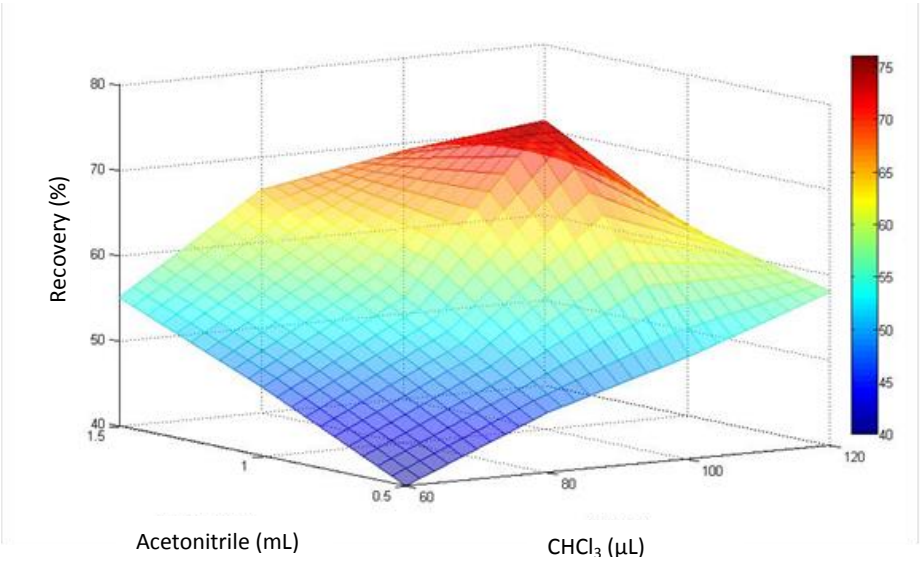
3.2.2. Influence of the disperser solvent volume

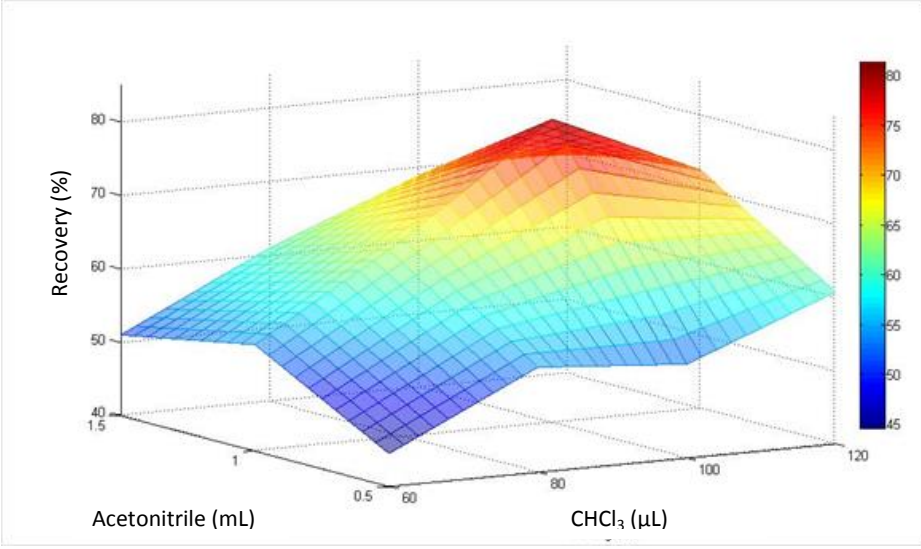
The miscibility of the disperser solvent with the extraction solvent and aqueous solution is a critical factor in DLLME. Although acetone, methanol and acetonitrile (with the polarity index of 5.1, 5.1 and 5.8, respectively) show adequate properties as disperser solvents, acetonitrile was selected with regard to its more close polarity of that with water. The effect of the acetonitrile volume on the extraction efficiency was investigated over the range of 0.5 – 1.5 mL in the interval of 0.5 mL. The results indicate that the extraction efficiency reached an optimum value at 1.0 mL (recovery range from 75 to 89%), but decreased by using the highest volume of disperser solvent (Figure 2). Hence, 1.0 mL of acetonitrile was chosen to achieve the best extraction efficiency.

3.2.3. Influence of salt addition

Adding of salt may have different results in DLLME like increasing [20], reducing [21] or no remarkable effect on the extraction efficiency [22]. To evaluate the effect of salt addition on DLLME performance, various experiments were carried out by adding different amounts of sodium chloride (from 0 to 0.3 g).

Figure 2: Response surfaces (expressed as recovery) of (A) DON and (B) mean trend of all analytes regarding influence of extraction ($CHCl_3$) and disperser (acetonitrile) solvent volumes.





The results showed that no significant statistical differences for a confidence interval of 95% were found in presence or absence of salt and therefore, salt was not added in further experiments.

3.2.4. Selecting the extraction time

Once the extraction and disperser solvents are mixed and rapidly injected into aqueous solution, a cloudy dispersion of droplets of the extraction solvent is produced resulting in a large increase in contact area between the phases. Consequently, analytes are easily transferred into the extraction phase [23]. The effect of extraction time on the process efficiency was investigated in the range of 10s to 70s. Results showed that equilibrium state is achieved quickly and thus the extraction is independent of time being in agreement with other DLLME procedures [24, 25].

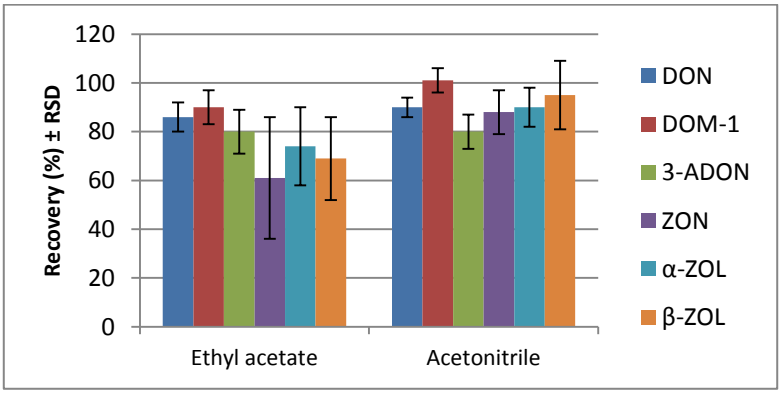
3.3. Optimization of SALLE procedure

Several experimental parameters, such as the extraction solvent type and its volume, the sodium chloride and C18 amounts, and the extraction time were investigated to reach high extraction efficiency.

3.3.1. Influence of the extraction solvent type

The selection of an appropriate solvent is a crucial step in the sample preparation. The extraction solvent must enable the extraction of the desired analytes and guarantee the minimal co-extraction interferences.

Figure 3: Effect of the extraction solvent type on the extraction efficiency. Extraction conditions: concentration of analytes, 100 µg/L; volume of solvent, 1 mL. NaCl amount, 0.3g; C18 sorbent amount, 30mg.



In this line, acetonitrile and ethyl acetate have been widely reported as preferred extraction solvent for multi-mycotoxin methods [26]. The extraction capabilities of the above solvents are compared in Figure 3. Under the same extraction conditions, acetonitrile provided the highest extraction efficiency (recovery range: 80 – 101%; RSDs <14%), whilst ethyl acetate showed low extraction efficiency (range from 61 to 90%; RSDs <25%). Therefore, acetonitrile was selected as extraction solvent for further experiments.

3.3.2. Influence of the extraction solvent volume

The effect of acetonitrile volume on the extraction of the mycotoxins contained in 1 mL of urine sample was investigated in the range of 0.5 – 1.5 mL in the interval of 0.5 mL. The use of 0.5 mL hindered suitable sampling of the organic phase after centrifugation due to the

phase boundary between acetonitrile and saline media was unclear and thus the removal of the upper organic layer was difficult. Optimal recoveries were achieved by using 1 mL (range: 84 – 96%) and 1.5 mL (range: 80 – 102%) of acetonitrile and no significant statistical differences for a confidence interval of 95% were found between them. Therefore, 1mL of acetonitrile was selected for extraction of mycotoxin and metabolites for the following experiments.

