VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

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Programa de Doctorado con Mención hacia la Excelencia en Ciencias de la Alimentación

EVALUACIÓN DE LA EXPOSICIÓN DE LA POBLACIÓN VALENCIANA A MICOTOXINAS A TRAVÉS DE UN ESTUDIO DE DIETA TOTAL

Tesis Doctoral

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CERTIFIQUEN QUE:

Dña. Dionisia Carballo Vera**,** ha realizado bajo nuestra dirección el trabajo que lleva por título: "Evaluación de la exposición de la población valenciana a micotoxinas a través de un estudio de dieta total", y autorizamos su presentación para optar al título de Doctor por la Universitat de València.

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

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La presente tesis doctoral ha dado lugar 6 artículos, publicados o que se publicaran en las siguientes revistas:

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- ✓ Red Nacional sobre las micotoxinas y hongos micotoxigénicos y de sus procesos de descontaminación (MICOFOOD).

Para la realización de la presente Tesis Doctoral, la estudiante de Doctorado Dionisia Carballo

Ha disfrutado de una beca predoctoral orientada a la formación del personal investigador en áreas de Ciencia y Tecnología (CyT) dentro del programa de Becas Carlos Antonio López (BECAL) del gobierno de Paraguay.

"Descubrir algo significa mirar lo mismo que está viendo todo el mundo, y percibirlo de manera diferente"

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A mis padres

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ACN	Acetonitrilo
AECOSAN	Agencia Española de Consumo, Seguridad Alimentaria y Nutrición
AFs	Aflatoxinas
AFB1	Aflatoxina B1
AFB2	Aflatoxina B2
AFG1	Aflatoxina G1
AFG2	Aflatoxina G2
AFM1	Aflatoxina M1
AFP1	Aflatoxina P1
AFQ1	Aflatoxina Q1
APPCC	Análisis de peligros y puntos críticos de control
AME	Alternariol methil eter
AOH	Alternariol
ATX	Altertoxinas
BEA	Beauvericina
C_{18}	Octadecilsílice
CaCo-2	Células de adenocarcinoma de colon humano
CIC	Comité interno de coordinación
CIT	Citrinina
CPA	Ácido cicloplazónico
BPA	Buenas prácticas agrícolas
BPH	Buenas prácticas de higiene
BPF	Buenas prácticas de fabricación
DAS	Diacetoxiscirpenol
DLLME	Micro-extracción dispersiva liquid-líquido
DON	Deoxinivalenol
DON GlcA	Deoxinivalenol glucoronido
DOM-1	Deepoxi-deoxinivalenol
EC	Comisión Europea
EDI	Ingesta Diaria Estimada
EDT	Estudio de dieta total
EFSA	Autoridad Europea de Seguridad Alimentaria

ENNs	Eniatinas
ENN A	Eniatina A
ENN A1	Eniatina A1
ENN B	Eniatina B
ENN B1	Eniatina B1
ESI	Ionización Electrospray
FAO	Organización de las Naciones Unidas para la Alimentación y la Agricultura
FBs	Fumonisinas
FB1	Fumonisina B1
FB2	Fumonisina B2
FDA	Administración de alimentos y medicamentos
FSAI	Autoridad de Seguridad Alimentaria de Irlanda
FSANZ	Normas Alimentarias de Australia Nueva Zelanda
FUS-X	Fusarenona X
GC	Cromatografía de gases
GLIO	Gliotoxina
HPLC	Cromatografía líquida de alta resolución
HT-2	Toxina HT-2
IARC	Agencia Internacional para la Investigación del Cáncer
IDA	Ingesta diaria admisible
IDTP	Ingesta diaria tolerable provisional
ISTP	Ingesta semanal tolerable provisional
IT	Trampa de iones
JECFA	Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios
LC	Cromatografía líquida
LC-MS-LIT	Cromatografía líquida-Espectrometría de Masas con trampa de iones lineal
LC-MS/MS	Cromatografía líquida-Espectrometría de Masas en tándem
LLE	Extracción líquida-líquida
LOD	Límite de detección
LOQ	Límite de cuantificación
LRM	Límite máximo de residuos
MAPAMA	Ministerio de Agricultura, Pesca y Alimentación

ME	Efecto matriz
MeOH	Metanol
MON	Moniliformina
MPA	Ácido micofenólico
MRM	Monitorización de Reacciones Múltiples
MSPD	Dispersión de matriz en fase sólida
NEO	Neosolaniol
NIV	Nivalenol
OTA	Ocratoxina A
OMS	Organización mundial de la salud
PAT	Patulina
PNCOCA	Plan Nacional de Control Oficial de la Cadena Alimentaria
PMTDI	Ingesta Diaria Tolerable Máxima Provisional
PMTWI	Ingesta Semanal Tolerable Máxima Provisional
PSA	Primary and Secondary Amine
QqQ	Triple cuádrupolo
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe extraction
RASFF	Sistema de Alerta Rápida para alimentos y piensos
RSD	Desviación estándar relativa
SCF	Comité Científico para la Alimentación
SCIRI	Sistema Coordinado de Intercambio Rápido de Información
STG	Esterigmatocistina
S/N	Relación señal-ruido de fondo
SPE	Extracción en fase sólida
SPME	Micro extracción en fase sólida
SSE	Alteración de la señal por supresión o aumento
T-2	Toxina T-2
TCs	Tricotecenos
TDI	Ingesta Diaria Tolerable
TeA	Tenuazonico
TEN	Tentoxina
TOF	Tiempo de vuelo
TR	Tiempo de Retención

UE	Unión Europea
Verru	Verruculogeno
ZAN	Zearalanona
ZEA	Zearalenona
α-ZAL	α-zearalanol
β-ZAL	β -zearalanol
α-ZOL	α-zearalenol
β-ZAL	β-zearalenol
3-AcDON	3-acetil deoxinivalenol
15-AcDON	15-acetil deoxinivalenol

RESUMEN

La dieta es la principal fuente de micotoxinas para el hombre. Los hongos micotoxigénicos pueden contaminar los productos vegetales en el campo, durante el almacenamiento o en el procesado. Aunque las micotoxinas son en su mayoría termostables, evaluar su presencia en los diferentes componentes de nuestra dieta incluyendo platos listos para su consumo de composición vegetal y animal, así como en productos lácteos, zumos y bebidas alcohólicas es un paso necesario para evaluar mejor los riesgos asociados a su presencia y adoptar medidas para proteger la salud de los consumidores.

En primer lugar, se realizó en esta tesis una revisión bibliográfica que incluyó dieciocho estudios de dieta total realizados en Canadá, China, Francia, Irlanda, Líbano, Nueva Zelanda, España, Paises Bajos, Viet Nam, y España, mostrando el creciente interés por la exposición a las micotoxinas a través de la dieta.

Así mismo, se desarrollaron y validaron diferentes procedimientos analíticos basados en la cromatografía de gases y liquida ambas acopladas a espectrometría de masas en tándem. Se han utilizado diferentes técnicas de extracción; como método QuEChERS que dio muy buenos resultados para cereales, vegetales, frutas, frutos secos, legumbres, carne, pescado y leche, mientras que para el café se usó la extracción liquida-liquida y para cerveza, vino, zumos y tés se seleccionó la microextracción liquida-liquida dispersiva. Las metodologías propuestas han sido validada de acuerdo a la normativa europea con resultados satisfactorios en cuanto a linealidad, exactitud, precisión y límites de detección y cuantificación.

Los resultados mostraron que el 49% de las 328 platos de menús listos para su consumo contenían al menos una micotoxina. Las micotoxinas más frecuentemente detectadas fueron deoxinivalenol, neosolaniol, toxina HT-2 y alternariol. EL 53% de las 110 bebidas alcohólicas han mostrado presencia simultánea de más de una micotoxina llegando hasta diez, principalmente de tricotecenos, aflatoxinas, zearalenona, patulina y toxinas *Alternarias* con sumas de concentraciones que oscilan entre 5.50 μg/L y 180.15 μg/L.

Las concentraciones de las micotoxinas detectadas en los alimentos se encuentran por debajo de los límites máximos establecidos a excepción de una muestra de vino que excedía los niveles establecidos para OTA (2 μg/L).

Se llevó a cabo una evaluación de la exposición teniendo en cuenta los resultados obtenidos y los datos de consumo, y se hizo una mención especial al consumo según las recomendaciones de la dieta mediterránea.

Se utilizó la técnica de análisis estadístico multivariado del análisis de componentes principales (PCA), para interpretar los resultados en términos de contribución de los alimentos analizados a la ingesta de micotoxinas estudiadas. Los resultados mostraron una mayor contribución de legumbres a la ingesta de HT-2 y βZAL , de carne a la ingesta de OTA, de cerveza a la ingesta de AOH, βZAL y DON, de zumos de frutas a la ingesta de PAT así como de frutos secos a la ingesta de NIV.

Además, se ha realizado la caracterización del riesgo mediante comparación de la ingesta diaria estimada con parámetros toxicológicos de ingesta diaria tolerable obteniendo un riego bajo para la mayoría de los alimentos listos para su consumo. Sin embargo, la presencia frecuente de micotoxinas

aunque a bajos niveles, pone de manifiesto la necesidad de incluir la monitorización de micotoxinas en estudios de dieta total.

SUMMARY

Diet is the main source of mycotoxins for man. Mycotoxigenic fungi can contaminate plant products in field, during storage or in processing. Although mycotoxins are mostly thermostable, evaluating their presence in the different components of our diet including ready-to-eat vegetable or animal dishes, as well as in dairy products, juices and alcoholic beverages is a necessary step for a better assessment of the risks associated with mycotoxin presence and to take measures to protect the health of consumers.

In the first place, a bibliographic review was carried out in this thesis including eighteen total diet studies carried out in Canada, China, France, Ireland, Lebanon, New Zealand, Spain, the Netherlands, Viet Nam, and Spain, showing the growing interest in exposure assessment to mycotoxins through diet.

Likewise, different analytical procedures based on gas chromatography and liquid both coupled to tandem mass spectrometry were developed and validated. Different extraction techniques have been used; QuEChERS method that gave very good results for cereals, vegetables, fruits, nuts, legumes, meat, fish and milk, while the liquid-liquid extraction was used for coffee and the liquid-liquid dispersive microextraction for beer, wine, juices and teas. The proposed methodologies have been validated according to European regulations with satisfactory results in terms of linearity, accuracy, precision and limits of detection and quantification.

The results showed that 49% of the 328 menu dishes ready for consumption contained at least one mycotoxin. The mycotoxins most frequently detected were deoxynivalenol, neosolaniol, HT-2 toxin and alternariol. 53% of

110 alcoholic beverages have shown simultaneous presence of more than one mycotoxin reaching up to ten, mainly of trichothecenes, aflatoxins, zearalenone, patulin and Alternaria toxins with sums of concentrations ranging between 5.50 µg / L and 180.15 µg / L.

The concentrations of the mycotoxins detected in the food are below the maximum limits established except for a sample of wine that exceeded the levels established for OTA (2 µg / L).

An evaluation of the exposure was carried out taking into account the results obtained and the consumption data, and a special mention was made to the consumption according to the recommendations of the Mediterranean diet.

The technique of multivariate statistical analysis of the principal components analysis (PCA) was used to interpret the results in terms of the contribution of the analyzed foods to the ingestion of mycotoxins studied. The results showed a greater contribution of legumes to the intake of HT-2 and βZAL, of meat to the intake of OTA, of beer to the intake of AOH, βZAL and DON, of fruit juices to the intake of PAT as well as of dried fruits to the intake of NIV.

In addition, the characterization of the risk has been made by comparing the estimated daily intake with toxicological parameters of tolerable daily intake, obtaining a low risk for most of the Food ready for consumption. However, the detection of mycotoxins, although at low levels, highlights the need to include mycotoxin monitoring in total diet studies.

1. INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Seguridad Alimentaria

El concepto de Seguridad Alimentaria ha adquirido diferentes significados con el paso del tiempo, haciéndose cada vez más completo. En la última Cumbre Mundial sobre Seguridad Alimentaria, la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO), relaciona la seguridad alimentaria con la accesibilidad física, social y económica de alimentos seguros, nutritivos y en cantidad suficiente para satisfacer sus requerimientos nutricionales y preferencias alimentarias, a fin de llevar una vida activa y saludable (FAO, 2018).

Este contexto plantea cuatro componentes: disponibilidad, acceso, utilización y estabilidad. El componente de mayor repercusión en los países desarrollados es la utilización de los alimentos, asociado habitualmente a la inocuidad o garantía de que un alimento no cause daño en la salud del consumidor cuando sea preparado y/o ingerido de acuerdo al uso que se destine.

Con el objetivo de garantizar que los alimentos sean seguros y no representen riesgos para la salud, la Unión Europea (UE) ha implementado un sistema de garantía de calidad para la aplicación en industrias alimentarias como sistema preventivo que incluye el análisis de peligros y puntos críticos de control (APPCC), buenas prácticas de agrícolas (BPA), buenas práctica de higiene (BPH) y buenas prácticas de fabricación (BPF).

Por otra parte, el sistema de control de alimentos, incluido en el Plan Nacional de Control Oficial de la Cadena Alimentaria (PNCOCA) realiza

controles en todas y cada una de las etapas de la cadena alimentaria (AECOSAN, 2016). Para ello, el plan persigue como objetivos principales el desarrollo y mejora de los planes de control oficial en el ámbito de la producción primaria para certificar, mediante el cumplimiento de la normativa en materia de seguridad y calidad alimentaria, que los alimentos sean seguros y de calidad; reducir los riesgos presentes en los alimentos que amenazan a las personas a niveles razonablemente posibles y aceptables; mantener un nivel elevado de lealtad de las transacciones comerciales y de la defensa integral de los derechos de los consumidores y contribuir a mantener la unidad de mercado y garantizar la consecución de un elevado nivel de calidad alimentaria de los productos agroalimentarios, cumpliendo de esta manera lo establecido en el Reglamento 2017/625 (EC, 2017).

El Sistema Coordinado de Intercambio Rápido de Información (SCIRI) dependiente de la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición AECOSAN, gestiona y coordina, a nivel nacional todas las incidencias en la cadena alimentaria que supongan un riesgo directo o indirecto para la salud de los consumidores (AECOSAN, 2017).

En la Comunidad Valenciana ha sido aprobado recientemente el Decreto 23/2017 por el que se regula el protocolo de gestión de emergencias alimentarias que afectan a la comunidad Valenciana dentro de su ámbito de competencia. Una vez declarada una situación de emergencia alimentaria, el Comité Interno de Coordinación (CIC) es el órgano responsable de la gestión, comunicación y evaluación de la emergencia. De acuerdo a los últimos datos disponibles, la AECOSAN, en el año 2017 gestionó un total de 259 notificaciones, siendo más numerosos los peligros químicos y biológicos en productos de origen animal.