3.3.3. Influence of sodium chloride amounts

Because acetonitrile and water are mixable at any ratio, phase separation of an acetonitrile-water solution can easily be induced through the addition of inorganic salts such as sodium chloride. Therefore, to evaluate salting-out effect different sodium chloride amounts on the performance of the proposed procedure were evaluated from 0.1 - 0.4 g in the interval of 0.1 g while the volume of extraction solvent (acetonitrile) was kept at 1 mL. The results revealed that the salt concentration has a great influence on the phase separation. The addition of salt from 0.1 to 0.3 g led to a gradual increase of the recoveries while the addition of salt at the highest tested amount resulted in a decrease in chromatographic signal. Figure 4 describes the MATLAB-based optimization procedure graphically.

3.3.4. Influence of C18 sorbent amount

C18 sorbent is widely employed in clean up step due to the fact that it is effective at removing nonpolar compounds. Other recently

developed SALLE-based protocols for the determination of mycotoxins in urine reported significant signal suppression due to matrix effects [16]. In order to limit the presence of interfering substances in the final extract from complex matrix such as urine, the addition of different amounts of C18 sorbent (10, 20, 30 and 40 mg) was investigated for the first time in SALLE-based protocols. A rise of C18 sorbent from 10 to 30 mg resulted in an increase of the recoveries of the studied analytes, and no differences were observed when the amount of sorbent was larger than 30 mg (Figure 4). Thus, 30 mg was selected as the optimum amount of C18sorbent.

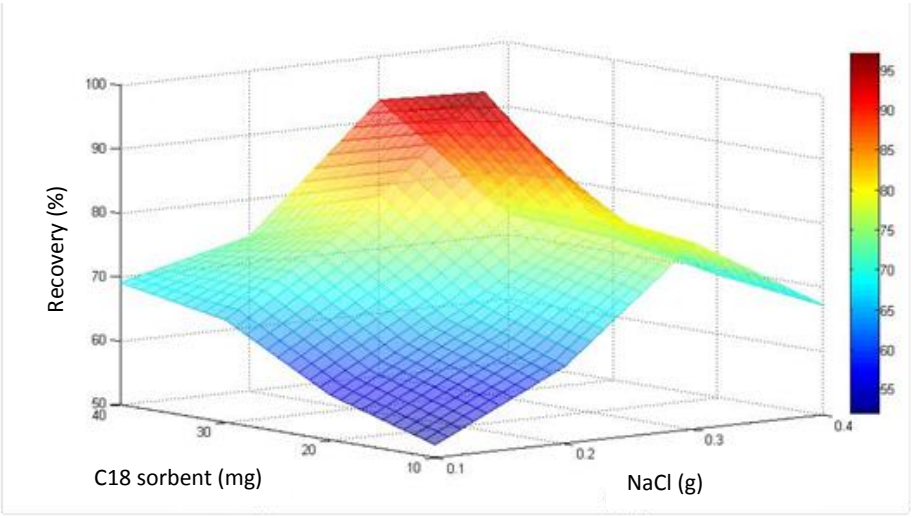
3.3.5. Influence of extraction time

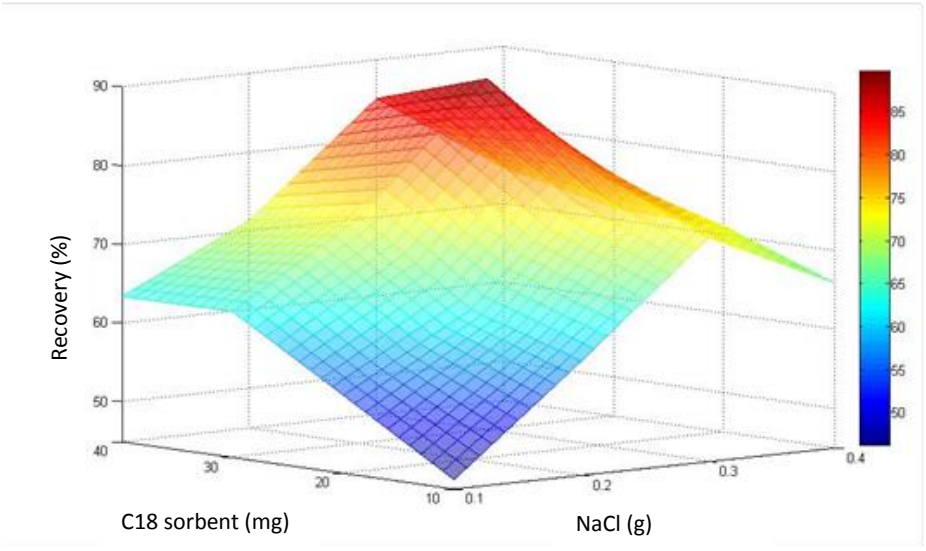
During the extraction time, mass transfer kinetics from matrix to extraction solvent occurs. Thus, the influence of the extraction time was investigated by increasing the time from 10s to 70s in the interval of 20s. The recoveries of the mycotoxins rose with the increase of extraction time from 10 to 30s, and remain stable with a further increase of extraction time from 30s to 70s indicating that the equilibrium state is achieved quickly (Figure 5). Thus, 30s was selected as the optimum extraction time.

3.4. Comparison of DLLME and SALLE

Based on the results obtained under optimum extraction conditions, DLLME showed slightly lower extraction efficiency (recovery range: 75 - 89%) than SALLE protocol (recovery range: 84 - 96%). Moreover, repeatability and reproducibility obtained in SALLE-based procedure was much higher (RSDs <14%) than in DLLME (RSDs <31%).

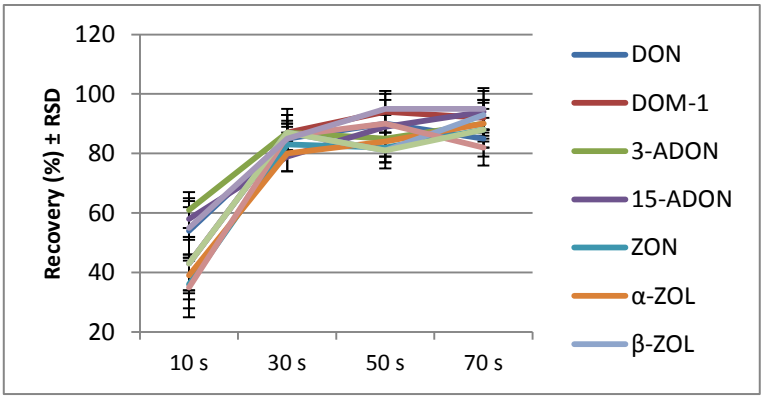
Figure 4: Response surfaces (expressed as recovery) of (A) DON and (B) mean trend of all analytes regarding influence of sodium chloride and C18 sorbent amounts.





Thus, SALLE is shown as a more appropriate sample preparation method and even it was faster than DLLME. In addition, SALLE methodology avoids the use of chlorinated solvents employed in DLLME. Therefore, SALLE was selected due to its great accuracy and precision, time- and cost-efficient as well as environmentally friendly sample preparation method, and further validation study was performed.

Figure 5: Effect of the extraction time on the extraction efficiency. Extraction conditions: concentration of analytes, 100 µg/L; volume of acetonitrile, 1 mL; NaCl amount, 0.3g; C18 sorbent amount, 30mg.



3.5. Evaluation of the method performance

3.5.1. Linearity and linear range

The calibration curves of all the studied mycotoxins in pure solvent and in matrix were obtained by plotting the peak areas against the concentration of the corresponding standards at eight concentration levels

ranging from LOQ and 100 µg/L. All calibration curves were performed in triplicate and the linearity was expressed by the square correlation coefficient (r^2). All studied mycotoxins gave r^2 >0.990 within the tested linear range.

3.5.2. Matrix effect

The co-extractives of complex samples can greatly affect the analyte signal or enhance background noise, which may compromise the quantitation of the analytes at trace levels. The matrix effect (ME) was calculated as the ratio between the slopes of the matrix-matched calibration curve and external calibration curve and expressed as percentage.

A value of 100% indicated that there was no matrix effect whereas values >100% indicated signal enhancement and values <100% signal suppression. The results showed that the matrix effects of the proposed method had signal suppression for all investigated analytes (from 65 to 95%) (Table 2). In order to achieve more reliable results, matrix-matched calibration curves were used for quantitation.

3.5.3. Limits of detection and limits of quantitation

The sensitivity of the method was assessed by the limits of detection and quantitation. The LODs and LOQs were calculated as the lowest matrix-matched calibration providing signal-to-noise ratios greater than 3 and 10, respectively. The LODs and LOQs of the proposed method

for ten mycotoxins were in the range of 0.12 – 4 µg/L and 0.25 – 8 µg/L, respectively (Table 2).

3.5.4. Specificity

The specificity of the method was evaluated with respect to interferences from endogenous compounds. Therefore, one blank sample was analysed using the above-mentioned procedures. The S/N ratio of a possible interfering peak in the blank sample had to be below the S/N ratio of the analytes in the same elution zone at the LOD level. GC–MS/MS chromatograms obtained by the analysis of spiked urine samples (8 µg/L) are illustrated in Figure 6.

3.5.5. Accuracy and precision

The accuracy was verified by measuring the recoveries from spiked blank samples at low (8 µg/L), medium (25 µg/L) and high (100 µg/L) concentration levels. Recovery studies were performed in triplicate in the same day as well as in three different days. Precision of the method (expressed as %RSD) was determined by repeatability (intra-day precision) and reproducibility (inter-day precision) at the same spiking levels than in the recovery study. Intra-day variation was evaluated in three determinations per concentration in a single day, whereas inter-day variation was tested on three different working days within 20 days. The results are shown in Table 2. At three spiking levels, recoveries ranged between 70 – 120% and 70 – 112% for intra-day and inter-day studies, respectively and RSDs were below 15% in all cases.

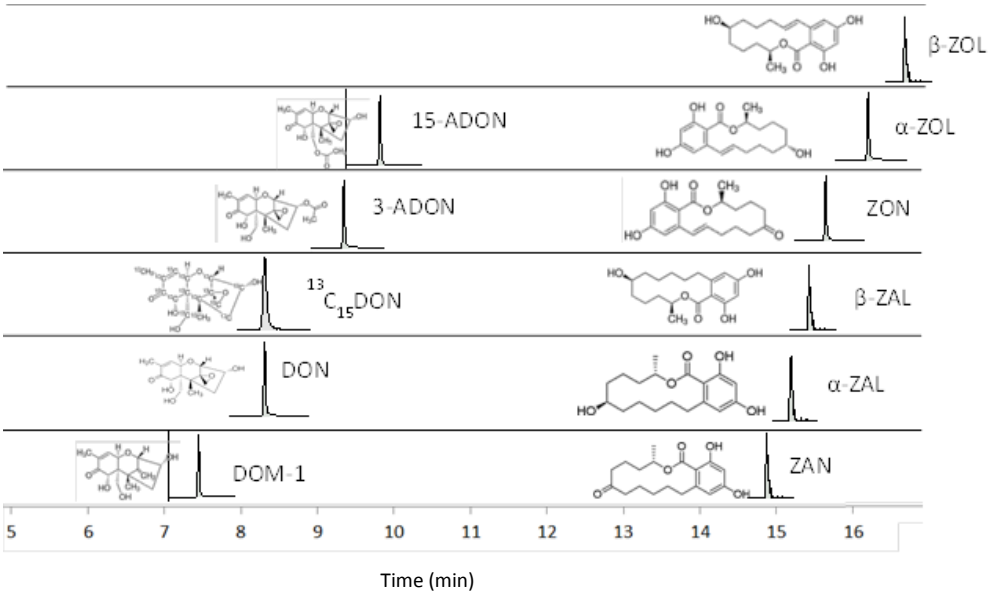
Table 2: Method performance for the SALLE-GC-QqQ-MS/MS.

Mycotoxin	LOD (µg/L)	LOQ (µg/L)	Intra-day (Rec ± RSD, %; *n = 3*)			Inter-day (Rec ± RSD, %; *n = 9*)			Matrix effect (%)
			Low	Medium	High	Low	Medium	High	
DON	0.12	0.25	83 ± 11	97 ± 6	106 ± 4	87 ± 12	91 ± 9	101 ± 6	88
DOM-1	0.25	0.50	92 ± 6	117 ± 7	120 ± 5	88 ± 7	110 ± 8	112 ± 6	92
3-ADON	0.25	0.50	87 ± 8	98 ± 5	92 ± 9	90 ± 6	92 ± 6	93 ± 10	75
15-ADON	0.25	0.50	70 ± 8	90 ± 4	96 ± 5	75 ± 9	96 ± 7	93 ± 7	79
ZON	3	6	81 ± 9	92 ± 7	82 ± 6	82 ± 10	89 ± 9	78 ± 8	72
α-ZOL	1	2	95 ± 8	75 ± 4	73 ± 4	91 ± 9	79 ± 8	77 ± 8	80
β-ZOL	2	4	84 ± 2	82 ± 13	96 ± 7	87 ± 5	88 ± 15	90 ± 9	87
ZAN	4	8	76 ± 13	78 ± 11	116 ± 8	70 ± 15	82 ± 12	105 ± 15	81
α-ZAL	4	8	91 ± 11	76 ± 10	74 ± 4	96 ± 11	79 ± 9	77 ± 6	95
β-ZAL	4	8	86 ± 6	79 ± 9	98 ± 8	84 ± 8	82 ± 8	92 ± 10	65

Spiking levels: low (8 µg/L); medium (25 µg/L); high (100 µg/L)

The results suggested that the proposed procedure was accurate, repeatable and reproducible for the determination of the studied mycotoxins in urine.

Figure 6: SRM chromatogram of a human urine sample spiked at 8 µg/L of the multi-mycotoxin standard solution.



3.6. Application of the SALLE to real samples

To evaluate the efficiency of the proposed procedure, it was successfully applied to perform the determination of the urine concentration levels of both free and total DON and its metabolites, in 12 volunteers of both genders. The detailed results of the survey are displayed in Table 3. Total DON (free + conjugated), obtained after enzymatic

hydrolysis, was detected in 10 out of 12 volunteers (83%) at average content of 31.6 µg/L (range from 4.1 to 84.1 µg/L).

Table 3: Free DON, total DON levels and conjugation rate in the analyzed human urine samples.

	Free DON (µg/L)	Total DON[a] (µg/L)	Conjugation rate (%)
Female *(n=6)*	<LOD	16.2	-
	21.3	84.1	75
	0.9	20.2	-
	1.9	7.9	76
	<LOD	19.0	-
	8.0	69.5	88
Male *(n=6)*	<LOD	4.1	-
	1.9	45.5	96
	<LOD	<LOD	-
	6.5	37.4	83
	<LOD	<LOD	-
	<LOD	12.1	-
Average	7.9 (50)[b]	31.6 (83)[b]	84

[a]*Total DON (free + conjugated),* [b]*Percentage of positive samples*

The levels here reported are consistent with those obtained in other European studies. For instance in a Portuguese study total DON was found in 69% individuals at mean content of 16.3 µg/L (range: 1.9 – 26.2 µg/L) [12] and in a French study total DON was found in 99% individuals at average contents of 6.8 µg/L (range: 0.5 – 28.8 µg/L) [27]. The mean glucuronidation rate in the volunteers was determined to be 84%, ranging from 75 to 96%. This result is in agreement with those reported in United Kingdom (mean: 91%, range: 85 – 98%; [28]) and Austria (mean: 86%,

range: 79 – 95%; [29]). These data suggest that the majority of DON is converted to the glucuronide, representing the major detoxification metabolites in humans.

Free DON was detected in 50% of participants ranging from 0.9 to 21.3 µg/L with a mean of 7.9 µg/L. Similar values were obtained in recent studies conducted in South Africa (mean: 4.9 µg/L; range: 0.4 – 53.4 µg/L) [30] and Italy (mean: 11.9 µg/L; range: <LOD – 67.4 µg/L) [31].

Neither DOM-1 nor 3-ADON nor 15-ADON (free or total) was detected in any analysed samples. Data on DOM-1 formation in humans is limited. Turner et al., (2011) [28] suggested that DOM-1 does not represent a major detoxification metabolite in humans and, if formed, is not significantly excreted via the urine. On the other hand, Eriksen et al., (2003) [32] reported that 3-ADON and 15-ADON could be rapidly deacetylated *in vivo* to DON as observed in pigs.

ZON (free and total) and ZON metabolites namely α-ZOL, β-ZOL, ZAN, α-ZAL and β-ZAL were not detected in any of the 12 analyzed samples being in agreement with the data reported by Rubert et al., (2011) [33] and Warth et al., (2014) [34].

4. Conclusions

There is a current trend toward the miniaturization of sample preparation procedures and a growing demand for simplification, time-efficient and cost-efficient methods. Two novel techniques namely DLLME

and SALLE were developed and compared for the quantitative determination of 10 *Fusarium* mycotoxins and metabolites in human urine samples. Under optimized conditions, SALLE showed higher extraction efficiency and thus a high-throughput biomarker method consisting of enzymatic hydrolysis, SALLE-based extraction and GC-QqQ-MS/MS determination was validated. Experiments conducted to evaluate matrix effect, accuracy and precision demonstrated that the proposed procedure was rugged, sensitive, selective, accurate, repeatable and reproducible achieving very low limits of detection and low matrix effect. In addition, the developed method has been successfully applied to 12 human urine samples and deoxynivalenol resulting from free or conjugated was quantified in 83% samples.