Por lo que respecta a la categoría del peligro detectado, los metales pesados fueron la causa primaria (108), en segundo lugar las notificaciones por la presencia de microorganismos patógenos (99) y en tercer lugar por la presencia de micotoxinas (70). Comparando estos datos con los reportados en años anteriores, en lo referente a micotoxinas en el año 2015 y 2016 se han reportado 495 y 551 notificaciones, lo que supone que los controles en cuanto a seguridad alimentaria han mejorado (AECOSAN, 2017). A nivel europeo, a través del Sistema de Alerta Rápida para Animales y Piensos, en su informe más reciente del año 2017, han registrado un total de 3032 notificaciones, 529 correspondieron a notificaciones relacionadas con la presencia de micotoxinas en alimentos (RASFF, 2017).

En la actualidad diversas autoridades nacionales e internacionales, como la Organización Mundial de la Salud (OMS), (FAO), la Autoridad Europea para la Seguridad Alimentari EFSA y Food and Drug Administration (FDA) prestan especial atención a la contaminación de alimentos por micotoxinas, abordando el problema global mediante la adopción de normativas y directrices para las principales clases de micotoxinas, contando actualmente con alrededor de 100 países que disponen de límites establecidos para estos contaminantes en alimentos (Alshanaq y Yu 2017).

1.2. Micotoxinas: Aspectos generales

Las micotoxinas son metabolitos secundarios producidos por determinados hongos filamentosos pertenecientes principalmente a los géneros *Aspergillus, Fusarium, Penicillium y Alternaria* (Alshanaq & Yu, 2017). Desde el descubrimiento de las aflatoxinas en la década de los sesenta (Bennet & Klich, 2003), la identificación de los hongos productores de micotoxinas, las diferentes

clases químicas de metabolitos secundarios y los problemas de salud asociados se han convertido en un reto difícil para los investigadores. En la actualidad, se conocen aproximadamente 400 metabolitos fúngicos secundarios pertenecientes al grupo de 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 (Fromme et al., 2016).

La gravedad de los efectos que producen las micotoxinas depende, en gran medida, de la potencialidad tóxica, las cantidades ingeridas, la duración de la exposición y las interacciones que pueden resultar de la ingestión simultánea de diferentes micotoxinas (Grenier and Oswald, 2011; Ruíz et al., 2011; Gómez et al., 2015). A señalar que las exposiciones crónicas a micotoxinas están relacionados con efectos cancerígenos, mutágenos, teratógenos e inmunodepresores (Bennett y Klich, 2003).

La contaminación natural de los alimentos por hongos toxigénicos depende principalmente de las condiciones óptimas de temperatura, oxígeno, pH, actividad de agua y humedad relativa (Bhat et al., 2010). La producción de micotoxinas en los cultivos puede ocurrir a lo largo de toda la cadena alimentaria (pre-cosecha, cosecha, secado y almacenamiento). En este sentido, una misma especie de hongo puede producir un amplio rango de micotoxinas y una misma micotoxina puede ser producida por diferentes tipos de hongos (Marín et al., 2013). La Tabla 1 muestra las principales especies de hongos que producen micotoxinas y los principales alimentos frecuentemente contaminados por estas micotoxinas.

Tabla 1: Tipos de micotoxinas, principales especies de hongos toxigénicos productores y alimentos implicados.

Micotoxina	Género	Especie	Presencia en alimentos
Aflatoxinas	*Aspergillus*	*A. flavus, A. parasiticus, A. bombycis, A pseudotamarii, A. ocharaceoroseus*	Cacahuete, maíz, trigo, arroz, colza, semilla de algodón, nueces, huevos, leche, queso, higos
Ocratoxina	*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	*Pinicillium*	*P. verrucosum, P. expansus, P. carneum, P. paneum, P. sclerotigenum*	Frutas, especialmente manzanas, zumos de frutas
	Apergillus	*A. clavi, A. terreus*	
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, 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
Emergentes	*Fusarium*	*F. sporotrichioides, F. poae, F. langsethiae, F. avenaceum, F. tricinctum, F. subglutinans*	Cebada, trigo, arroz, avena, maíz, centeno, productos a base de cereales y frutos secos
Toxinas *Alternarias*	*Alternari*	*A. alternata, A. tenuissima, A. arborescens, A. radicina, A. brassicae, A. brassicicola, A. infectoria.*	Frutas y derivados de frutas, cereales, legumbres, vegetales

Algunas micotoxinas pueden resistir a los tratamientos físicos, químicos y biológicos aplicados durante el procesado de alimentos destinado al consumo humano o animal (pan, pasta, galletas, cerveza, vino, zumos, piensos). Por tanto, una vez la materia prima alimentaria este contaminada por micotoxinas, es muy difícil la eliminación de las mismas (Delledonne, 2006; Estiarte et al., 2018).

Además de la ingestión directa de micotoxinas a través de alimentos contaminados de origen vegetal, existe creciente preocupación sobre la ingestión de estos compuestos tóxicos a través de alimentos de origen animal que pueden contener residuos de micotoxinas o de sus metabolitos (Alshannaq and Yu, 2017 Tolosa et al., 2017; Montanha et al., 2017).

Los mecanismos de acción de las diferentes micotoxinas son muy variables, principalmente debido a la diversidad de estructuras químicas que presentan. La Figura 1 muestra la estructura química de las principales micotoxinas.

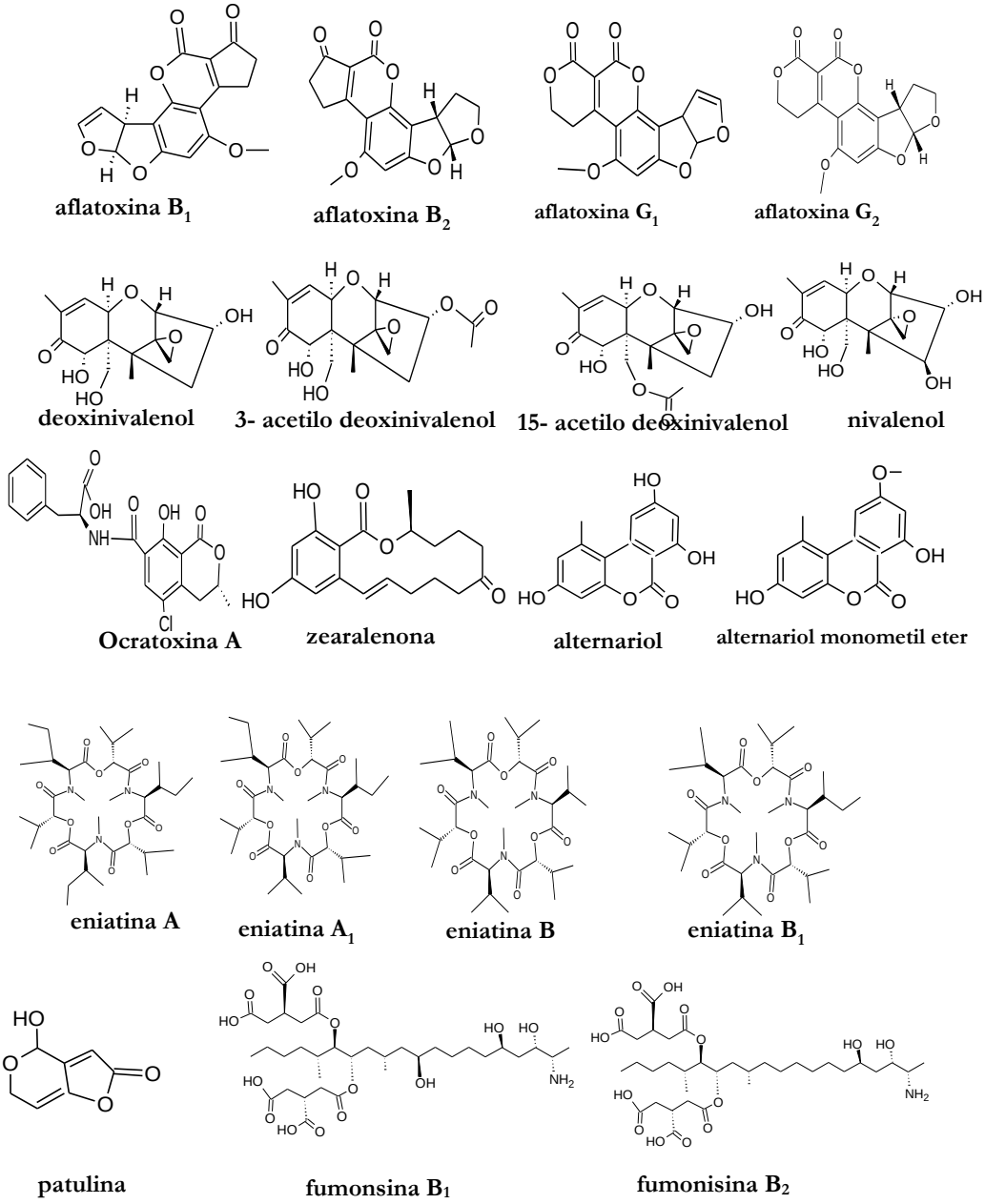

Figura 1: Estructura química de las principales micotoxinas.

1.2.1 Aflatoxinas

Las aflatoxinas (AFs) son producidas principalmente por las cepas de *Aspergillus flavus* y *Aspergillus parasiticus*. Las más importantes se denominan B_1, B_2, G_1, G_2 y M_1. La aflatoxina B_1 (AFB_1) es metabolizada principalmente en AFM_1, AFM_2, AFB_1-N7 guanina, AFQ_1, AFP_1 que se excretan por la leche y la orina respectivamente (Yao et al., 2015).

Los principales alimentos asociados a la contaminación por AFs son el maíz, frutos secos (cacahuetes, pistachos y nueces), especias, frutas desecadas y café (Jeyaramraja et al., 2017; Gambacorta et al., 2018; Shepard et al., 2018). La AFM1 está presente principalmente en productos lácteos y derivados (Scaglioni et al., 2014; Campagnollo et al., 2016).

La AFB_1 y la AFM_1 son consideradas las más tóxicas del grupo. Se han observado efectos sobre el hígado, el miocardio y los riñones, siendo el hígado el órgano más afectado por su toxicidad. Cada año se detectan nuevos casos de cáncer hepático de los que podrían estar relacionados con la ingesta de AFs (Chiewchan et al., 2015). La IARC clasifica la AFB_1 y la AFM_1 en el Grupo 1, compuestos carcinógenos para el hombre (IARC, 2012).

1.2.2. Ocratoxina A

La ocratoxina A (OTA) se produce en climas tropicales por hongos del genero *Aspergillus* y en áreas templadas o frías por hongos del genero *Penicillium*.

Se encuentra normalmente en cereales, principalmente en arroz, soja, café, cacao, bebidas alcohólicas (especialmente cervezas y vino) y otros

alimentos como zumo de uva, legumbres, carnes y frutos secos (De Girolamo et al., 2019; Ostry et al., 2015).

Los mecanismos de toxicidad de la OTA son inhibición de la síntesis de proteínas por competición con la fenilalanina e inducción de estrés oxidativo por peroxidación lipídica, formación de radicales libres, interferencia en la fosforilación oxidativa e incremento de la apoptosis en diferentes líneas celulares (Marín et al., 2013). La IARC la clasifica en el grupo 2B, posible carcinógeno humano (IARC, 2012). Entre sus efectos crónicos, la OTA se destaca como agente hepatotóxico, nefrotóxico, neurotóxico, inmunosupresor, citotóxico y genotóxico (Pfohl-Leszkowicz et al., 2007; Malir et al., 2016; Tao et al., 2018).

1.2.3. Patulina

La patulina (PAT) es producida por ciertas especies de *Penicillium*, *Aspergillus*, *Byssochlamys* y *Paecylomyces*, siendo principalmente producida por *Penicillium expansum*.

La PAT se encuentra generalmente en las frutas, en particular en manzana y productos a base de manzana como zumo, compotas y purés (Barreira et al., 2010; Zaied et al., 2013; Iqbal et al., 2018). La contaminación se produce principalmente por el uso de frutas dañadas (Li et al., 2018). También está presente en alimentos procesados a base de cereales (Assunçao et al., 2016).

Respecto a su toxicidad, la PAT se estudió inicialmente como un antibiótico potencial, aunque posteriormente fueron descubiertos sus efectos negativos sobre la salud. La ingesta crónica de dosis elevadas de PAT produce trastornos inmunitarios, neurotóxicos y gastrointestinales graves (Puel et al.,

2010). Aunque la IARC ha expresado mucha preocupación por la posible carcinogenicidad de la PAT, ésta ha sido clasificada dentro del grupo 3 (IARC, 2012).

1.2.4. Fumonisinas

Las fumonisinas (FBs) son producidas principalmente por especies de *Fusarium Liseola. F. verticillioides* (*F. moniliforme*) y *F. proliferatum*. Otras especies como *F. napiforme, F. dlamini* y *F. nygamai,* también producen FBs.

Actualmente se han aislado más de 28 fumonisinas que se clasifican en cuatro grupos: fumonisinas A (A_1, A_2 y A_3), fumonisinas B (B_1, B_2 y B_3), fumonisinas C (C_4, C_3 y C_1) y fumonisinas P (P_1, P_2 y P_3), aunque solo los grupos A y B se consideran importantes desde el punto de vista toxicológico. La Fumonisina B1 (FB_1) es la Fumonisina que se encuentra con más frecuencia en los alimentos y comprende el 70-80% de la familia total de FBs. Los alimentos comúnmente contaminados por FBs son el maíz y sus derivados, cerveza, sorgo, trigo, cebada, soja, espárragos, higos, té negro y plantas medicinales (Scott, 2012; Bryla et al., 2013; Alshanaq & Yu 2017).

FB_1 es la fumonisina más tóxica del grupo y está clasificada en el grupo 2B como probable carcinógeno para humanos (IARC, 2012). La ingestión de cereales contaminados con FBs en animales se ha asociado con pérdida de apetito, peso reducido en la camada, desarrollo óseo bajo en el feto, mortalidad fetal, problemas respiratorios, edema pulmonar porcino, lesiones hepáticas, fibrosis, neurotoxicidad, carcinoma hepatocelular e inmunosupresión (Escrivá et al., 2015). Además, se han descrito otras enfermedades como cáncer de hígado en ratas, hemorragia en el cerebro de conejos y nefrotoxicidad en algunos

animales (Ahangarkani et al., 2014). Los riesgos para los humanos planteados por las FBs son indeterminados en la actualidad; sin embargo, algunos autores han descrito relación entre la ingesta elevada de FBs y el cáncer de esófago (Silva et al., 2014).

1.2.5. Tricotecenos

Los tricotecenos (TCs) simples tipo A y B son producidos por varias especies de *Fusarium*. Los TCs se dividen en cuatro tipos, A, B, C, D, según sus grupos funcionales presentes en la molécula, siendo los tipos A y B los más comunes. Los tricotecenos de tipo A incluyen la toxina T-2 y HT-2, el diacetoxyscirpenol (DAS), el neosolaniol (NEO) y se diferencian de los tricotecenos de tipo B, como nivalenol (NIV), fusarenon-X (FUS-X), deoxinivalenol (DON) y sus formad acetiladas, 3-acetildeoxinivalenol (3-AcDON)y 15-acetildeoxinivalenol (15-AcDON) por la ausencia de un grupo carbonilo en la posición C-8 (Pinton and Oswal, 2014).

Los principales productores de DON son *F. graminearum, F. culmorum y F. cerealis*. Algunas especies de *Trichoderma*. *F. sporotrichioides, F. langsethiae, F. acuminatum y F. poae* producen principalmente las toxinas T-2 y HT-2. Son hongos de suelo e importantes patógenos de plantas de cultivo en campo (Yazar and Omurtag, 2008; Mc Cormick et al., 2011).

TCs contaminan cereales como el trigo, la cebada, la avena, el centeno, el maíz y el arroz. También pueden estar presentes en la soja, la patata, las semillas de girasol, cacahuetes y alimentos procesados como cereales de desayuno, pan, pasta y cervezas (Cano-Sancho et al., 2012; Stanciu et al., 2018; Rodriguez-Carrasco; 2015; Alshanaq and Yu 2017). Además se consideran

compuestos muy estables, tanto durante el almacenamiento, molienda, como en el procesamiento, cocción de alimentos, ya que no se degradan por las altas temperaturas (EFSA, 2011a).

Los TCs han sido clasificados en el grupo 3, como no carcinógenos para humanos (IARC, 2012). Los efectos adversos de los TCs incluyen emesis, náuseas, anorexia, retraso del crecimiento, cambios neuroendocrinos e inmunosupresión. Los TCs tienen múltiples efectos sobre las células eucariotas, siendo el más importante la inhibición de la síntesis de proteínas, ARN y ADN (Awad et al., 2012; Mishra et al., 2014). El mecanismo de inhibición de la síntesis de ADN no se conoce con detalle, pero está unido a la inhibición de la síntesis de proteínas y a la apoptósis (Yazar & Omurtag, 2008; Escrivá et al., 2015; Payros et al., 2016). Las toxinas T-2 y HT-2 se han asociado a aleukia tóxica alimentaria, daño en el ADN, inducción de apoptosis y muerte celular (Adhikari et al., 2017).

1.2.6. Zearalenona

La zearalenona (ZEA) es una micotoxina estrogénica no esteroidea producida principalmente por *F. graminearum, F. culmorum, F. cerealis, F. semitectum*. Se encuentra con frecuencia contaminando el maíz, el trigo, la cebada, el sorgo y el centeno (Calori-Domingues et al., 2016; Pleadin et al., 2017). La producción de ZEA está favorecida por condiciones de alta humedad y bajas temperaturas (Alshannaq & Yu, 2017; Franco et al., 2019).

Varios estudios han demostrado el efecto toxicológico de la ZEA *in vivo* en el sistema reproductivo, incluidas las alteraciones en el tracto reproductivo, el agrandamiento del útero, la disminución de la fertilidad, el aumento de

reabsorciones embrionarias, cambios en los niveles séricos de progesterona y estradiol (Abrunhosa et al., 2014; Escrivá et al., 2015). La ZEA está clasificada por la IARC en el Grupo 3 (IARC, 2012).

1.2.7. Micotoxinas emergentes de *Fusarium*

Las micotoxinas emergentes son producidas principalmente por las especies de *Fusarium: F. proliferatum, F. subglutinans, F. poae, F. avenaceum, F. oxysporum, F. sambucinum*, *F. tricinctum* y *F. vertillioides.* Se denominan emergentes debido a que han sido descubiertas con posterioridad a las descritas anteriormente. Dentro del grupo de las micotoxinas emergentes se encuentran las enniatinas (ENs), beauvericina (BEA) fusaproliferina (FUS) y moniliformina (MON).

Se conocen al menos 29 análogos de las ENs, agrupados en diferentes series (A, B, C, D, E, F, G, H, I, J, K, L, M, N, O y P) en función de los grupos funcionales que presenta su estructura. Sin embargo, tan sólo siete ENs han sido detectadas en alimentos, presentando una mayor incidencia las ENs A, A1, B y B1, consideradas por lo tanto de mayor interés (EFSA, 2014; Tittlemier et al., 2013). Las ENs A, A_1, B, B_1 y BEA se han detectado principalmente en cereales como trigo, arroz, cebada, centeno y avena detectándose en muy eleva frecuencia y concentración (Sifou et al., 2011; Luz et al., 2017; Kim et al., 2019).

Estas micotoxinas presentan diversas actividades biológicas, que incluye una serie de propiedades antimicrobianas, insecticidas y herbicidas, así como actividad antibiótica sobre diferentes organismos patógenos (Klaric et al., 2010).

Las ENs son citotóxicas en diferentes líneas celulares en concentraciones micromolares, pudiendo originar muerte celular por apoptosis, bien a través de la vía mitocondrial o por la inducción de la necrosis vinculada al daño lisosómico (Prosperini et al., 2017; Fraeyman et al., 2018). Asimismo, la BEA ha sido relacionada con alteración de las enzimas antioxidantes y citotoxicidad (Mallebrera et al., 2014; Klaric et al., 2010).

1.2.8. Toxinas de *Alternaria*

Las especies de *Alternaria* producen más de 70 toxinas, pero una pequeña proporción de ellas se ha caracterizado por su alta incidencia en alimentos como alternariol (AOH), alternariol monometil éter (AME), tentoxin (TEN), ácido tenuazónico (TeA) y altertoxinas (ATX) (Ostry et al., 2008).

Las toxinas de *Alternaria* han sido detectadas en el trigo, el sorgo, la cebada, girasol, el tomate, las manzanas, aceitunas, así como en frutas cítricas y verduras. *Alternaria alternata* es la especie de *Alternaria* más común en las frutas y hortalizas cosechadas. Debido a su crecimiento incluso a bajas temperaturas, las especies de *Alternaria* se asocian con el deterioro de estos productos durante el transporte y almacenamiento de los alimentos refrigerados (EFSA, 2011b; Rodríguez-Carrasco et al., 2016; Escrivá et al., 2017).

Las micotoxinas de *Alternaria* presentan diversas actividades biológicas como propiedades antimicrobianas, fitotóxicas y citotóxicas. Además se han estudiado como agente quimioprotector del cáncer y por su potencial antitumoral. TeA y TEN se han estudiado como posibles herbicidas (Lou et al., 2013).

Las micotoxinas producidas por *Alternaria* son tóxicas *in vivo* habiéndose estudiado su toxicidad en ratas y embriones de pollo (Fraeyman et al., 2017). En estudios *in vitro*, las toxinas de *Alternaria,* en especial el AOH y AME de relacionaron con efecto citotóxico en líneas celulares Caco-2 (Fernández-Blanco et al., 2014).

1.3. Análisis de micotoxinas

El establecimiento de un protocolo de muestreo adecuado es el primer paso crítico para garantizar la fiabilidad y la veracidad de los resultados. La UE ha establecido un procedimiento de muestreo común referido en el Reglamento nº 401/2006 para el control oficial de los niveles de micotoxinas en los productos alimenticios (EC, 2006), modificado por UE 519/2014 (EC, 2014).

Con el fin de poder determinar bajas concentraciones de micotoxinas en matrices complejas, como alimentos, es necesario realizar procesos de extracción y purificación que permitan eliminar interferencias de la matriz y concentrar el analito (Pereira et al., 2014). La elección de una técnica de extracción depende de varios factores, como el tipo de matriz, las propiedades fisicoquímicas del analito (s) y el método de detección. Las técnicas de extracción convencional como la extracción liquida-líquida (LLE) y la extracción en fase sólida (SPE) siguen siendo ampliamente utilizadas, y además, se utilizan otras técnicas como Micro-Extracción en Fase Sólida (SPME, Solid Phase Micro-Extraction) y Dispersión de Matriz en Fase Sólida (MSPD, Matrix Solid Phase Dispersion). En los últimos años, los avances se han centrado en disminuir las cantidades de solventes, especialmente aquellos que son peligrosos para el medio ambiente y la salud humana, así como la automatización de extracción on line (Capriotti et al., 2013). También han surgido nuevas metodologías como QuEChERS (Quick,

Easy, Cheap, Effective, Rugged and Safe) o la micro-extracción líquido-líquido dispersiva (DLLME) (Meneely et al, 2011; Pereira et al., 2014),

El método QuEChERS es un procedimiento analítico simple, rápido, seguro y válido para llevar a cabo análisis múltiples de residuos que proporciona resultados satisfactorios y en la actualidad es una de las técnicas más empleadas que permite realizar la extracción y purificación en presencia de sales inorgánicas (González-Curbelo et al., 2015). Consiste en una extracción con ACN y sales, generalmente sulfato de magnesio o sulfato de sodio, seguido de un paso de purificación por dispersión en fase sólida (d-SPE). La fase de purificación se lleva a cabo mediante la adición de PSA (Primary and Secondary Amine) la cual se aplica generalmente en combinación con C_{18} para eliminar pigmentos polares, ácidos orgánicos y otros productos presentes en la matriz (Zhang et al., 2012; Rejczak and Tuzimski, 2015). El método QuEChERS ha sido empleado para la extracción de multi-micotoxinas en diferentes alimentos (Anastassiades et al., 2003; González-Jartín et al., 2019; Sartori et al., 2015).

DLLME es una técnica de micro-extracción simple basada en el uso de un solvente de extracción mezclado con un solvente dispersante (Zgola-Grzéskowiak & Grzéskowiak, 2011). Esta técnica es ampliamente utilizada en la actualidad en análisis de muestras de alimentos, por ser una metodología fácil, eficiente, económica y ambientalmente responsable debido a la poca cantidad de disolvente que se utiliza (Arroyo-Manzanares et al., 2015; Serrano et al., 2016; Pallarés et al., 2018).

El reglamento de la Comisión Europea (CE 657/2002) establece los principios básicos que rigen la legislación europea sobre el control analítico de calidad y procedimiento de validación de la metodología analítica e

interpretación de los resultados como linealidad, efecto de matriz, exactitud, precisión, repetibilidad, reproducibilidad, límite de detección (LOD) y límite de cuantificación (LOQ).

Existen diferentes técnicas para la determinación de micotoxinas en alimentos, pero las más empleadas son la cromatografía líquida (LC) y la cromatografía de gases (GC) acopladas a diferentes detectores, siendo las más comunes es la espectrometría de masas en tandem (MS/MS) con triple cuádrupolo (QqQ), trampa de iones (IT) o con tiempo de vuelo (TOF) debido a su alta selectividad y sensibilidad (Di Stefano et al., 2012; Turner et al., 2015).

La LC-MS/MS es el método de referencia en el campo de análisis de micotoxinas, permitiendo la identificación y cuantificación altamente precisa y especifica de compuestos no volátiles. Dado su alta precisión y especificidad se ha aplicado a la determinación de múltiples-micotoxinas en diferentes productos alimenticios (Stanciu et al., 2017; Meerpoel et al., 2018; Pascari et al., 2018).

Generalmente la GC-MS/MS se emplea para la determinación de micotoxinas del grupo de los tricotecenos tipo A y B (Rodríguez-Carrasco et al., 2014; Juan et al., 2017; Bouafifssa et al., 2018). Así también se utiliza para otras micotoxinas como ZEA, PAT, OTA y AFs en matrices de cereales, vino y cerveza, alimentos infantiles (Rahmani et al., 2009, Sadok et al., 2019; Stanciu et al., 2018). Para volatilizar la micotoxina es necesario realizar un paso previo de derivatización, en la cual se logra una modificación química pasando los grupos hidroxilo, carboxilo y amino en sus derivados éster, acilo y alquilo con la finalidad que los compuestos sean lo suficientemente volátiles para su análisis por GC.

1.4. Legislación de micotoxinas

La UE ha establecido niveles máximos de algunas micotoxinas en determinados productos alimenticios. El reglamento (CE) nº 1881/2006 de la Comisión del 19 de diciembre de 2006, fija el contenido máximo de determinados contaminantes, incluyendo las micotoxinas AFB1, AFB2, AFG_1, AFG_2, AFM_1, OTA, DON, FB_1, FB_2, ZEA y PAT en diferentes productos alimenticios. Esta normativa ha sido modificada sustancialmente por los siguientes reglamentos.

❖ Reglamento (UE) 1126/2007, por el que se fija el contenido máximo de micotoxinas de *Fusarium* en el maíz y los productos del maíz.

❖ Reglamentos (UE) 105/2010, 594/2012 y el 2015/1137 por los que se fijan el contenido máximo de OTA en los productos alimenticios.

❖ Reglamentos (UE) 165/2010 y 1058/2012 en lo concerniente al contenido máximo de AFs en los productos alimenticios.

❖ Reglamento (UE) 212/2014 de la Comisión, en relación al contenido de citrinina (CIT) en complementos alimenticios basados en arroz fermentado con levadura roja *Monascus purpureus*.

EL rango de contenidos máximos de las principales micotoxinas en diferentes alimentos destinados al consumo humano se recoge en la Tabla 2.

Tabla 2: Contenido máximo de micotoxinas en alimentos

Micotoxina	Alimentos	Contenidos máximos (µg/kg)
AFB_1	Cereales, derivados de cereales, maíz, frutos secos, especias, alimentos infantiles, alimentos dietéticos	0.1-8
AFs	Cereales, derivados de cereales, maíz, frutos secos, especias, alimentos infantiles, alimentos dietéticos	4-15
AFM_1	Leche cruda, alimentos para lactantes	0.025-0.05
OTA	Cereales, derivados de cereales, café, vino, zumo de uva, frutos secos, cerveza, cacao y derivados, productos cárnicos, especias y regaliz, alimentos infantiles, alimentos dietéticos	0.5-10
PAT	Zumos y néctares de frutas, derivados de manzana, alimentos infantiles	10-50
DON	Cereales, derivados de cereales, alimentos infantiles	20-1750
ZEA	Cereales, derivados de cereales, maíz, alimentos elaborados a base de maíz, alimentos infantiles	20-200
FB_1+FB_2	Maíz y alimentos elaborados a base de maíz	20-2000

Además la comisión europea ha establecido algunas recomendaciones para ciertas micotoxinas:

❖ Recomendación 2013/165 en la cual se ha fijado diferentes niveles indicativos de límites máximos de las toxinas de T-2 y HT-2 en cereales y productos a base de cereales.

❖ Recomendación 2006/583) por la cual se establece medidas de prevención y reducción de las toxinas de *Fusarium* en cereales y productos a base de cereales.

❖ Recomendación 2003/598 por la cual se fijan medidas de prevención de la contaminación por patulina del zumo de manzana y los ingredientes de zumo de manzana en otras bebidas.

❖ Recomendación 2012/154 sobre el control de la presencia de alcaloides de cornezuelo en los alimentos y los piensos.

El Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) ha establecido la estimación de la dosis consideradas como seguras para algunas micotoxinas, como la ingesta diaria tolerable (Tolerable Daily Intake TDI), o la ingesta diaria tolerable máxima provisional (Provisional Máximum Tolerable Weekly Intake PMTDI). La JECFA y la EFSA ha fijado IDT para DON y sus formas acetiladas 3-ADON y 15-ADON (JECFA, 2001), NIV (EFSA, 2013), ZEA (EFSA, 2011), PAT (SFC, 2000), FBs (JECFA, 2001) OTA (EFSA, 2006) y las toxinas T2 y HT2 (EFSA, 2011) (Tabla 1).