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4. General discussion

Discusión general

4. DISCUSIÓN GENERAL

El trabajo de investigación llevado a cabo a lo largo de esta Tesis Doctoral ha abordado el desarrollo, optimización y validación de diferentes procedimientos analíticos basados en GC-MS/MS para la determinación de micotoxinas de *Fusarium* en distintas matrices alimentarias. Una vez puestas a punto las metodologías propuestas se aplicaron a un elevado número de muestras con la finalidad de evaluar la exposición de los consumidores, incluyendo grupos de población sensibles, a estos contaminantes alimentarios combinando los datos de consumo de las matrices ensayadas con datos sobre las concentraciones encontradas de micotoxinas. Las matrices alimentarias que se evaluaron fueron principalmente productos a base de cereales tanto en crudo, cocinados como comercializados listos para el consumo tras un procesado tecnológico.

No obstante, y con la finalidad de realizar una mejor aproximación de la exposición a estos contaminantes, se planteó un estudio para conocer la posible correlación entre los niveles de micotoxinas consumidos a través de la dieta y excretados vía renal. Para ello, previamente fue necesario desarrollar una estrategia analítica para la determinación de micotoxinas y sus metabolitos en orina.

Finalmente se llevó a cabo una monitorización de los niveles de micotoxinas a través del análisis de orina de distintos grupos de población, también, para evaluar el grado de exposición a las mismas.

4.1. Estudio analítico de las micotoxinas de *Fusarium*

4.1.1. Optimización de las condiciones de la Cromatografía Gaseosa y Espectrometría de Masas en Tándem triple cuadrupolo

La instrumentación que se utilizó a lo largo de la Tesis Doctoral fue el equipo de cromatografía gaseosa Agilent 7890A acoplado con un espectrómetro de masas tiple cuadrupolo Agilent 7000A. El espectrómetro de masas operó tanto en el modo de barrido completo (*full-scan*), como en modo escaneo del ion producto (*product ion scan*) y en modo de monitorización de reacciones múltiples (MRM). Este modo MRM permitió cuantificar las micotoxinas estudiadas con la más alta selectividad y sensibilidad. En el modo MRM, el primer cuadrupolo filtra un ion precursor específico; luego el segundo, concretamente la celda de colisión, genera iones producto que se filtran en el tercer cuadrupolo.

El presente trabajo de investigación se inició con la puesta a punto de un procedimiento analítico para la determinación de diez micotoxinas (PAT, DON, 3-ADON, FUS-X, DAS, NIV, NEO, HT-2, T-2 y ZON) mediante GC-MS/MS (Rodríguez-Carrasco, et al., 2012). En posteriores actualizaciones del método se incluyeron nuevos compuestos tales como DOM-1, $^{13}C_{15}$-DON, α-ZOL, β-ZOL, ZAN, α-ZAL, β-ZAL permitiendo finalmente una determinación simultánea de dieciocho micotoxinas y metabolitos (Rodríguez-Carrasco, et al., 2014a). A pesar de las diferentes propiedades físico-químicas de estos compuestos se alcanzó una situación de compromiso permitiendo el análisis de los mismos.

La optimización de las condiciones espectrométricas de las micotoxinas y sus metabolitos se abordó mediante la inyección individual de cada analito en el equipo y posterior monitorización de los iones en modo *full-scan*. Tras la selección de los iones que presentaron una mayor abundancia (iones precursores) se procedió a evaluar su fragmentación generando así sus iones producto (*product ion*) y escogiendo los más abundantes. Posteriormente se combinaron diferentes valores de energía de colisión y de tiempo de lectura (*dwell time*) para optimizar la fragmentación y consecuentemente aumentar la sensibilidad del método. Aun cuando se cuenta con gran selectividad en la detección utilizando el modo MRM, siempre existe un riesgo potencial de falsos positivos o negativos debido a la interferencia de señales de la matriz. Para minimizar este riesgo, se monitorizó una segunda transición por analito obteniendo así cuatro puntos de identificación por compuesto tal y como especifica la legislación para la adecuada confirmación de muestras positivas (Commission Decision 2002/657/EC, 2002). Además, se calculó para cada micotoxina el ratio iónico específico del método desarrollado, definido como el cociente de las respuestas entre la transición utilizada para cuantificar (Q) y aquella para identificar (q) debiéndose cumplir este parámetro para confirmar la presencia de la micotoxina en una muestra.

Una vez optimizados todos los parámetros espectrométricos se inyectó una mezcla con todos los analitos para poder definir con mayor precisión las ventanas de tiempo en el método. Se tuvo especial consideración en la agrupación del menor número de transiciones por

ventana de tiempo para garantizar la mayor sensibilidad. Así pues se agrupó únicamente más de un analito cuando el tiempo de retención entre éstos impidió crear diferentes ventanas.

Con respecto a la cromatografía se emplearon distintas rampas de tiempo/temperatura para tratar de resolver los picos cromatográficos en el menor tiempo. La elección final de la rampa supuso un tiempo de cromatografía de 17 min para separar un total de 18 compuestos.

Tras la puesta a punto del método, se estudió la respuesta del instrumento a distintas concentraciones observándose una respuesta lineal para todas las micotoxinas en el intervalo estudiado. En cuanto a los límites de detección y cuantificación instrumentales se consiguieron alcanzar niveles del orden de pocos µg/L comparables a otras metodologías analíticas (Beltrán, et al., 2011; Ferreira, et l., 2012; Gregori, et al., 2013; Juan, et al., 2014).

Seguidamente, se desarrollaron distintos métodos de extracción y purificación para llevar a cabo el análisis de estos contaminantes en distintas matrices.

4.1.2. Optimización de los distintos protocolos de preparación de la muestra

A lo largo de la Tesis Doctoral se ensayaron distintos protocolos de preparación de la muestra y se optimizaron sus condiciones para aplicarlos a diferentes matrices. Posteriormente se llevaron a cabo estudios de linealidad, exactitud y precisión. Otros parámetros incluidos en los estudios

de validación fueron los límites de detección y cuantificación y el estudio del efecto matriz.

4.1.2.1. Método QuEChERS

El procedimiento utilizado para la extracción de las micotoxinas estudiadas se basa en una modificación del método publicado por Cunha and Fernandes (2010). Para la optimización del QuEChERS, se han estudiado diferentes volúmenes de acetonitrilo (utilizado como disolvente de extracción), pH del medio y tipo de fases utilizadas en la etapa de purificación (Rodríguez-Carrasco, et al., 2012). Al analizar muestras con un contenido de agua inferior al 40%, se requiere un proceso de "humidificación" previo a la extracción. Por ello, cuando se analizaron cereales o productos a base de cereales se adicionó agua para facilitar la homogeneización entre la muestra y el disolvente. Se usó una cantidad de muestra, previamente triturada, de 5 g y un volumen de agua de 25 mL. En el estudio del polen apícola se utilizaron 10 mL de agua para hidratar la muestra (Rodríguez-Carrasco et al., 2013a), mientras que en el caso de la extracción de micotoxinas a partir de muestras líquidas no fue necesaria la adición de agua (Rodríguez-Carrasco, et al., 2014a). Se realizó un estudio para evaluar el efecto del pH del medio en la eficacia de la extracción. Para ello, se probaron distintos pH (pH=3, pH=6 y pH=8). Adicionalmente también se evaluó la eficacia de la extracción utilizando agua destilada. Los resultados mostraron mejores recuperaciones para los analitos estudiados cuando se empleó agua destilada sin previo ajuste del pH. Por otra parte

también se evaluó el efecto del volumen de acetonitrilo en la eficacia del proceso de extracción. Los volúmenes óptimos del disolvente de extracción se seleccionaron en base a los datos de recuperación obtenidos y, en caso de no existir diferencias significativas en los estudios de exactitud, se seleccionó el mínimo volumen de disolvente que generó resultados satisfactorios. Así pues los volúmenes optimizados fueron: 5 mL para cerveza y orina, 7,5 mL para cereales y productos a base de cereales y 8 mL para polen apícola. Finalmente, se evaluó la eficacia de la fase de purificación. Con la finalidad de minimizar el tiempo de preparación de muestra se omitió en un primer momento dicha etapa. No obstante, los resultados obtenidos no fueron satisfactorios hecho por el que se ensayaron distintas combinaciones de fases empleadas. El QuEChERS clásico utiliza generalmente PSA para la eliminación de ácidos, pigmentos polares y azúcares. En este sentido se requirió el uso de una cantidad pequeña de esta fase (300 mg) junto con C18 en polvo (300 mg) para la purificación del extracto obtenido a partir del polen apícola (Rodríguez-Carrasco et al., 2013a). Para el resto de matrices ensayadas, el uso de PSA no fue necesario utilizándose únicamente C18 en polvo comúnmente empleado para la eliminación de lípidos y componentes no polares. En ambas combinaciones se empleó $MgSO_4$ (900 mg) para la disminución del contenido de agua presente en el extracto. Con este método se consiguieron unas recuperaciones para la amplia mayoría de los analitos en todas las matrices ensayadas entre 80 y 110% a diferentes puntos de fortificación. Los resultados de precisión mostraron en todos los casos que

la metodología propuesta fue repetible y reproducible al obtener, generalmente, unas RSDs <15%. Por su parte, se obtuvieron unos límites de cuantificación en muestra que oscilaron entre 0,1 y 16 µg/L para la totalidad de los analitos y matrices evaluadas. El efecto de la matriz se evaluó en cada caso dada la complejidad y composición de las muestras estudiadas. Para evaluar dicho parámetro, la pendiente de la curva de calibrado obtenida al adicionar el estándar al extracto se comparó con la pendiente de la curva de calibrado del estándar. Se observó generalmente una supresión de la señal para la mayoría de las micotoxinas; no obstante para algún analito se mostró un ligero aumento de la señal (SSE: 101 – 144%) en alguna matriz (Rodríguez-Carrasco, et al., 2012). Consecuentemente se utilizaron los estándares preparados en los extractos de las matrices evaluadas para compensar las variaciones de señal producidas por el efecto matriz y permitir una cuantificación adecuada. Por otra parte, la veracidad del método se comprobó mediante el uso de material certificado de referencia (BRM003004) consistente en trigo artificialmente contaminado por DON (1062 ± 110 µg/Kg). Este material de referencia se sometió al proceso de extracción optimizado (*n = 6*) y se obtuvo una concentración promedio de 1025 ± 23 µg/Kg (Rodríguez-Carrasco, et al., 2014b).

El método QuEChERS permite disminuir el uso tanto de disolventes, como de reactivos y de materiales respecto a los métodos tradicionales de extracción y purificación. También permite disminuir los tiempos de análisis, a la vez que ofrece resultados seguros. Estos hechos

hacen que sea valorado como una alternativa eficaz y robusta para su utilización en matrices alimentarias complejas.

4.1.2.2. Dispersión de matriz en fase sólida

El método de dispersión de matriz en fase sólida previamente desarrollado en el laboratorio por Blesa et al. (2004) se utilizó como punto de partida para llevar a cabo una evaluación de la eficacia de la extracción de micotoxinas a partir de productos a base de cereales y compararlo con el método QuEChERS. Si bien la cantidad de muestra utilizada se redujo a una quinta parte (1 g), el volumen de disolvente (mezcla acetonitrilo-metanol) se incrementó hasta 15 mL con el consiguiente aumento del tiempo necesario para llevar a sequedad el extracto obtenido. Por otra parte los resultados del estudio de exactitud y precisión no fueron satisfactorios al obtener unas recuperaciones entre 46 – 89% y unas RSDs ≤ 37% (Rodríguez-Carrasco, et al., 2014b). En base a esto se decidió utilizar otras técnicas de preparación de muestras atendiendo a las ventajas ofrecidas.

4.1.2.3. Técnicas de microextracción

El uso de técnicas de microextracción en el presente trabajo de investigación surgió ante la necesidad de desarrollar un protocolo de preparación de muestra que permitiera la extracción de los analitos a partir de un volumen reducido de matriz. Estas técnicas se desarrollaron y optimizaron en este trabajo para la determinación de micotoxinas a partir de muestras biológicas, como la orina. Se utilizó 1 mL como tamaño de

muestra. Este requisito fue indispensable puesto que previamente a la etapa de extracción se desarrolló una etapa de hidrólisis enzimática para separar los conjugados de micotoxinas formados como consecuencia de la fase II del metabolismo. En esta etapa previa, se incubó 1 mL de orina con 250 µL de solución de β-glucuronidasa durante 18h a 37°C siguiendo las condiciones descritas por Cunha and Fernandes (2012).

4.1.2.3.1. Microextracción líquido-líquido dispersiva

Para la optimización de esta técnica, se evaluaron distintos parámetros tales como la influencia del tipo de extractante utilizado y su volumen, la influencia del volumen de acetonitrilo (utilizado como dispersante), la influencia de la adición de cloruro sódico así como el efecto del tiempo de extracción.

Se evaluaron dos disolventes halogenados como agentes extractantes, concretamente diclorometano y cloroformo. La combinación de 1 mL de acetonitrilo y 100 µL de cloroformo ofreció los mejores resultados en cuanto a la eficacia del proceso de extracción (intervalo de recuperaciones: 75 – 89%; RSDs: 10 – 31%). En esta técnica la adición de sal puede resultar en un incremento, descenso o no tener un efecto significativo respecto a la eficiencia de la extracción (Leong, et al., 2014). De los resultados obtenidos en el presente trabajo se deduce que la adición de sal no tuvo un efecto significativo en la eficacia de la extracción. Similarmente, se llevó a cabo un estudio para evaluar el efecto del tiempo de extracción. Los resultados mostraron que, bajo las condiciones

experimentales, el equilibrio se alcanza rápidamente como consecuencia de la elevada superficie de contacto generada en el proceso de dispersión y, por tanto, la extracción resultó ser independiente del tiempo (Rodríguez-Carrasco et al., 2015a).

4.1.2.3.2. Extracción líquido-líquido asistida por sales

Los parámetros que se investigaron con el objetivo de optimizar esta extracción fueron el tipo y volumen del disolvente, la cantidad de cloruro sódico y de fase C18 así como el tiempo de extracción.

En este protocolo se evaluaron dos disolventes, acetonitrilo y diclorometano, comúnmente utilizados en metodologías para la extracción simultánea de micotoxinas (Monbaliu, et al., 2009; Devreese, et al., 2012). Se obtuvieron unos intervalos de concentraciones del 80 – 101% y del 61 – 90% cuando se utilizó acetonitrilo y diclorometano, respectivamente. Junto con los mejores resultados de exactitud, el acetonitrilo también mostró resultados más precisos (RSDs <14%) frente al diclorometano (RSDs < 25%). Por otra parte, de los diferentes volúmenes utilizados de acetonitrilo, las mejores recuperaciones se obtuvieron cuando se utilizó 1 mL de dicho disolvente.

El efecto conocido como *salting-out* se evaluó mediante la adición de diferentes cantidades de cloruro sódico (0,1 – 0,4 g) en la muestra conteniendo el disolvente. Se observó un aumento de la eficiencia de la extracción cuando se incrementó la adición de sal en el intervalo de 0,1 a 0,3 g mientras que se obtuvieron recuperaciones ligeramente inferiores

cuando se empleó 0,4 g. Similarmente se evaluó el efecto de la adición de cantidades crecientes de fase C18 (10 – 40 mg) para minimizar la presencia de sustancias interferentes. Los resultados obtenidos mostraron una mejora de la eficiencia del proceso a medida que se incrementaba la cantidad de C18 hasta 30 mg no observándose diferencias significativas cuando continuó incrementándose. Finalmente se evaluó el efecto del tiempo de extracción en un intervalo de tiempo de 10 – 70 seg alcanzándose el equilibro a los 30 seg (Rodríguez-Carrasco et al., 2015a).

4.1.3. Derivatización

El análisis de las micotoxinas estudiadas en el presente trabajo se ha realizado por GC-MS/MS después de llevar a cabo una reacción de derivatización mediante una mezcla de agentes sililantes (BSA + TMCS + TMSI). La derivatización es una estrategia ampliamente utilizada en GC que tiene por objetivo la eliminación de los hidrógenos activos de los grupos reactivos permitiendo un aumento de la volatilidad de los analitos (Wells, 1999).