Tabla 3: IDT de algunas micotoxinas

Micotoxina	**Ingestas diarias tolerables µg/kg**	**Referencia**
Σ (DON+3ADON+15ADON)	1	JECFA, 2001
Σ (T2+HT2)	0.1	EFSA, 2011[a]
NIV	120	EFSA, 2013
ZEA	0.25	EFSA, 2011c
FBs	2	JECFA, 2001
PAT	0.4	SCF, 2000
OTA	0.017	EFSA, 2006

1.5. Estudios de dieta total

El concepto de Estudio de Dieta Total (EDT) surgió a fines de los años cincuenta en Estados Unidos en respuesta a dos tipos de contaminantes ambientales que se habían introducido en la cadena alimentaria, los radionúclidos

provenientes de ensayos de armas nucleares y los residuos de pesticidas químicos procedentes de aplicaciones agrícolas (Egan, 2013). Posteriormente, la lista de analitos se amplió para incluir otros residuos de pesticidas, micotoxinas y compuestos químicos industriales (Pennington, 1996; Sirot et a., 2013; Betsy et al., 2012). En la actualidad, en los estudios EDT se han incluido, además de los anteriormente citados, diferentes analitos debido a su naturaleza tóxica y carcinogénica que incluyen acrilamida, benceno, furano, nitratos/nitritos, bisfenol A, anilina y aditivos alimentarios (Hulin et al., 2014; Lee et al., 2015).

El enfoque de EDT ha sido promovido y respaldado por la OMS junto con la FAO desde la década de 1960 y más recientemente en 2011 por la EFSA (EFSA/FAO/OMS, 2011). En los EDT los alimentos a analizar representan una muestra representativa de la dieta total de la población, los alimentos se analizan tal como se consumen y se evalúa la exposición dietética de la población a contaminantes de estudio, lo que los diferencia de otros estudios en los que se analizan niveles de contaminantes en alimentos no procesados de manera individual (EFSA/FAO/OMS, 2011; Lee et al., 2015).

Los EDT consisten en la selección, recolección y análisis de los alimentos comúnmente consumidos. Esta metodología está diseñada para determinar la cantidad de sustancias químicas y nutrientes de interés ingeridas por distintos grupos de población (EFSA/FAO/OMS, 2011). Desde el punto de vista de la salud pública, un EDT puede ser un enfoque complementario valioso y rentable para los programas de vigilancia y monitorización de contaminantes en alimentos que proporciona datos confiables para realizar evaluaciones de riesgo en los distintos grupos de población de un determinado país (Devlin et al., 2014).

Por lo general, los EDT proporcionan información directa sobre la contribución de diferentes alimentos o grupos de alimentos a la exposición de contaminantes, teniendo en cuenta el procesado de alimentos para el consumo, no solo a través de la cocción, sino también por la posible migración desde el almacenamiento hasta el alimento envasado (Kroes et al., 2002; Kim et al., 2015).

Los datos resultantes tras la aplicación de un EDT se pueden usar para establecer prioridades y políticas de seguridad alimentaria. Además, los EDT por su diseño, proporcionan concentraciones precisas de contaminantes en los alimentos analizados (Moy, 2013).

Hasta la actualidad, los principales países europeos que han realizado EDT son Bélgica, República Checa, Finlandia, Francia, Irlanda, Italia, España, Suecia, Países Bajos y el Reino Unido (Dofcova et al., 2016), para diferentes contaminantes como dioxinas (Zhang et al., 2013), bifenilos policlorados (PCBs) (Bramwel et al., 2017), pesticidas (Chen et al., 2015) y metales pesados (Rose et al., 2010). Por lo general, los estudios han sido financiados por agencias gubernamentales y organismos internacionales como la FAO y/o OMS.

Se distinguen dos tipos de EDT, los EDT en sentido estricto y los EDT-like, en los cuales el estudio no cubre la dieta total, sino que se centra en grupos de alimentos específicos que contribuyen de manera importante a la exposición de las sustancias químicas consideradas (EFSA/FAO/OMS, 2011).

1.5.1. Metodologías utilizadas en estudio de dieta total

Tradicionalmente, los EDT han sido clasificados de acuerdo al tipo, selección y agrupación de los alimentos en:

1.5.1.1. Cesta de mercado

Los alimentos comúnmente consumidos son adquiridos y preparados teniendo en cuenta los hábitos culinarios de cada país. Los alimentos se pueden combinar ya analizar en forma de "composite" y se obtiene información de ingesta medias de la población, aunque cuando se detectan concentraciones elevadas de sustancias químicas es necesario realizar el análisis de alimentos de forma individual. Los alimentos recolectados deben cubrir al menos uno de los dos aspectos principales de la representatividad: estacionalidad y variación geográfica (EFSA/FAO/OMS, 2011). La cobertura geográfica es importante debido a las posibles diferencias regionales en los patrones dietéticos y en los niveles de sustancias químicas en los alimentos. Incluso si no se hace una diferenciación en la evaluación de la exposición entre regiones, el muestreo debe cubrir las posibles diferencias geográficas. La estacionalidad es importante, particularmente para los alimentos en los que los niveles de sustancias pueden variar debido a las condiciones climáticas o las variaciones estacionales del suministro de alimentos (Kroes et al., 2002; Marín et al., 2013; de Nijs et al., 2016).

1.5.1.2. Alimentos individuales

Los alimentos son adquiridos, preparados de acuerdo a las tradiciones culinarias locales y analizados de forma individual. Aplicando este método de análisis se obtiene mayor información. Si el nivel de contaminación en el

alimento resulta muy alto se realiza un estudio de monitorización, normalmente en varios puntos de la cadena de suministro, con la finalidad de rastrear el contaminante (de Nijs et al., 2016). Particularmente este método se aplica solo para cierto tipo de alimentos que representan una dieta global de una población debido al alto coste que supone su realización (Kroes et al., 2002; Bakker et al., 2009). El tamaño de la muestra, la estrategia de muestreo y la preparación de la muestra deben documentarse, ya que pueden influir en los resultados de la exposición (De Rijk et al., 2015; de Nijs et al., 2016).

1.5.1.3. Dieta duplicada

Inicialmente, los métodos de dieta duplicada proporcionan información sobre las ingestas individuales. Son particularmente útiles para estimar la exposición cuando no se dispone de datos de consumo nacional o cuando se está realizando una investigación de la exposición de un subgrupo de población en particular (Kroes et al., 2002). Se realiza una copia exacta de todos los alimentos consumidos por una persona en un período de tiempo determinado (24 horas), recogido por voluntarios con un diario de alimentación. Como resultado, los estudios de dieta duplicada siempre están limitados en el número de participantes y días de muestreo, lo que dificulta la extrapolación de los resultados a poblaciones más grandes. Dado que los alimentos a analizar son una combinación de varios alimentos que se consumen, la contaminación rara vez se debe a un ingrediente o alimento específico, lo que hace que el método no sea adecuado para el cumplimiento de la legislación, pero permite comparar los datos de exposición con valores de niveles de referencia establecidos por legislaciones actuales orientación basados en la salud (de Nijs et al., 2016; Jekel and Van Egmond, 2014; Tomerlin et al., 2002).

A modo de estandarizar la metodología de un EDT, la EFSA/FAO/OMS ha desarrollado una guía para facilitar su aplicación que incluye una descripción de los conceptos de EDT y proporciona principios sobre la metodología, desde la planificación hasta la recopilación de resultados analíticos, la evaluación de la exposición y la comunicación de los resultados, con el objetivo de proponer un enfoque general para facilitar el uso de la información de EDT a nivel internacional (EFSA/FAO/OMS, 2011).

Además, la OMS ha convocado diversos talleres internacionales de EDT para alentar a los países a implementar una metodología confiable y armonizada. La OMS ha desarrollado una lista de sustancias químicas prioritarias en la realización de EDT como pesticidas, metales pesados, micotoxinas, radionúclidos, elementos traza, incluyendo parámetros analíticos como límites de detección, límites cuantificación e instrumentación que se recomienda utilizar para el análisis (WHO, 2002; WHO, 2006; WHO, 2015).

1.5.2. Componentes de un EDT

Para la realización de un EDT es necesario tener en cuenta varios procedimientos (Figura 2).

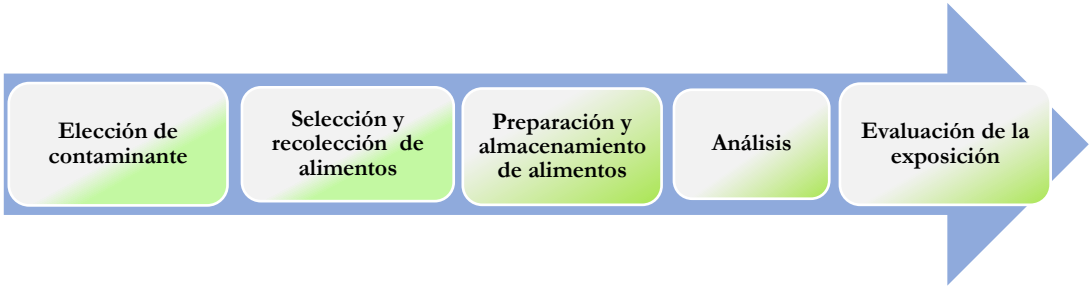

Figura 2: Principales componentes de EDT.

1.5.2.1. Elección de contaminantes

El proceso de establecimiento de prioridades es de suma importancia y debe ser realizado por los gestores de riesgos en estrecha consulta con los asesores de riesgos y con la participación de todos los interesados claves (EFSA/FAO/OMS, 2011). La OMS establece como contaminantes prioritarios las micotoxinas (AFs, PAT, FB1 y OTA) debido a su efecto perjudicial para la salud humana (WHO, 2006).

1.5.2.2. Selección y recolección de los alimentos representativos de la dieta de la población

Los alimentos se seleccionan de acuerdo con los patrones de consumo de alimentos de cada país donde pretende realizar el estudio. El número de lugares de recolección generalmente se determina primero dentro del límite de presupuesto y el período de tiempo de la EDT. La selección de los sitios de recolección puede basarse en el tamaño de la población, la distancia entre los sitios y la condición geográfica (Kim et al., 2015).

1.5.2.3. Preparación y almacenamiento de alimentos

Algunos alimentos requieren de poca preparación (frutas, vegetales) y otros requieren de una cocción previa (carnes). Las partes no comestibles (huesos, piel de pescado, semillas, cáscaras no comestibles) siempre se eliminan antes o después de la cocción. Dependiendo del enfoque de EDT, los alimentos pueden prepararse en alimentos individuales, "*composites*" o en grupos de alimentos (Vannort et al., 2013). Algunas sustancias pueden requerir condiciones específicas, aunque por lo general los alimentos deben almacenarse

en congelación (-20ºC), y descongelarse en un refrigerador antes de la preparación de la muestra. Los alimentos no perecederos (bebidas, frutos secos, alimentos enlatados) se pueden almacenar a temperatura ambiente en lugares secos y frescos (EFSA/FAO/OMS, 2011).

La OMS proporciona una lista de métodos e instrumentación para la realización del EDT se caracterizan en gran medida por su selectividad y sensibilidad para poder determinar los niveles bajos de analitos (WHO, 2015).

El objetivo principal es estimar los niveles probables de exposición de la población a contaminantes teniendo en cuenta la dieta total y el nivel de riesgo a la salud (Petersen, 2013).

1.6. Biomonitorización

Evaluar la exposición a través del uso de biomarcadores es una forma alternativa que se está implantando los últimos años. La variación individual en la absorción, distribución, metabolismo y excreción se integra cuando se usan biomarcadores, por lo que lleva a una evaluación más precisa de la exposición a nivel individual (Heyndrickx et al., 2015).

La exposición a micotoxinas puede ser evaluada por biomarcadores de exposición, es decir a través de la determinación micotoxinas y/o sus metabolitos principales de fase I y fase II (conjugados de glucurónido) en orina. En contraste, los biomarcadores de efecto pueden usarse para evaluar el resultado después de la exposición a micotoxinas e incluyen, por ejemplo, cambios en el nivel de proteínas específicas (incluidas las enzimas), metabolitos celulares (metabolómica) o perfiles de expresión génica (toxicogenómica) que

resultan de la alteración específica en las vías metabólicas (Valencia-Quintana et al., 2014; De Nijs et al., 2016).

El análisis de las micotoxinas en orina humana es una alternativa muy adecuada para la evaluación de la exposición a las micotoxinas. Además, la recolección de orina es un método de muestreo fácil y no invasivo. Se han propuesto en la literatura varios biomarcadores de exposición para algunas de las micotoxinas de mayor preocupación toxicológica, por ejemplo la AFM_1, AFM_2 y aflatoxina-N7-guanina, aflatoxina-albúmina sérica (Egner eta et al., 2006; Turner et al., 2012). Aunque también se han reportado otros metabolitos como AFP_1, AFQ_1 (Vidal et al., 2018).

En el caso del DON y considerando la corta vida media de excreción, solo los niveles urinarios de DON o sus formas de glucurónido se han propuesto como biomarcadores. Se 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). se encontró una relación entres los niveles de DON urinario y la ingesta de DON vía consumo de cereales y alimentos a base de cereales (Turner et al., 2010). Posteriormente un método directo para la cuantificación de los metabolitos de DON, como el desoxinivalenol-3-glucurónido (DON-3-glucurónido) fue desarrollado por Warth et al. (2012), apoyado por la alta proporción de este metabolito en la orina (Shephard et al., 2013). En cuanto a las micotoxinas y metabolitos como 3ADON, 3-GlcA, 15-GlcA, DOM-GlcA, también se han detectado en muestras de orina (Vidal et al., 2016; Warth et al., 2016).

Asi mismo, la exposición puede evaluarse convirtiendo las concentraciones de micotoxinas urinarias en niveles de ingesta, teniendo en cuenta su cinética, como la tasa de excreción (es decir, el porcentaje de las micotoxinas ingeridas excretadas como el compuesto o metabolitos en la orina), la producción diaria de orina (definida por creatinina) y peso corporal (Gratz et al., 2014; Rodríguez-Carrasco et al., 2014; Sarkanj et al., 2013; Solfrizzo et al., 2014). Este recálculo solo es posible para las toxinas que muestran una eliminación rápida y un tiempo de residencia medio corto (como por ejemplo el DON) (Papageoglou et al., 2018).

Con respecto a la OTA, la OTα, formada por hidrólisis en el tracto gastrointestinal, es el principal metabolito de la OTA en los seres humanos. El producto de desintoxicación OTα se metaboliza aún más a un glucurónido y posiblemente a sulfato; estos metabolitos se eliminan mucho más eficientemente que OTA y se excretan fácilmente con la orina (Duarte et al., 2011). Por lo tanto, después de la hidrólisis enzimática con ß-glucuronidasa/arilsulfatasa, OTα se encuentra a menudo en concentraciones considerablemente altas en la orina, y puede servir como biomarcador adicional de la exposición a las micotoxinas (Muñoz et al., 2010; Coronel et al., 2011; Klapec et al., 2012; Ali et al., 2018). También se ha demostrado la presencia de conjugados de OTA y OTB en varias muestras de orina (Muñoz et al., 2017; Vidal et al., 2018) lo que indica que la hidrólisis enzimática de los metabolitos de fase II es altamente recomendable en estudios de biomonitoreo en humanos.