La mezcla de BSA + TMCS + TMSI es una de las que más capacidad sililante presenta, siendo aparentemente capaz de derivatizar todos los grupos hidroxilos indistintamente de la posición en la que se encuentren. La reacción general de un analito con la mezcla de agentes sililantes se muestra en la figura 1:

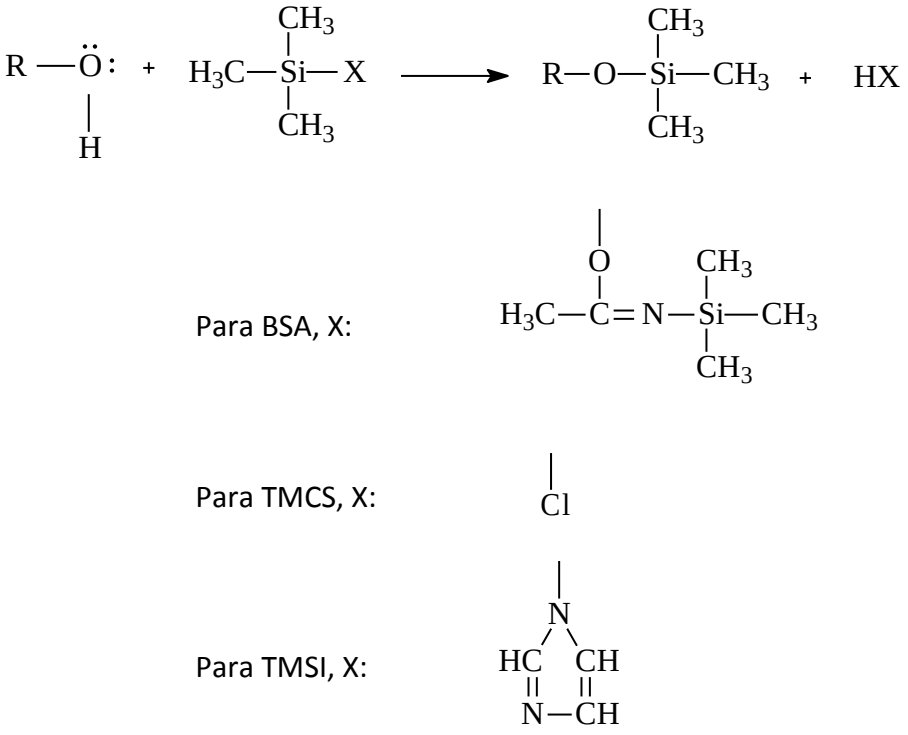

Figura 1: Esquema de la reacción de derivatización con la mezcla comercial de agentes sililantes.

Como producto de la reacción se generan derivados trimetilsililo (TMS) los cuales presentan una mayor estabilidad térmica respecto al compuesto original. En el presente trabajo se evaluó el efecto del binomio tiempo – temperatura en la reacción de derivatización. Se sometió la reacción bajo diferentes condiciones de temperatura (ambiente, 40°C, 60°C y 80°C) y tiempo (15 min, 30 min y 45 min) pero no se observaron diferencias en cuanto a las temperaturas aplicadas siendo 30 min el tiempo mínimo para completar la reacción.

4.2. Presencia de micotoxinas en diversas matrices y evaluación de la exposición a las mismas

4.2.1. Muestreo

La presencia de micotoxinas a lo largo de esta Tesis Doctoral se ha evaluado en un total de 418 muestras de alimentos y 55 muestras de orina. La clasificación de las muestras se indica a continuación:

- Productos a base de cereales que requieren tratamiento previo para su consumo y contienen gluten: avena (*n = 8*), espelta (*n = 8*) y trigo (*n = 119*).
- Productos a base de cereales y otros alimentos sin gluten: tapioca (*n = 3*), soja (*n = 4*), maíz (*n = 17*) y arroz (*n = 23*).
- Productos procesados a base de cereales listos para el consumo: rosquilletas (*n = 61*) y cerveza (*n = 154*).
- Suplemento dietético: polen apícola (*n = 15*).
- Productos a base de cereales consumidos en 24 h por un individuo sano (*n = 6*) y su orina 24 h (*n = 1*).
- Orina de diferentes grupos de población: niños entre 8 – 14 años (*n = 16*), adultos entre 18 – 28 años (*n = 16*) y adultos mayores de 28 años (*n = 22*).

4.2.2. Presencia de micotoxinas en matrices alimentarias

Los resultados muestran una incidencia de micotoxinas relativamente alta, ya que 246 de las 418 matrices alimentarias analizadas estaban contaminadas al menos con una micotoxina.

La incidencia y los niveles de micotoxinas varían según la matriz evaluada. Las micotoxinas que se detectaron con mayor frecuencia fueron el DON (58,8 %) y la toxina HT-2 (15,4 %); y pese a la incidencia destacable que presentaron estas toxinas, las concentraciones encontradas fueron generalmente bajas (< 50 µg/Kg).

El DON se ha encontrado principalmente en trigo y productos a base de trigo, concretamente en el 80% de las 119 muestras de trigo analizadas presentaban DON, con un promedio de las muestras positivas de 12,7 µg/Kg y una concentración máxima de 83,2 µg/Kg (Rodríguez-Carrasco, et al., 2014c). Pese a la importante incidencia encontrada de DON los valores máximos permitidos para estas matrices (750 µg/Kg) son muy superiores a las concentraciones obtenidas. Se obtuvieron concentraciones similares de DON en el 64% de las 61 muestras de rosquilletas analizadas con un valor promedio de 32 µg/Kg y un máximo de 60 µg/Kg (Rodriguez-Carrasco, et al., 2014b). Para este tipo de productos la legislación permite un contenido máximo de 200 µg DON/Kg aún bastante superior a las concentraciones halladas en las muestras evaluadas. Por otra parte, los resultados obtenidos en el análisis de muestras de cerveza revelan una contaminación por DON en el 60% de las 114 muestras evaluadas y se

encontró una concentración promedio de 28,9 µg/L y una concentración máxima de 47,7 µg/L. No obstante, teniendo en cuenta la clasificación hecha en las cervezas evaluadas, aquellas elaboradas a base de trigo presentaron incidencias y valores promedio de DON superiores a las cervezas elaboradas a base de otras materias primas (Rodríguez-Carrasco et al., 2015). Otras matrices como los productos a base de tapioca, espelta, maíz o arroz presentaron una incidencia de DON menor respecto al trigo (Rodríguez-Carrasco, et al., 2014c).

Los datos sobre la presencia de estas toxinas en cereales llevados a cabo en otros países como Alemania (Gottschalk, et al., 2009), Túnez (Bensassi, et al., 2010), Japón (Tanaka, et al., 2009) o Canadá (Lombaert, et al., 2003) están en la misma línea que los hallados en este trabajo de investigación.

Por otro lado, la cerveza y los alimentos a base de trigo mostraron contaminación por la toxina HT-2 en el 9% y 17% de las muestras respectivamente. Los promedios calculados a partir de las muestras positivas fueron 10,2 µg/Kg para alimentos a base de trigo (Rodríguez-Carrasco, et al., 2014c) y 30,9 µg/L para cerveza (Rodríguez-Carrasco et al., 2015b). Cabe mencionar que todas las muestras de cerveza que presentaron contaminación por HT-2 pertenecían a cervezas elaboradas a partir de trigo. Algunas muestras de otras matrices como la avena, el maíz (Rodríguez-Carrasco, et al., 2014) o las rosquilletas (Rodriguez-Carrasco, et

al., 2014b) mostraron también presencia de HT-2 aunque presentaron una incidencia menor.

A pesar del predominio de DON y HT-2, se detectó también presencia de NIV en el 4,5% de las 418 matrices alimentarias analizadas (intervalo: 4,9 – 61,8 μg/Kg) siendo los alimentos a base de trigo los que significativamente mostraron una mayor incidencia (84% del total). Esto coincide con los resultados encontrados en otros trabajos que afirman que el NIV es un problema menor que DON en cereales europeos (Bernhoft, et al., 2010). En las muestras analizadas se detectaron otras micotoxinas como T-2 y ZON aunque a niveles e incidencias menores.