La información disponible sobre la presencia de ZEA y sus principales metabolitos α-ZOL y β-ZOL en muestras de orina es escasa pero varios estudios coinciden en el análisis de ZEA libre, α-ZOL, β-ZOL y algunos de los productos

más comunes de hidroxilación y glu-curonidación. En este sentido, algunos estudios recientes han demostrado la presencia de conjugados de ZEA en muestras de orina como 8-OH-ZEN, 13 -OH-ZEA, 15-OH-ZEA y ZEA-14-glucurónido. También han sido descritos otros metabolitos, concretamente ZAN, α-ZAL y β-ZAL (Heyndrickx et al., 2015; Li et al., 2018).

Otras micotoxinas también han sido investigadas en muestras de orina como diacetoxyscirpenol (DAS), 15- monoacetoxyscirpenol (15-MAS), FUS-X, CIT, deihidrocitrinina (HO-CIT) y las toxinas T-2, HT-2 y HT-2-4-GlcA (Gerding et al., 2015; Huybrechts et al., 2015; Heyndrickx et al., 2015; Vidal et al., 2018).

1.7. Evaluación de riesgos

El análisis del riesgo es la base de la política de la seguridad alimentaria y se sostenta en tres componentes principales: i) evaluación de riesgos (asesoramiento científico y análisis de datos); ii) gestión de riesgos (reglamentación y control); y iii) comunicación de riesgo para comunicar la información obtenida (Figura 3).

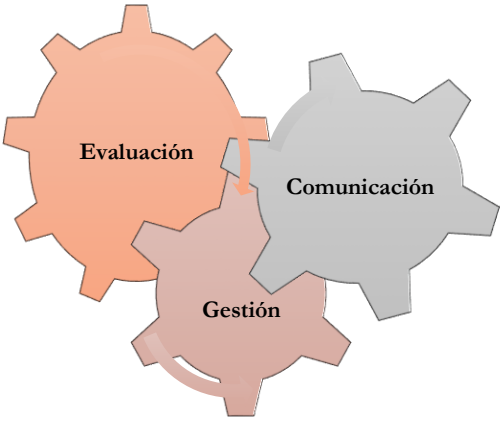

Figura 3: Componentes del análisis de riesgo.

La evaluación de riesgos ocasionados por peligros presentes en los alimentos debe llevarse a cabo de forma transparente, estructurada y multidisciplinar, y para ello se encargan diferentes instituciones a nivel internacional como los expertos de la FAO/OMS a nivel europeo, la EFSA actúa como órgano científico de referencia principal, mientras que a nivel nacional se encargan las agencias de seguridad alimentaria y centros de investigación correspondientes.

La metodología puede variar en función de la clase de peligro (químico, biológico o físico), el escenario de inocuidad de los alimentos (peligros conocidos, emergentes, o resultado de la aplicación nuevas tecnologías de procesado) así como el tiempo y los recursos disponibles. (Figura 4)

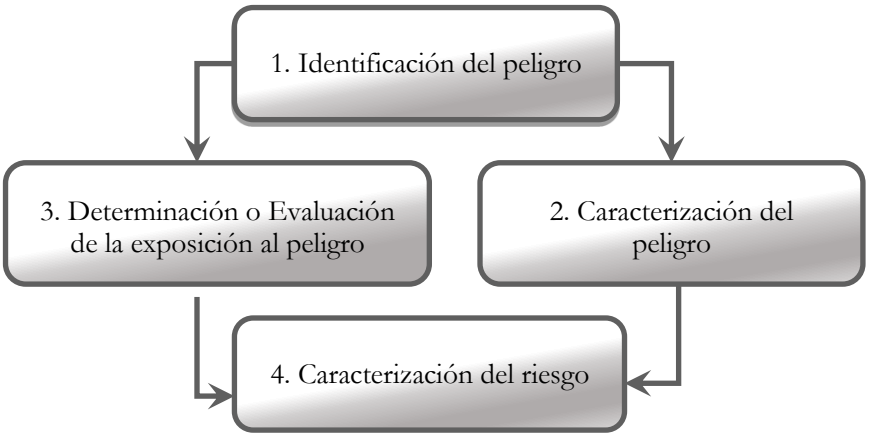

Figura 4: Fases de la evaluación de riesgos.

La finalidad de una evaluación de riesgo es integrar los resultados de la identificación del peligro, su caracterización y de la evaluación de la exposición. El resultado general será una estimación de la probabilidad de aparición de los efectos adversos del tóxico en la población objeto del estudio, incluyendo las incertidumbres de todas las estimaciones anteriores.

Las evaluaciones de exposición combinan los datos de consumo de alimentos con datos sobre la concentración de productos químicos en los alimentos. En general, los datos disponibles sobre los datos de consumo de alimentos se recopilan de forma independiente y se utilizan junto con los resultados analíticos, obteniéndose la estimación de la exposición (IPCS, 2009; Verger et al., 2013; Petersen, 2013).

Las estimaciones de la exposición dietética se interpretan en comparación con un punto final toxicológico o un valor de referencia nutricional para el alimento. La exposición dietética media se comparará con un valor de referencia toxicológico crónico (a largo plazo) o las dosis de referencia como la

ingesta diaria admisible (IDA) o IDT en el caso de las micotoxinas. (FAO & OMS, 2008).

1.7.1. Metodologías utilizadas en la evaluación de riesgos

Según la FAO/OMS (2008) la estimación de la exposición se puede abordar desde una *aproximación básica* usando métodos de screening a *estimaciones refinadas* con datos de consumo de alimentos y de concentración de contaminantes en alimentos, tales como los EDTs (Figura 5).

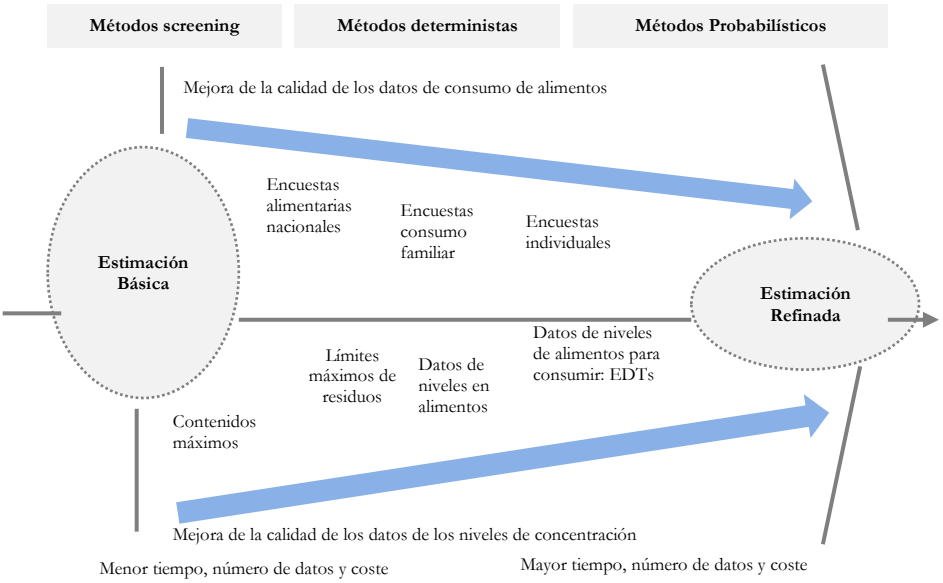

Figura 5: Métodos para estimar la exposición (FAO/OMS, 2008).

1.7.1.1. Métodos screening

Son realizados por organizaciones internacionales como el Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios (JECFA) y reuniones conjuntas sobre residuos de plaguicidas (JMPR) para reflejar los detalles de las

exposiciones que deben considerarse. Difieren en gran medida según la categoría de sustancia, son diferentes para aditivos alimentarios, pesticidas y medicamentos veterinarios. Su finalidad no es evaluar la exposición real de la dieta, sino detectar contaminantes en alimentos para las cuales es necesaria una evaluación refinada de la exposición alimentaria.

1.7.1.2. Métodos deterministas

En las evaluaciones de estimación puntual, un único valor de nivel de contaminante se combina con un único parámetro de consumo. El resultado es una estimación de exposición única con un alto grado de incertidumbre. El aporte para la concentración a menudo proviene de un estudio de monitoreo de alimentos o un EDT (De Nijs et al., 2016).

Las exposiciones calculadas por producto alimenticio se pueden combinar para evaluar la exposición de la dieta total en exposiciones múltiples. La principal ventaja de este enfoque es que es fácil de realizar y se usa a menudo para una primera identificación de riesgo (FAO/OMS, 2008). Con este enfoque, se genera una distribución de las exposiciones promedio diarias para diferentes individuos dentro de una población (EFSA, 2012; 2015).

1.7.1.3. Método probabilístico

Con el uso del enfoque probabilístico, se puede estimar la exposición aguda y crónica a través de los alimentos. La exposición aguda se calcula combinando los patrones de consumo individual diario de una encuesta de consumo de alimentos con niveles seleccionados al azar por producto alimenticio de un banco de datos con niveles de micotoxinas en muestras

individuales (De Nijs et al., 2016). La EFSA utiliza actualmente este método para evaluar la exposición crónica a contaminantes y aditivos alimentarios. Las exposiciones individuales resultantes por producto alimenticio se suman para obtener la exposición por día y posteriormente se dividen por el peso corporal del individuo. Este procedimiento se repite varias veces dando como resultado estimaciones de exposición diarias individuales que reflejan todas las combinaciones de las concentraciones admisibles y consumos diarios de una población (EFSA, 2012).

La parte superior de la distribución representa a los consumidores con una alta ingesta del compuesto (consumidores altos), lo cual es importante para evaluar el riesgo agudo. Con el estudio probabilístico se puede caracterizar la exposición de toda la población de una forma más precisa, diferenciando los grupos de consumidores, es decir, teniendo en cuenta los diferentes percentiles de consumo en la población: 90th, 95th, 97.5th. Además permite obtener resultados más manejable para ser usado posteriormente, para compararlo con la IDT y hacer una aproximación del riesgo de la población (EFSA, 2011d).

1.8. Datos de consumo de alimentos

En las evaluaciones de exposición dietética, es tan importante obtener información precisa sobre los niveles de contaminantes en los alimentos como obtener información precisa sobre el consumo de alimentos.

Los datos de consumo de alimentos reflejan lo que los individuos o grupos consumen en términos de alimentos sólidos, bebidas, incluyendo agua potable y suplementos. El consumo de alimentos se puede estimar a través de encuestas de consumo de alimentos a nivel individual o doméstico o se puede

aproximar a través de estadísticas de producción de alimentos (FAO & OMS, 2008). Existen tres categorías amplias de datos de consumo de alimentos: (i) hojas de balance de alimentos, (ii) encuestas de hogares (iii) encuestas de patrones de consumo de alimentos individuales. Estos se describen brevemente a continuación.

Hojas de balance de alimentos: proporcionan estimaciones anuales brutas de la disponibilidad nacional de productos alimenticios. Los datos de suministro de alimentos se calculan en balances de alimentos, que son datos a nivel nacional, de la producción anual de alimentos, cambios en las existencias, importaciones y exportaciones, y uso agrícola y uso industrial. Existen algunas limitaciones en el uso de las hojas de balance para estimar las exposiciones. No se consideran los residuos a nivel doméstico e individual. Generalmente solo proporcionan datos para productos crudos y algunos alimentos semielaborados, por lo tanto hay poca información sobre alimentos procesados o alimentos de componentes múltiples. Según la OMS, las estimaciones de consumo tienden a ser aproximadamente un 15% más altas que las estimaciones de consumo derivadas de encuestas de hogares o encuestas dietéticas nacionales.

Encuestas de hogares: los alimentos disponibles a nivel de hogar pueden estimarse mediante encuestas de presupuesto y encuestas de consumo. El primer tipo de encuesta proporciona información sobre las compras de alimentos en términos de gastos y se utiliza para la política económica. En las encuestas de consumo de los hogares, también se registran las cantidades de alimentos y bebidas. En su mayor parte, solo se anotan los gastos de las compras de alimentos destinadas al hogar. Sin embargo, estos datos no proporcionan información sobre la distribución del consumo de alimentos entre los miembros individuales del hogar (Kroes et al., 2002; Petersen 2013).

Encuestas individuales: proporcionan datos sobre el consumo de alimentos por individuos específicos. Los métodos para evaluar el consumo de alimentos de individuos pueden ser retrospectivos (registro 24 horas, cuestionarios de frecuencia de alimentos e historias de dieta), prospectivos (diarios de alimentos, registros de alimentos o porciones duplicadas), o una combinación de ellos. Los estudios más utilizados son aquellos que utilizan una combinación de los métodos de registro y el método de frecuencia de alimentos (Petersen, 2013). Las cantidades de cada alimento consumido pueden o no ser registradas, dependiendo de los objetivos del estudio. Si se deben calcular las ingestas de nutrientes o las exposiciones químicas a los alimentos, las cantidades consumidas deben medirse con la mayor precisión posible. Las cantidades pueden determinarse pesando o midiendo el volumen.

Diversas autoridades nacionales e internacionales, como Ministerio de Agricultura, Pesca y Alimentación (MAPAMA), Food Agriculture Organization (FAO), Food and Drug Administration (FDA) proporcionan datos de producción de alimentos y consumo dentro y fuera del hogar.

El patrón alimentario seguido en la comunidad valenciana es la dieta Mediterránea que se caracteriza por un modelo nutricional basado en un alto consumo de cereales, frutas y verduras y una cantidad moderada de pescado, productos lácteos y carne. (Nomikos et al., 2018; CIEAM, 2015).

No obstante los datos de consumo de alimentos de la población proporcionados por los organismos oficiales en España (MAPAMA) difieren de las recomendaciones de dieta saludable basados en la dieta Mediterránea. En la Figura 5 muestra la pirámide representativa de los alimentos y raciones que componen la alimentación saludable de la dieta Mediterránea (Figura 6)..