Otro aspecto importante a tener en consideración es la presencia simultánea de micotoxinas ya que, como cualquier mezcla de productos activos, los efectos tóxicos de la mezcla pueden resultar aditivos o sinérgicos. Respecto a las muestras de los productos a base de los principales cereales (trigo, arroz y maíz), se encontró presencia simultánea de entre 2 y 5 micotoxinas en el 15,7% de las 159 muestras, siendo el trigo el cereal que presentó un mayor porcentaje de muestras multi-contamiandas. Las principales combinaciones de micotoxinas han sido DON y HT-2 y en menor medida DON y NIV (Rodríguez-Carrasco, et al., 2014c). La presencia simultánea de micotoxinas se ha detectado también en otros trabajos (Serrano, et al., 2012; Marin, et al., 2013). En los análisis de rosquilletas se detectaron resultados similares al encontrar presencia simultánea de DON y HT-2 en el 3,3% de las muestras. De forma similar, en

cerveza el 9,1% de las muestras presentó presencia simultánea de DON y HT-2.

Con respecto al polen apícola, se detectó presencia de NEO en 2 de las 15 muestras analizadas (promedio: 26 µg/Kg). También se detectó NIV en estas mismas muestras aunque a niveles próximos a los del LOQ (1 µg/Kg) (Rodríguez-Carrasco, et al., 2013a). Cabe destacar que las muestras contaminadas se trataban de polen apícola fresco y, por tanto, con un contenido de humedad elevado, hecho por el que podría haber favorecido el crecimiento del hongo y la posterior producción de micotoxinas; mientras que todas las muestras que resultaron negativas se trataban de polen apícola comercial (< 8% humedad) (Magrama, 1980).

4.2.3. Presencia de micotoxinas en muestras biológicas

Los resultados de orina revelaron la presencia de micotoxinas en un porcentaje importante de muestras (68,5%; *n = 54*). Los resultados por grupo mostraron una incidencia de micotoxinas en adultos superior a la encontrada en la orina de los niños, no obstante las concentraciones promedio halladas fueron comparables entre los grupos estudiados, siendo 14,8, y 32,9 µg/g de creatinina en adultos >28 años y adultos entre 18 y 28 años, respectivamente, frente a los 27,8 µg/g de creatinina encontrada en niños. Éstos resultados son comparables a los obtenidos por otros autores (Cunha and Fernandes, 2012; Shephard, et al., 2013; Solfrizzo, et al., 2014). En las muestras analizadas se detectó también presencia de DOM-1, metabolito de fase I del DON, aunque únicamente se cuantificó en dos

muestras y a niveles muy bajos. En las orinas analizadas se detectó también HT-2 y NIV en el 7,4% y 13% de las muestras y valores promedio similares a los encontrados para DON (Rodríguez-Carrasco, et al., 2014d).

Tras los resultados obtenidos en orina se investigó la presencia de posibles conjugados de micotoxinas como consecuencia de las reacciones del metabolismo de fase II. Para ello se aplicó una hidrólisis enzimática a las orinas problema con una solución de β-glucuronidasa, siguiendo las condiciones optimizadas por Cunha and Fernandes, (2012) y posteriormente siendo extraídas por el método desarrollado en nuestro laboratorio (Rodríguez-Carrasco et al., 2015a). En el 50% de las 12 muestras de orina analizadas previamente a la hidrólisis se detectó un promedio de 6,7 µg/L de DON (intervalo: 0,9 – 21,3 µg/L) mientras que en las mismas muestras analizadas tras la hidrólisis enzimática se detectó DON en el 83% con un valor promedio de 31,6 µg/L (intervalo: 1,9 – 26,2 µg/L). Los valores encontrados concuerdan con aquellos obtenidos por otros autores (Turner, et al., 2010a; Warth, et al., 2012a). Así mismo se obtuvo un ratio de conjugación de DON promedio del 84% (intervalo: 75 – 96%) siendo comparable con los resultados obtenidos en Reino Unido (promedio: 91%; intervalo: 85 – 98%) por Turner et al. (2011) y en Austria (promedio: 86%; intervalo: 79 – 95%) por Warth et al. (2012b).

Respecto a la ZON, no se encontró presencia de esta micotoxina en su forma libre y, tras hidrólisis enzimática de las muestras, tampoco se detectó ZON procedente de la liberación de su forma conjugada. Estos

resultados se corresponden con los obtenidos en la monitorización de las matrices alimentarias ensayadas, donde no fue significativa la presencia de ZON.

Por otro lado, dado el perfil de contaminación de las muestras analizadas, se evaluó la posible correlación entre la ingesta de micotoxinas a través de los alimentos y su excreción vía urinaria. Para ello se recogieron raciones idénticas a las consumidas de aquellos alimentos a base de cereales que formaron parte de la dieta 24 h de un individuo sano. Del mismo modo se recogió la orina 24 h del individuo en el periodo de estudio. Se cuantificó una ingesta total de DON de 49,2 ± 5,6 µg/día y una excreción de DON de 35,2 ± 4,3 µg/día, representando un ratio de excreción de DON del 72% (Rodríguez-Carrasco et al., 2015c). Los resultados obtenidos en el estudio muestran por tanto una correlación entre la ingesta y excreción de DON siendo comparables a otros estudios en donde se estimó un ratio de excreción de DON del 68% y 72% (Turner, et al., 2010b; Warth, et al., 2012b).

4.2.4. Evaluación de la exposición

Para evaluar la exposición a los riesgos alimentarios, antes se debe identificar el riesgo y efectuar su caracterización. Así pues, se realizó una evaluación de la exposición basada en un enfoque determinista para llevar a cabo una estimación de la probabilidad y severidad de los efectos adversos sobre la población a causa de la presencia de micotoxinas. Ello requiere la estimación de la ingesta de un contaminante por parte de los

consumidores en un determinado tiempo. Con este fin, la ingesta diaria estimada se obtuvo multiplicando los datos de consumo de alimentos por los datos de contaminación de los mismos y se expresó en base al peso corporal del individuo (Rodríguez-Carrasco, et al., 2013b). La información sobre el consumo de las matrices alimentarias se obtuvo a partir de la base de datos de consumo en hogares españoles del Ministerio de Agricultura, Alimentación y Medio Ambiente (Magrama, 2014), así como de los balances alimentarios de la FAO (FAOSTAT, 2015). A parte de evaluar la exposición a micotoxinas en el consumidor medio (utilizando datos de consumo promedio para población general), se tuvieron también en cuenta grupos de población susceptibles. Por otra parte, se incluyeron alimentos, como las rosquilletas, de los que no se dispone de datos de consumo. En este caso se asumió un consumo diario equivalente a la totalidad del producto comercializado en el envase clásico (35 g). Cabe mencionar que en el caso de obtener unos resultados por debajo del límite de detección superiores al 10 – 15% de las muestras, los datos se procesaron de acuerdo a las recomendaciones de la FAO/WHO (IPCS, 2009). En este sentido, en las muestras en las que no se detectó presencia de micotoxinas se asumió el valor LOD/2 (Rodríguez-Carrasco, et al., 2013b). Otra estrategia utilizada fue definir dos escenarios posibles de exposición: nivel de exposición bajo, asignando un valor de cero a aquellas muestras <LOD o LOQ; nivel de exposición alto, asignando el valor del LOD para muestras <LOD y el valor del LOQ para muestras >LOD pero <LOQ (Rodríguez-Carrasco et al., 2015b).

Las ingestas diarias de micotoxinas calculadas demostraron que la contribución individual de las matrices alimentarias ensayadas fue inferior a las ingestas diarias tolerables. En el caso de la ingesta de micotoxinas a partir del consumo de los principales cereales, se obtuvo una caracterización del riesgo (expresada como % de PMTDI) para DON del 1% para adultos; mientras que para niños entre 5 y 12 años el aporte de micotoxinas a través del consumo de estas matrices supuso un 6% de la PMTDI del DON y para niños <3 años ascendió al 8%. No obstante, pese a encontrar una incidencia de las toxinas T-2 y HT-2 significativamente menor con respecto al DON, se calculó una exposición a las mismas significativamente mayor y especialmente destacable en grupos susceptibles de población como los niños (10% TDI para adultos, 79% TDI para niños de 5 – 12 años y 86% TDI para niños <3años) (Rodríguez-Carrasco, et al., 2013b).

Por otra parte, para evaluar la exposición en muestras que presentaron contaminación simultánea de micotoxinas se propuso una fórmula para llevar a cabo una caracterización del riesgo combinada (Rodríguez-Carrasco, et al., 2013b). Los resultados mostraron que para las muestras que presentaban presencia simultánea de cuatro y cinco micotoxinas la ingesta de éstos contaminantes era superior a la ingesta tolerable en el caso de los niños. No obstante, estas muestras suponen el 1,9% de las 159 muestras analizadas de los principales cereales.