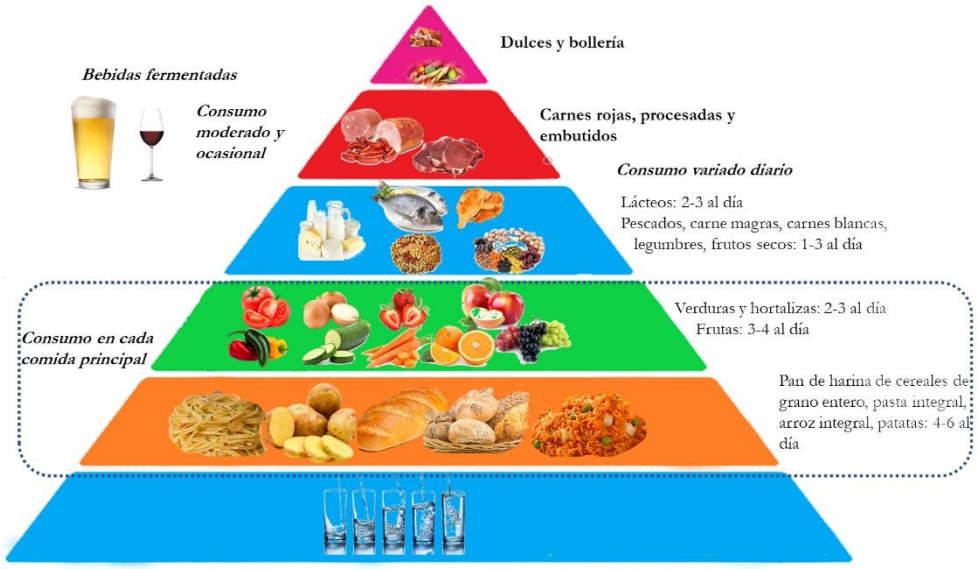

Figura 6: Pirámide representativa de los alimentos y raciones que componen la dieta Mediterránea.

En los últimos años, se han realizado en varios países EDTs referentes a micotoxinas. La Tabla 4 resume los estudios realizados y las micotoxinas determinadas. Las micotoxinas analizadas en los EDT dependen del enfoque del estudio aunque la mayoría incluyen análisis de multi-micotoxinas que comprende micotoxinas clásicas y micotoxinas emergentes como esterigmatocistina (STE), citrinina (CIT), ácido cicloplazónico (CPA), moniliformina (MON), gliotoxina (GLIO), ácido micofenolated (MPA), verruculogen (Verru), las alternarias y los ergot alcaloides.

Tabla 4: EDT sobre análisis de micotoxinas.

País	Micotoxinas analizadas	Referencia
Canadá	OTA	Tam et al., 2011
China	AFB_1, AFB_2, AFG_1, AFG_2, PAT, ZEA, DON, FB_1, FB_2, FB_3, ZEA, α-ZOL, βZOL, DON, 3ADON, 15ADON	Yau et al., 2016
China	STC, CIT, CPA, MON, GLIO, MPA, Verru	Qiu et al., 2017
Francia	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FB_1, FB_2, DON, NIV, 3ADON, 15ADON, T-2, HT-2, NEO, FUS-X, DAS, MAS	Leblanc et al., 2005
Francia	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, FB_1, FB_2, OTA, PAT, ZEA, DON, NIV, 3ADON, 15AcDON, T-2, HT-2	Sirot et al., 2015
Irlanda	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FB_1, FB_2, DON, DAS, NIV, 3ADON, 15ADON, T-2, HT-2	FSAI, 2016
Líbano	AFB_1, AFM_1, OTA, DON	Raad et al., 2014
Nueva Zelanda	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2	FSANZ, 2001
Nueva Zelanda	AFB_1, AFB_2, AFG_1, AFG_2, OTA	FSANZ, 2003
Nueva Zelanda	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FB_1, FB_2, DON	FSANZ, 2011
España	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FBs, DON, T-2, HT-2	Cano-Sancho et al., 2012
España	AFM_1	Urieta et al., 1991; Urieta et al., 1996

España	AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEN, FB_1, FB_2, DON, NIV, 3ADON, 15ADON, T-2, HT-2, T-2 triol, NEO, FUS-X, DAS	Beltrán et al., 2013
Países Bajos	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AOH, AME, BEA, ENA, ENA_1, ENB, ENB1, OTA, PAT, ZEA, α-ZOL, β-ZOL, STE, FB_1, FB_2, FB_3, DON, DON 3G, FUS-X, NEO, DAS, NIV, 3ADON, 15ADON, T-2, HT-2, MON, MPA, NPA, PeA, ROC, Agro	López et al., 2016; Sprong et al., 2016a Sprong et al., 2016b
Viet Nam	AFB_1, FBs, OTA	Huong et al., 2016

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2. OBJETIVOS

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El objetivo general de la presente tesis doctoral es la evaluación de la exposición de la población valenciana a micotoxinas a través de un estudio de dieta total.

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

1. Realización de una revisión bibliográfica para investigar los estudios de dieta total llevados a cabo en diferentes países centrados en la determinación de micotoxinas.

2. Validación de métodos de análisis multi-micotoxina en diferentes platos listos para su consumo de composición vegetal y animal
3. Validación de métodos de análisis multi-micotoxina en zumos y bebidas alcohólicas.

4. Evaluación de la presencia de micotoxinas en menús listos para consumo.

5. Evaluación de la exposición teniendo en cuenta datos de consumo y las recomendaciones de la dieta mediterránea.

6. Caracterización del riesgo mediante comparación de la ingesta diaria estimada con parámetros toxicológicos de ingesta diaria tolerable.

7. Interpretar los resultados en términos de contribución de los alimentos analizados en relación a las micotoxinas estudiadas para futuras recomendaciones.

3. RESULTADOS

3.1. Dietary exposure assessment to mycotoxins through total diet studies. A review

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Dietary exposure assessment to mycotoxins through total diet studies. A review

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Abstract

Mycotoxins are secondary metabolites of fungi that contaminate food in several stages and their increasing presence in food chain demand further control. Assessment of mycotoxins human exposure through processed diet is an important component of food safety strategies. The present review explores and summarizes total diet studies (TDS) carried out in different countries focusing on mycotoxins determination. TDS were classified by samples preparation, mycotoxins analysis and dietary exposure evaluation. Most of reviewed TDS performed multi-mycotoxins analysis in composite samples mainly, prepared taking into account local culinary habits. High performance liquid chromatography coupled with fluorescence detector was the predominant and the most sensitive technique used for determination. Ochratoxin A was the most analyzed mycotoxin, followed by trichothecenes, aflatoxins, zearalenone, fumonisins, patulin, enniatins, and beauvericin respectively. *Alternaria* toxins and ergot alkaloids were also included. Food commonly analyzed were cereals, meat, vegetables, fruits, nuts and beverages. The findings in food were in below the current European legislation, except for some sporadic samples of wine and milk meaning less than 1% of total analyzed samples. Dietary exposure was evaluated, through the estimated daily intake mycotoxin evaluation and risk assessment concluded that relatively scarce toxicological concern was associated to mycotoxins exposure. However, a special attention should be paid to meat and cereal products high percentile consumers.

1. Introduction

In the last years, food safety policies based on scientific evidence are being implemented in food industries to protect consumer's interest (Lee et al.,

2015). The European Food Safety Authority (EFSA) through the Council and European Parliament established several scientific advice and technical support in all areas impacting on food safety sharing information, data and best practices, for emerging risks identification and coherent communication development (EC 178/2002; EFSA 2009). The monitoring or surveillance data often focus on individual chemical substances in raw food commodities and don't often provide a direct assessment of dietary population exposure. There is scarce data in literature behaving toward ready-to-eat foods analysis, leading sometimes to an over-estimation of the amount of contaminants presents in food chain (EFSA, FAO & WHO, 2011). In this sense, De Nijs et al., (2016) suggested food consumption surveys, food monitoring studies, duplicate diet studies and total diet studies (TDS) as useful strategies to be used for mycotoxin exposure estimation.

Many countries regularly conduct TDS to evaluate population exposure to different food contaminants. (Moy & Vannoort, 2013; Betsy et al., 2012; Lee et al., 2015). In this sense, international committees EFSA, FAO & WHO, (2011) published harmonized guidance document to improve dietary exposure assessment's efficiency and accuracy. Selection of representative food of the overall diet and culinary preparation of ready-to-eat dishes are critical steps in TDS implementation which very often combined analytical results with food consumption data for relatively accurate estimation of dietary exposure to such contaminants. The wide varieties of chemical substances present in diet make the establishment of priorities in TDS approach necessary. In particular, for mycotoxins, analysis is recommended in selected food items (Figure 1) (WHO, 2002; WHO, 2015).

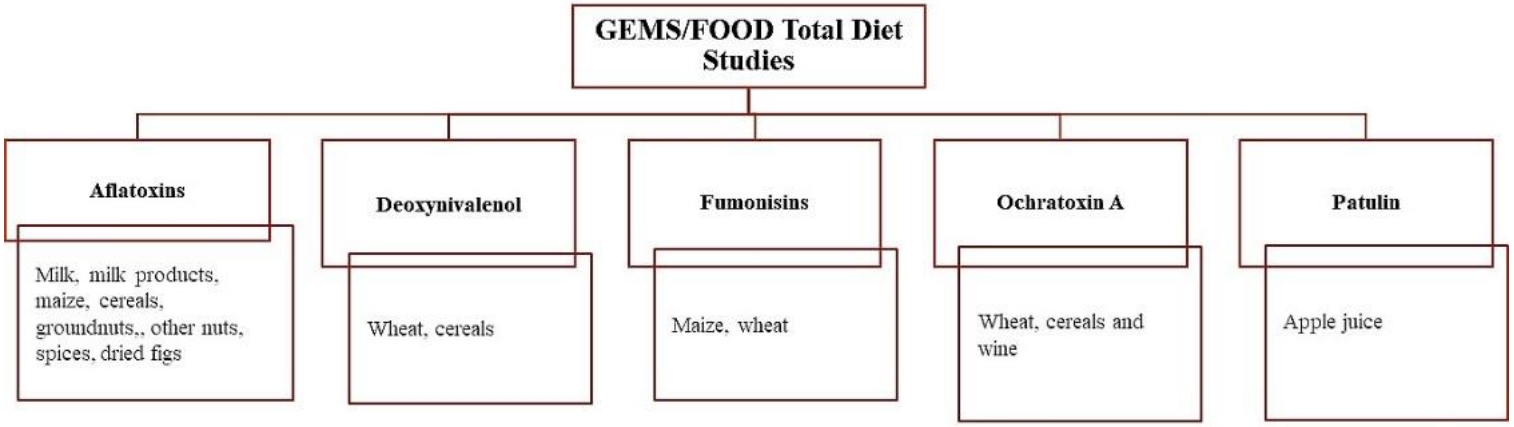

Fig. 1. Mycotoxins analysis suggested in the main food in TDS workshop (WHO, 2002; WHO, 2015).

Mycotoxins are compounds produced by fungi growing mostly on plant products during production and storage. The Rapid Alert System for Food and Feed of the European Union reports mycotoxins on the second position according to the total number of hazard notifications (RASFF, 2017). The most important mycotoxins in food and animal feed are: aflatoxins (AFs), produced by *Aspergillus* species; Ochratoxin A(OTA), produced by both *Aspergillus* and *Penicillium*; trichothecenes (TCTs) [HT-2, T2 toxin and deoxynivalenol (DON)], nivalenol (NIV), zearalenone (ZEA), fumonisins (FBs). Emerging mycotoxins such as fusaproliferin (FUS), moniliformin (MON), beauvericin (BEA), and enniatins (ENNs), altenuene (ALT), alternariol (AOH), alternariol methyl ether (AME), altertoxin (ALTs), and tenuazonic acid (TeA) produced mainly by *Fusarium* and *Aternaria* toxins (Kralj & Prosen, 2009; Yang et al., 2014; Marin et al., 2013).

The presence of mycotoxins has been highly investigated in different foodstuff such as cereals products (Juan et al., 2017; Saladino et al., 2017), coffee and tea beverages (Pallarés et al., 2017; García-Moraleja et al., 2015), vegetables (Dong et al., 2019; Rodriguez-Carrasco et al., 2016) and some studies have also been carried out in cooked food (Sakuma et al., 2013; Carballo et al., 2018).
Table 1 summarises mycotoxins subject of study in foodstuffs extracted from different reviewed TDS. Mycotoxin's exposure evaluation through TDS have been conducted in several countries; even countries in process of development such as Benin, Cameroon, Mali and Nigeria and some countries have conducted several TDSs like France, Spain and New Zealand (Ingenbleek et al., 2017). The number of mycotoxins varies from one mycotoxin (Tam et al. 2011; Urieta at al., 1996) to 38 (López et al., 2016).

This review offers for the first time a compilation data about mycotoxin occurrence and dietary exposure assessment through TDS studies. Also it features an overview of the different methodologies reported on mycotoxin analysis in food, published in the last two decades, focusing on samples preparation, analytical techniques used and estimation of the potential contribution to the dietary exposure.

2. Methodology

A systematic literature review was conducted using the databases Web of Science, PubMed and Scopus with the focus on the following keywords: total diet studies, mycotoxin, occurrence, dietary exposure etc. The period of time framed was of last two decades. Eighteen articles, which met the criteria to be included into the study, were analyzed and classified. To facilitate data presentation four groups were established based on selection and collection of samples, samples preparation, analytical determination techniques and dietary exposure assessment. The information was double-checked to select bibliographies of relevant literature and summarize the information about, analytical methodology, studied mycotoxins, limits of detection and quantitation, incidence and concentration levels of mycotoxin. Finally, data available was used to estimate exposure dietary to mycotoxins.

3. Results and discussion

3.1. Selection and collection of samples

Several tools were applied to obtain population consumption data, such as food frequency questionnaires, 24-hour recalls, and family budget surveys

(Kim et al., 2015). Dietary intake data from national surveys was one of the most frequently used on TDS. Some countries disposed of detailed information available from national surveys for individual consumption, like France (Leblanc et al., 2005; Sirot et al., 2013), Ireland (Food Safety Authority of Ireland) (FSAI, 2016) and New Zealand (Australian National Nutrition Survey) (Food Standard Authority New Zealand, 2011).

In this sense, the number of collection sites is usually determined first within budget limit and the duration of the TDS is sometimes based on population size (national or regional/local), distance between sites and geographical conditions (Kim et al., 2015). Food is often collected three to four times a year and randomly from different locations (Betsy et al., 2012) and during various seasons of year (Elegbede et al., 2017).

In general, samples were analysed in form of individual food, composite samples and food groups samples (Leblanc et al., 2005; Sirot et al., 2013; Raad et al., 2014; Huong et al., 2016). Collected samples were from fast-food chain to supermarkets, representing typical products mostly consumed (Tam et al., 2011; López et al., 2016). The different food categories and the number of samples collected in reviewed TDS are listed in Table 1.

Table 1: Total diet studies revised for mycotoxin analysis

Country	Analyzed food	Composite (n)	Individual food (n)	Analyzed mycotoxins	Reference
Canada	Cereal and cereal products; alcohol drinks; coffee; tea; beans; fruits; sugars; chocolate; cheese; milk; eggs; dessert; meat; herb and spices; dried fruits; soya products; mixed dishes	140	_	OTA	Tam et al., 2011
China	Cereal and their products; vegetables; legumes, nuts, seeds; fruits; meat and poultry; fats, oils; alcoholic beverages; non-alcoholic beverages; mixed dishes; snacks; sugars; condiments, sauces	600	1800	AFB_1, AFB_2, AFG_1, AFG_2, PAT, ZEA, DON, FB_1, FB_2, FB_3, ZEA, α-ZOL, βZOL, DON, 3AcDON, 15AcDON	Yau et al., 2016
China	Cereal products; beans; potatoes; meat; eggs; aquatic products; milk; vegetables; fruits; saccharides; beverages; condiments	240	-	STC, CIT, CPA, MON, GLIO, MPA, Verru	Qiu et al., 2017
France	Vegetarians food; biscuits; breakfast cereals; breads; pasta; rice; cakes; chocolates; desserts; nuts and oilseeds; vegetables; pulses; eggs; sugars; breads, buns; butter; dairy products; coffee; meat; offal; fruits; soft drinks; alcoholic beverages; pizzas, salt cakes, quiches; sandwiches; soup; prepared dishes; salads; compotes	456	2280	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FB_1, FB_2, DON, NIV, 3AcDON, 15AcDON, T-2 and HT-2, NEO, FUS-X, DAS, MAS	Leblanc et al., 2005

France	Breads; breakfast cereals; pasta; rice; croissants; pastries; biscuits; cakes; milk; dairy products; eggs; butter; offal; delicatessen meat; vegetables; fruits; dried fruits; nuts and seeds; chocolate; non-alcoholic beverages; alcoholic beverages; coffee; pizzas; sandwiches; snacks; mixed dishes; desserts; compotes	577	1319	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, FB_1, FB_2, OTA, PAT, ZEA, DON, NIV, 3AcDON, 15AcDON, T-2, HT-2	Sirot et al., 2005
Ireland	Cereals; milk; dairy products; eggs; meat; fish; potatoes; vegetables; fruits; dried fruits; nuts; seeds; herbs spices; soups; sauces; sugars; beverages; fats; oils; snacks; pizza	141	1043	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FB_1, FB_2, DON, DAS, NIV, 3AcDON, 15AcDON, T-2, HT-2	FSAI, 2016
Lebanon	Breads and toasts; biscuits; croissants; cakes; pastries; pasta; pizza; pies; rice; pulses; olive oils, sesame oils; nuts, seeds, olives and dried dates; cheese; milk and milk-based beverages; milk-based ice cream and pudding; yogurt and yogurt-based products; caffeinated beverages; alcoholic beverages	47	705	AFB_1, AFM_1, OTA, DON	Raad et al., 2014
New Zealand	Alcoholic and non-alcoholic beverages; cereal and cereal products; condiments; dairy products; eggs; fats and oils; fish; fruits; meat; nuts; seeds; snacks; sugars; vegetables; infant food	48	–	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2	FSANZ, 2001

New Zealand	Alcoholic non-alcoholic beverages; cereal and cereal products; condiments; dairy products; eggs; fats; oils; fish; seafood; fish products; fruits; meat products; nuts and seeds; snacks; sugars; vegetables; infant food	65	–	AFB_1, AFB_2, AFG_1, AFG_2, OTA	FSANZ, 2003
New Zealand	Alcoholic and non-alcoholic beverages; cereal products; condiments; dairy products; eggs; fats; oils; fish; fruits; meat; nuts; seeds; snacks; sugars; vegetables; infant food, beverages; fast food	570	–	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FB_1, FB_2, DON	FSANZ, 2011
Spain	Cereal and cereal products; milk; cheeses; dried fruits; sweet corns; breakfast cereals; corn snacks; alcoholic beverages; coffee; vegetables; baby food; apple juice; jams and apple sauce; ethnics food; gluten-free food	1690	3447	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, OTA, PAT, ZEA, FBs, DON, T-2, HT-2	Cano-Sancho et al., 2012
Spain	Milk; dairy products	60		AFM_1	Urieta et al., 1991; Urieta et al., 1996
Spain	Cereal and cereal products; olives; pickles; apple; pear; eggs; milk; milk shakes; custards; soya products; cheeses; grapes; alcoholic beverages; juices; oils	240	–	AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEN, FB_1, FB_2, DON, NIV, 3AcDON, 15AcDON, T-2, HT-2, T-2 triol, NEO, FUS-X, DAS	Beltrán et al., 2013
The Netherlands	Alcoholic, non-alcoholic beverages; sugars; dairy products; eggs; fish; fruits; cereal products; legumes; meat; offal; nuts; seeds;	88	-	AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AOH, AME, BEA, ENN A, ENN A_1, ENN B, ENN B1, OTA, PAT, ZEA, α-	López et al., 2016; Sprong et al., 2016a Sprong et al., 2016b

	oils; fats; soy products; tuber; vegetables			ZOL, β-ZOL, STE, FB_1, FB_2, FB_3, DON, DON 3G, FUS-X, NEO, DAS, NIV, 3AcDON, 15AcDON, T-2, HT-2, MON, MPA, NPA, PeA, ROC,	
Viet Nam	Rice; wheat and products; tubes root and products; beans products; tofu; oily seeds; vegetables; sugars; seasoning; fats; oils; meat products; egg and milk; fish; other aquatic products	42	1134	AFB_1, FBs, OTA	Huong et al.,2016

3.2. Samples preparation

As a wide variety of food is collected for TDS conduction, some samples require preparation such as peeling (e.g. orange or banana) and cooking for example. (e. g. beef and chicken) (Abbey & Mooney, 2013). Depending on TDS's approaches, namely the composite or individual food approach, steps for food preparation, as normally consumed in home, are recommended (Vannort et al., 2013). The accuracy and comprehensiveness of the manual procedure is critical as it influences the manner in which food samples are handled, prepared and stored before analysis. These decisions can influence the quality and representativeness nature of the overall TDS (Abbey & Mooney, 2013).

3.3. Analysis of mycotoxins

3.3.1. Analytical methods

Instrumental determinations driven on TDS samples are largely characterized by their selectivity and sensitivity to meet the TDS challenge for measuring the trace levels of chemical residues in the most varied food matrices (Sack, 2013).

A list of the sensitivity of the methods and the instrumentation required was already provided (WHO, 2015) even a verification of the capabilities to replicate the key method's characteristics, including precision, limit of detection (LOD), limit of quantification (LOQ) and specificity, as well as ongoing monitoring of the accuracy during use is recommended.

Table 2 summarises the analytical methods used as well as LOD and LOQ reached for the reported mycotoxins. Liquid chromatography (LC) and gas chromatography (GC) were widely used because of its superior performance

and reliability, coupled to different detectors as fluorescence (FD), ultra-violet (UV), electron capture detector (ECD), diode-array (DAD), mass spectrometry (MS) and tandem spectrometry (MS/MS).

The most used technique for determination was HPLC-FD, achieving LODs between 0.003 to 1.95 μg kg^{-1} and LOQs of 0.03 to 15.6 μg kg^{-1} (Cano-Sancho et al., 2012), followed by HPLC-UV method with LODs between 0.02 to 62.5 μg kg^{-1} and LOQs 0.2 to 125 μg kg^{-1} (FSAI, 2016; Raad et al., 2014). UHPLC-MS/MS reached LODs from 0.05 to 2.5 μg kg^{-1} and LOQs 0.1 to 5 μg kg^{-1} (Yau et al., 2016; Beltrán et al., 2013) and LC-MS/MS LODs from 0.2 to 6 μg kg^{-1} and LOQs from 0.5 to 20 μg kg^{-1} (Sirot et al., 2013). HPLC-DAD presented the highest LOQs reported from 9.0 to 153 μg kg^{-1}, being the less sensitive technique mainly for trichothecenes screening in cereal products (Cano-Sancho et al., 2012).GC coupled to different detectors as ECD, MS and MS/MS presented LODs from 0.8 to 30 μg kg^{-1} and LOQs from 0.2 to 153 μg kg^{-1} (Leblanc et al., 2005; Cano-Sancho et al., 2012; López et al., 2016). Finally, enzyme-linked immunosorbent assay (ELISA), allowed specific evaluations of one mycotoxin or small related group of them, reached LOQs up to 0.005 μg kg^{-1} (Cano-Sancho et al. 2012), but none of them in multimycotoxin mode.

Table 2: Analytical methods, limit of detection (LOD) and quantification (LOQ)

Analytical technique	Mycotoxin	Foodstuffs	LOD (µg/kg)	LOQ (µg/kg)	Reference
HPLC-FD	AFs	Cereal products, dried fruit peppers, ethnic food	0.025-0.1	2.64	Cano-Sancho et al., 2012
HPLC-FD	AFS	Chocolate	0.05	0.2	Sirot et al., 2013
HPLC-FD	AFs	Cereal products, seeds, olives, dried dates	0.01	0.03	Raad et al., 2014
HPLC-FD	AFM_1	Milk and milk based beverages	0.01	0.03	Raad et al., 2014
HPLC-FD	AFM_1	Dairy products	-	0.025	Urieta et al., 1996
HPLC-FD	OTA	Cereal products	0.25	1	Leblanc et al., 2005
HPLC-FD	OTA	Cereal products, beer, dessert wine, coffee, peanuts, pistachios, baby food	0.003-1.160	-	Cano-Sancho et al., 2012
HPLC-FD	OTA	Cereal products	0.01-0.06	0.05-0.025	Tam et al., 2011
HPLC-FD	OTA	Cereal products, beverages	0.05	0.21	Raad et al., 2014
HPLC-FD	ZEA	Cereal products, vegetables, nuts, oilseeds	0.25	1	Leblanc et al., 2005
HPLC-FD	ZEA	Cereal products, beer, baby food, ethnics food	0.070-1.95	-	Cano-Sancho et al., 2012
HPLC-FD	FBs	Corn flakes, corn snacks, baby food, gluten-free food, ethnics food	-	15.6	Cano-Sancho et al., 2012
HPLC-FD	FBs	Beer	-	11.7	
HPLC-UV	AFs	Dried fruits, seeds	-	1	FSNAZ, 2001
HPLC-UV	OTA	Cereals, dairy products, eggs, vegetables, meat, fish, fruits, dried fruits, sugars, beverages	0.02-0.05	0.2	FSAI, 2016
HPLC-UV	FBs	Cereal products, vegetables, beverages, soft drinks, offal	10.0-20	20-40	Leblanc et al., 2005
HPLC-UV	PAT	Fruits, alcoholic and non-alcoholic beverages	1.5-4	5	FSAI, 2016
HPLC-UV	DON	Cereals products	62.5	125	Raad et al., 2014
HPLC-UV	PAT	Alcohol beverages, soft drinks	20	40	Leblanc et al., 2005
HPLC-UV	PAT	Jams	-	6.25	Cano-Sancho et al., 2012

UPLC-MS/MS	AFs	Cereal products, legumes, seeds, fats, oils	0.05	0.10	Yau et al., 2016
UPLC-MS/MS	OTA	Cereals, legumes, seeds, meat, oil, mixed diches, sugars	0.05	0.10	Yau et al., 2016
UPLC-MS/MS	OTA	Cereal products	0.09	0.29	Beltrán et al., 2013
UPLC-MS/MS	ZEA	Cereals, meat, fats, oil, sugars, condiments	2.5	5	Yau et al., 2016
UPLC-MS/MS	DON	Cereal products, non-alcoholic beverages, mixed diches, sugars	2.5	5	Yau et al., 2016
UPLC-MS/MS	DON	Cereal products	0.7	2.4	Beltrán et al., 2013
UPLC-MS/MS	FBs	Cereal products	2.5	5	Yau et al., 2016
UPLC-MS/MS	FBs	Breakfast cereals	1.05	3.5	Beltrán et al., 2013
UPLC-MS/MS	FBs	Beer	0.39	1.3	

3.4. Mycotoxins occurrence in foodstuffs

OTA, DON, NIV, T-2 and HT-2 followed by AFs, ZEA FBs and PAT, were the most frequently reported mycotoxins (Table 3 to 8).

3.4.1. Ochratoxin A

OTA has been reported in wide variety of agricultural commodities such as cereal products, oats, rye, beans, coffee, rice, peas and meat, and was notably present in wine, beer, grape juice and dried vine fruits (Alshannaq & Yu, 2017; Neme & Mohamed, 2017). OTA was linked to immunotoxicity, genotoxicity, neurotoxicity and teratogenicity and was classified by the International of Research on Cancer (IARC) in Group 2B as possible human carcinogen (IARC, 2012; Marin et al., 2013). EU established OTA limits in several foodstuffs, in the ranges of 0.50-10 μg kg^{-1} (EC, 1881/2006).

OTA was evaluated in 55% of the studied TDS, incidences and mean concentrations reported are listed in table 3. (Leblanc et al., 2005; Cano-Sancho et al., 2012; Beltrán et al., 2013; Sirot et al., 2013; Raad et al., 2014; Yau et al., 2016; Huong et al., 2016; Tam et al., 2011; FSAI, 2016; López et al., 2016). The highest and lowest OTA prevalence were reported in Spain with 73% and 13% of breakfast cereals samples contaminated in Valencian and Catalonia region (Beltrán et al., 2013; Cano-Sancho et al., 2012). The highest concentration was reported in Viet Nam (Huong et al., 2016) with mean levels of 9.68 μg kg^{-1} in bean samples. The lowest concentration was reported in alcoholic beverages in French, ranging from 0.01 to 0.025 μg L^{-1} (Sirot et al., 2013).

The highest incidence and concentration of OTA were reported in meat products (64%) at level of 2.68 μg kg^{-1} from Viet Nam (Huong et al., 2016), while the TDS performed in France in 2005 showed the lowest OTA incidence (7%) (Leblanc et al., 2005). The OTA prevalence data in alcoholic beverages, was only included in four TDSs (Leblanc et al., 2005; Cano-Sancho et al., 2012; Sirot et al., 2013; Raad et al., 2014). Cano-Sancho et al., (2012), reported the highest incidence in beer samples; about 89% were OTA contaminated and Sirot et al., (2013) reported the lowest concentrations ranging from 0.017 to 0.025 μg L^{-1}. Similar contents were detected in alcoholic beverages samples from France TDS at mean levels of 0.04 μg L^{-1} (Leblanc et al., 2005).

In coffee beverages, the highest incidence was found in Spain with 49% of coffee samples contaminated (Cano-Sancho et al., 2012) while lower incidences were observed in TDS performed in France, where only 7% of coffee samples contained OTA (Leblanc et al., 2005). All findings ranged from 0.04 μg L^{-1} (Leblanc et al, 2005) to 2.17 μg L^{-1} (Cano-Sancho et al., 2012; Raad et al., 2014).

None reported coffee products did exceed the maximum limit (ML) established by EU for OTA of 10 μg L^{-1}. OTA was also reported in beans products at concentrations between 0.05 μg kg^{-1} to 9.68 μg kg^{-1} in 20% and 50% of the analyzed samples (Yau et al., 2016; Huong et al., 2016). Similar values were reported in legume composite samples at mean levels of 1.7 μg kg^{-1} (López et al., 2016). OTA was also detected at high incidence at sugar samples and aquatic product samples at mean levels of 0.22 μg kg^{-1} and 4.85 μg kg^{-1} respectively (Yau et al., 2016; Huong et al., 2016).

OTA was also detected in specific food such as fat and oil, mixed dishes with a incidence of 11% to 25% respectively, ranging from 0.01 to 0.06 μg kg^{-1} in Chinese TDS (Yau et al., 2016). Relatively high OTA incidence at eggs and milk samples was described with mean contents of 3.16 μg kg^{-1} in TDS performed in Viet Nam (Huong et al., 2016). Concentrations lower than 0.25 μg kg^{-1} were reported by Cano-Sancho et al. (2012) in peanuts (42%), pistachios (3%) and baby food (7%).