En el caso de las rosquilletas, se obtuvieron resultados similares ya que la ingesta de DON representó el 1,6% de la PMTDI del DON en adultos mientras que para niños se obtuvo un valor de 4,5% (Rodríguez-Carrasco, et al., 2014b). Por su parte la ingesta de micotoxinas para la población europea a través del consumo promedio de cerveza (0,19 L/día) representó un 5% de la PMTDI del DON, mientras que se obtuvo un intervalo del 7 – 12% de la TDI de las toxinas T-2 y HT-2 en base a los escenarios de exposición definidos anteriormente (Rodríguez-Carrasco et al., 2015b). No obstante, la exposición a micotoxinas en la población con un elevado consumo de cerveza podría ser significativa. Un ejemplo de ello se plasmó cuando se asumió un consumo diario de 1L de la cerveza que presentó un contenido de micotoxinas más elevado (DON: 42 µg/L y HT-2: 33 µg/L). Bajo estas condiciones se obtuvo una ingesta de DON equivalente al 60% de la PMTDI mientras que la ingesta de HT-2 excedería hasta cinco veces los niveles de ingesta tolerable.

Habida cuenta de la correlación existente entre los niveles de micotoxinas en la dieta y en los fluidos biológicos, se propuso evaluar la exposición a estas a través de su determinación en orina, reduciendo consecuentemente la incertidumbre asociada al análisis de alimentos y permitiendo llevar a cabo una evaluación individual. Para transformar los valores de micotoxinas excretadas vía renal a niveles ingeridos se siguió la ecuación propuesta por Rodríguez-Carrasco et al. (2014a). Los resultados obtenidos mostraron valores de exposición superiores a los obtenidos a partir del análisis individual de alimentos. Para más del 50% de los

individuos expuestos se obtuvo una ingesta de DON ≥ 50% de su PMTDI e incluso el 8,1% de los individuos superaron los niveles de ingesta tolerable siendo la población infantil el grupo más susceptible (Rodríguez-Carrasco, et al., 2014d). Estos resultados concuerdan con investigaciones previas llevadas a cabo por otros autores, los cuales también indican niveles de exposición a micotoxinas superiores a los tolerables utilizando la orina como vía de evaluación de exposición a estos contaminantes alimentarios (Warth, et al., 2012b; Solfrizzo, et al., 2014).

Así pues, en base a los resultados, se propone la evaluación de la exposición a las micotoxinas a través de sus biomarcadores de exposición en orina como alternativa a los métodos tradicionales permitiendo una mayor aproximación a la exposición real tanto a nivel individual como por grupos de población (Meky, et al., 2003).

4.3. Referencias

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5. Conclusions

Conclusiones

1. El procedimiento basado en GC-MS/MS, desarrollado en esta Tesis Doctoral, es una herramienta analítica muy útil para la determinación de 18 micotoxinas en las distintas matrices ensayadas alcanzando límites de detección a concentraciones del orden de unidades de µg/Kg.

2. En este trabajo de investigación se han desarrollado métodos rápidos y sencillos, principalmente basados en QuEChERS y extracción líquido-líquido asistida por sales, para la determinación simultánea de deoxinivalenol, deoxinivalenol marcado con isótopos estables ($^{13}C_{15}$DON), de-epoxideoxinivalenol, 3-acetildeoxinivalenol, 15-acetildeoxinivalenol, fusarenona X, diacetoxiscirpenol, nivalenol, neosolaniol, toxina HT-2, toxina T-2, zearalenona, α-zearalenol, β-zearalenol, zearalanona, α- zearalanol, β- zearalanol y patulina en matrices alimentarias y orina.

3. De un total de 418 muestras de alimentos, se ha encontrado deoxinivalenol y HT-2 en un 58,8% y 15,4% de las muestras, respectivamente; no obstante las concentraciones medias encontradas fueron relativamente bajas (<50 µg/Kg).

4. El deoxinivalenol se encontró en un 67,6% de los productos a base de trigo con un promedio de las muestras positivas de 12,7 µg/Kg en pasta, 28,9 µg/Kg en cerveza y 32,0 µg/Kg en rosquilletas.

5. En el 10,8% de las 418 muestras de alimentos analizadas se han encontrado de forma simultánea varias micotoxinas. Las combinaciones de micotoxinas más frecuentes han sido DON y HT-2 y en menor medida DON y NIV.

6. La evaluación de la exposición, realizada bajo un enfoque determinista, mostró que las ingestas diarias de micotoxinas fueron inferiores a las ingestas diarias tolerables y por consiguiente no supondrían un riesgo para el consumidor medio. No obstante, para ciertos grupos de población como niños y grandes consumidores de cereales o de sus productos derivados, la ingesta de micotoxinas podría superar los niveles de seguridad.

7. Los resultados obtenidos a partir del análisis de la orina revelaron la presencia de deoxinivalenol en un 69% de las muestras analizadas y concentraciones promedio entre 14,8 y 27,8 µg/g de creatinina según el grupo de población. Así mismo se detectó presencia de nivalenol y HT-2 aunque presentaron una incidencia inferior al 15%.

8. Las orinas analizadas también mostraron presencia de metabolitos de deoxinivalenol, tanto de fase I como de fase II, estos últimos fueron los más importantes cuantitativamente. El ratio de conjugación medio obtenido experimentalmente fue superior al 70%

9. Los resultados de evaluación de la exposición calculados a partir de orina mostraron que más del 50% de los individuos presentan una ingesta de DON ≥ 50% de su PMTDI y un 8,1% de los sujetos superaron los niveles de ingesta tolerable, siendo la población infantil el grupo más susceptible.

10. La correlación entre los niveles de DON ingeridos a partir de la dieta y los encontrados en la orina en el mismo periodo de estudio proporcionó un ratio de excreción de DON del 72%.

11. Finalmente, y en base a todo ello, se propone evaluar la exposición a las micotoxinas a través de los biomarcadores de exposición en orina, para reducir la incertidumbre asociada a la evaluación de la exposición obtenida a partir del análisis de los alimentos.

1. The proposed procedure developed in this PhD Thesis based on GC-MS/MS is a suitable analytical tool for the determination of 18 mycotoxins in different matrices reaching limits of detection at low µg/Kg range.

2. In this research project, rapid and easy sample preparation procedures mainly based on QuEChERS and salting-out liquid-liquid extraction were developed for the simultaneous determination of deoxynivalenol, isotopic-labeled $^{13}C_{15}$-deoxynivalenol, de-epoxy-deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon X, diacetoxyscirpenol, nivalenol, neosolaniol, HT-2 toxin, T-2 toxin, zearalenone, α-zearalenol, β- zearalenol, zearalanone, α- zearalanol, β- zearalanol and patulin in food matrices and uriune.

3. Deoxynivalenol and HT-2 were quantified in 58.8% and 15.4%, respectively, in a total of 418 food samples. Nonetheless, average concentrations found were relatively low (<50 µg/Kg).

4. Deoxynivalenol was found in 67.6% of analyzed wheat-based products at average concentrations in positive samples of 12.7 µg/Kg in pasta-related products, 28.9 µg/Kg in beer and 32.0 µg/Kg in breadsticks.

5. Co-occurrence of mycotoxins was found in 10.8% of analyzed food matrices (*n = 418*). Deoxynivalenol and HT-2 toxin was the most common combination followed by DON and NIV.

6. Exposure assessment carried out by a deterministic approach showed daily mycotoxin intakes lower than the established tolerable daily ones, and thus they would not suppose a risk for average consumers. However, mycotoxin intakes for some

population groups such as children and heavy consumers of cereals or their derivative products could exceed the safety levels.

7. The results obtained from urine analysis revealed occurrence of deoxynivalenol in 69% of samples at average concentrations between 14.8 and 27.8 μg/g creatinine depending on the population group. In addition, nivalenol and HT-2 were also found but at incidences lower than 15%.

8. Deoxynivalenol phase I and phase II metabolites were also found in the analyzed samples, the latter being the most important. An average DON conjugation rate ≥70% was experimentally obtained.

9. Exposure assessment calculated from urine showed a DON intake ≥ 50% of the PMTDI for more than 50% of subjects. 8.1% individuals exceed the DON PMTDI, being children the most susceptible population studied group.

10. Correlation between levels of DON intake from the diet and those calculated in urine at the same period, showed a DON excretion rate of 72%.

11. Finally, and based on all the data before mentioned, the exposure assessment to mycotoxins through biomarkers in urine is here proposed to reduce the uncertainty associated with the exposure obtained from food analysis.