In general, OTA contents reviewed were very similar although a slight difference was observed in prevalence.

Table 3: ***Ochratoxin A***: Occurrence and dietary exposure assessment

Matrix	n	Incidence (%)	Mean (μg kg^{-1})	EDI (ng kg^{-1} bw day^{-1})	TDI (%)	Reference
Beans and products	108	50	9.68	0.2	1.176	
Meat and products	297	64	2.68	5	29.411	
Egg and milk	135	75	3.16	1.4	8.235	Huong et al.,
Fish	54	50	2.24	1.4	8.235	2016
Other aquatic products	54	100	4.85	0.3	1.764	
Corn flakes	72	3	0.73			
Wheat flakes	28	21	0.31	0.37-0.53	2.17-3.11	Cano-Sancho
Whole wheat breads	70	13	0.28			et al., 2012
Peanuts	72	42	0.21			

Pistachios	70	3	0.23			
Baby food	69	7	0.23			
Beer	71	89	0.02			
Dessert wine	141	57	2.85			
Red wine	120	15	0.51			
Coffee	72	49	2.17			
Bread and toast	45	-	0.29	0.59	3.47	Raad et al., 2014
Biscuits and croissants	45	-	2.84	0.76	4.52	
Cakes and pastries	45	-	0.15	0.05	0.30	
Pizza and pies	45	-	0.223	0.15	0.88	
Rice	15	-	0.68	0.46	2.73	
Pasta and other cereal products	60	-	0.18	0.08	0.47	
Dried fruits	45	-	0.07	0.01	0.05	
Alcoholic beverages	15	-	1.472	0.64	3.80	
Caffeinated beverages	30	-	0.508	1.48	8.71	
Cereals and their products	76	38	0.07-0.10	1.3-5.4	7.3-31.7	Yau et al., 2016
Legumes, nuts and seeds	24	20	0.05-0.09			
Meat and poultry	36	8	0.01-0.5			
Fats and oils	4	25	0.02-0.05			
Mixed dishes	44	11	0.01-0.06			
Sugars	4	100	0.22			
Cereal products	28	57	0.48	2.19[a]	12.90	Tam et al., 2011
Breakfast cereals and bread	30	73	0.06-0.29	0.24-1.20[a]	1.4-7.05	Beltrán et al., 2013
Bread rusk	18	39	0.43	0.71	4.176	Leblanc et al., 2005
Breakfast cereals	12	17	0.29	0.02	0.117	
Biscuits	14	15	0.69	0.14	0.823	
Cakes	12	17	0.29	0.17	1	
Delicatessen meat	20	7	0.34	0.08	0.470	
Soft drinks	4	50	0.11	-	-	
Alcoholic beverages	18	28	0.04	0.05	0.294	
Coffee	14	7	0.04	0.08	0.470	
Breads	14	-	0.129-0.393	0.172-0.580	1.01-3.41	Sirot et al., 2013
Breakfast cereals	6	-	0.033-0.25	0.003-0.014	0.01-0.08	
Pasta	4	-	0.1-0.35	0.006-0.120	0.03-0.70	
Rice and wheat products	6	-	0.067-0.3	0.007-0.084	0.04-0.47	
Delicatessen meat	80	-	0.047-0.253	0.017-0.099	0.1-0.58	
Alcoholic beverages	6	-	0.017-0.025	0.065-0.077	0.38-0.45	
Cereal products	-	-	1.5	0.47-3.11	2.7-18.2	FSAI, 2016
Pizza	-	-	0.20			

Seeds	-	-	1.8			
Coffee	-	-	1.1			López et al.,
Liquorice	-	-	1.3	0.73-1.01	4.29-5.9	2016; Sprong
Legumes	-	-	1.7			et al., 2016

[a]The estimate daily intake was calculated in this study based on the mean concentration reported with consumption data of cereals products and assuming 70 kg as the average body weight for the Canadian and Spanish population.

3.4.2. Trichothecenes

TCs are structurally related mycotoxins produced by *Fusarium* species. DON is probably the most commonly detected trichothecene in cereals followed by T-2 and HT-2 toxins (Pereira, Fernandes & Cunha, 2014; Covarelli et al., 215; Bryla et al., 2018). DON is mainly associated to toxicological effects on gastrointestinal tract, immune, and endocrine systems (Juan-García et al., 2013). For instance, EU has established limits for DON in several foodstuffs, in the range of 200-1750 μg kg^{-1} (EC, 1881/2006).

DON was found contaminating a variety of foodstuffs; mainly cereal based products with an overall incidence between 3% and 100% (Table 4). The higher incidence of DON was reported in TDS from Spain, 100% of bread samples (Cano-Sancho et al., 2012) and the highest concentration was published in Lebanese TDS, with mean level of 524.17 μg kg^{-1} in bread and toast samples (Raad et al., 2014). However, the lowest incidence of DON was observed in TDS conducted also in Spain with only 3% of corn samples contaminated (Cano-Sancho et al., 2012) and the lowest contents (between 8.5 to 10.8 μg kg^{-1}) were detected in breakfast cereals samples from TDS study performed in France (Sirot et al., 2013). DON reported values did not exceed the EU maximum limits of 750 μg kg^{-1} for cereals (EC, 1881/2006).

DON was also detected in 22% of the mixed dishes at levels ranging from of 10.57 to 14.49 μg kg^{-1} (Yau et al., 2016), even at Leblanc study, only 6% of the prepared dishes contained DON at mean concentration of 15.4 μg kg^{-1} (Leblanc et al., 2005). Concerning beverages, DON was detected in 13% of analyzed samples of non-alcoholic beverages at concentrations between 1.64 to 6.02 μg L^{-1} (Yau et al., 2016). Furthermore, DON was also present in 5% of nuts and oilseed samples with mean content of 16.2 μg kg^{-1} (Leblanc et al., 2005).

NIV was detected in rice, bread, nuts, oil seed and vegetarian food samples even at incidences and concentrations lower than 5% and 20 μg kg^{-1} respectively (Table 4) (Sirot et al., 2013; Leblanc et al., 2005).

T-2 and HT-2 toxins were mainly reported in cereal products (Table 4). The most prevalence was observed in cornflakes samples and the highest concentration was quantified in sweet corn up to 144.8 μg kg^{-1} (Cano-Sancho et al., 2012). The TDS study performed in Netherlands also referred that apple juice composite contained T-2 at mean level of 14 μg L^{-1}. Therefore, cross-contamination during manufacturing of composites preparation could explain the presence of T-2 in apple juice samples (López et al., 2016). Therefore, for TCTs the highest incidences and concentrations were observed for DON.

Table 4: ***Deoxynivalenol:*** Occurrence and dietary exposure assessment

Matrix	N	Incidence (%)	Mean (μg kg^{-1})	EDI (ng kg^{-1} bw day^{-1})	TDI (%)	Reference
Pasta	70	74	226	370-560	37-56	Cano-Sancho et al., 2012
Corn flakes	65	75	109			
Wheat flakes	27	74	190			
Corn snacks	71	79	154			
Sweet corns	72	3	114			
Whole bread	72	17	68			
Breads	31	100	247			

Baby food	30	40	131			
Gluten-free food	18	11	216			
Ethnic food	35	40	406			
Cereals products	76	37	29.95-33.11			
Mixed dishes	44	22	10.57-14.49			
Sugars	4	25	3.65-7.40	86.1-142.6	8.61-14.2	Yau et al., 2016
Non-alcoholic beverages	16	13	1.64-6.02			
Cereal products	60	95	2.4-203	9.94-841[a]	0.99-84.1	Beltrán et al., 2013
Breads and toasts	45	-	524.17	1052	105.2	
Biscuits and croissants	45	-	340.33	92	9.2	
Rice	15	-	322	230	0.23	
Pizza and pies	45	-	121.16	80	0.8	
Cakes and pastries	45	-	109.67	37	0.3	Raad et al., 2014
Pasta and other cereal products	60	-	62.50	28.8	2.82	
Dried fruits	45	-	62.50	12.55	1.25	
Alcoholic beverages	30	-	52.08	23.53	2.35	
Vegetarians food	35	9	16.3	-	-	
Breads	18	45	108.9	188	18.8	
Rice and semolina	8	25	58.8	9.51	0.951	
Viennese bread and buns	12	42	57.9	18.3	1.83	
Biscuits	14	29	46.4	9.52	0.952	Leblanc et al., 2005
Nuts and oilseeds	22	5	16.2	0.83	0.083	
Pizzas, salt cakes and quiches	6	17	22.5	9.81	0.981	
Sandwiches	12	25	23.8	4.73	0.473	
Prepared dishes	24	6	15.4	22.2	2.22	
Breads	14	-	132.1-132.1	226.3-226.3	22.63-22.3	
Breakfast cereal	6	-	8.5-10.8	0.5-0.6	0.05-0.06	
Pasta	4	-	56.3-56.3	30.6-30.6	3.06-3.06	
Rice	6	-	57.5-58.5	5.7-6.6	0.57-0.66	
Croissants	6	-	73.3-73.3	11.6-11.6	1.16-1.16	Sirot et al., 2013
Biscuits	8	-	58.4-61-9	6.7-7.1	0.67-0.71	
Pastries and cakes	18	-	54.2-55.0	38.2-38.5	3.82-3.85	
Pizza, quiches	4	-	101.3-101.3	23.8-23.8	2.38-2.38	
Sandwiches	28	-	83-83	16.1-16.1	1.61-1.61	
Mixed dishes	65	-	20.7-23.4	12.1-13.4	1.21-13.4	

Beer	-	-	9.1			
Biscuits and cookies	-	-	55			
Breads	-	-	28	95.8-107	9.58-10.7	López et al., 2016; Sprong et al., 2016
Breakfast cereals	-	-	29			
Pasta	-	-	35			
Rye and maize products	-	-	30			
Nivalenol						
Vegetarians food	35	3	15.4	-	-	Leblanc et al., 2005
Breads	18	-	15.8	27.7	2.30	
Nuts and oilseeds	22	-	16	0.79	0.06	
Rice and rice products	6	-	18.3-19.3	1.88-2.80	0.15-0.23	Sirot et al., 2013
Mixed dishes	65	-	7.7-10.6	3.47-5.0	0.28-0.41	
T-2 Toxin						
Pasta	70	20	27			
Corn flakes	65	11	24.4			
Wheat flakes	27	22	41.2	27-38	27-38	Cano-Sancho et al., 2012
Corn snacks	71	8	32.6			
Sweet corns	72	8	144.8			
Apple juice	-	-	14	2.6-10.3	2.6-10.3	López et al., 2016; Sprong et al., 2016
HT-2 Toxin						
Pasta	70	46	24.8			
Corn flakes	65	54	23.4			
Wheat flakes	27	52	40.3	27-38	27-38	Cano-Sancho et al., 2012
Corn snacks	71	34	82.8			
Sweet corns	72	11	31.6			
Whole wheat breads	72	22.2	39.2			

[a]The estimate daily intake was calculated in this study based on the mean concentration reported with consumption data of cereals products (FAOSTAT, 2016) assuming 70 kg as the average body weight for the Spanish population.

3.4.3. Aflatoxins

Fungi producing AFs grow on variety of food mainly cereal products, dried fruits spices, beer and milk and dairy products (Alshannaq & Yu, 2017).

AFs linked to genotoxicity, carcinogenic, teratogenic, hepatotoxic, mutagenic and immunosuppressive effects (Theumer et al., 2018). AFB_1 is classified by IARC in Group 1 as carcinogen to human (IARC 2012). For instance, EU has established limits AFs in several foodstuffs, in the ranges of 4-15 μg kg^{-1} (EC, 1881/2006).

AFs were reported in variety of foodstuffs mainly cereal products, seed and dried fruit, ranging from 3% to 100% (Table 5). A relatively high incidence (67%) was found in rice and products samples (Huong et al., 2016). However, only 3% of cereals and their products were AFs positives at levels ranging from 0.05 to 0.24 μg kg^{-1} (Yau et al., 2016). Incidence of 9% was reported by Cano-Sancho for ethnic food analysis even at mean concentrations of 8.19 μg kg^{-1} (Cano-Sancho et al., 2012). In other TDS study performed in Ireland, 21% of the analyzed food, including cereals, nuts and pizza were contaminated by AFs even at levels lower than 1.5 μg kg^{-1} (FSAI, 2016). AFs were reported in 20% of dried fruits at concentrations ranging from 2.66 to 8.85 μg kg^{-1} (Cano-Sancho et al., 2012). However, lower contents ranging from 0.180 to 0.260 μg kg^{-1} were detected in nuts, seed, and dried date (Raad et al., 2014). TDS study performed in New Zealand showed also that roasted salted peanuts samples contained AFB_2 and AFB_1 at mean levels of 6 μg kg^{-1} and 38 μg kg^{-1} respectively exceeding the maximum permitted concentrations fixed in 15 μg kg^{-1} (FSANZ, 2001; EC 1881/2006).

100% of vegetables and seasoning samples were contaminated by AFs at mean levels of 2.4 μg kg^{-1} and 3.8 μg kg^{-1} respectively in Viet Nam's TDS (Huong et al., 2016). Spain's TDS also reported AF's incidence of 57% for pepper samples even at mean level of 2.22 μg kg^{-1} (Cano-Sancho et al., 2012). AFs were also present in fats and oil products; AFB_1 incidence of 100% were reported for

oil seed and oil fat samples in Viet Nam and 44% in fats and oils in Hong Kong at mean levels lower than 4 μg kg^{-1} and (Huong et al., 2016; Yau et al., 2016). 50% of fish and 100% of other aquatic products were also reported AFB_1contaminated at mean level of 2.3 and 2.5 μg kg^{-1} respectively, while 91% of meat samples contained AFs at mean concentrations of 4 μg kg^{-1} (Huong et al., 2016). In this same study AFs were also reported in 100% of sugar samples at mean contents of 4 μg kg^{-1}. AFs were also detected in dark chocolate samples in French TDS even at trace levels (Sirot et al., 2013).

AFM_1 was reported in high incidence (94%) in milk samples even at mean contents of 0.0097 μg L^{-1}, while it was only detected in 3% of yogurt samples with mean levels of 0.0038 μg kg^{-1} (Cano-Sancho et al., 2012). Similar levels of AFM_1 were reported in Lebanon in milk based beverages 0.11 to 0.18 μg L^{-1}, exceeding the maximum permitted concentrations established in 0.05 μg kg^{-1} for milk (Raad et al., 2014; EC 1881/2006).

Table 5: ***Aflatoxins:*** Occurrence and dietary exposure assessment

Matrix	n	Incidence (%)	Range (μg kg^{-1})	EDI (ng kg^{-1} bw day^{-1})	Reference
Rice and products	81	67	2.6	22.2	Huong et al., 2016
Corn	27	50	3.2	1	
Tubes, root and products	54	50	2.1	0.1	
Beans and products	135	75	2.9	0.1	
Oily seeds	27	100	4	0.4	
Vegetables	54	100	2.4	1.6	
Sugars	135	100	4	0.1	
Seasoning	27	100	3.8	0.8	
Fats and oils	54	100	3.3	0.6	
Meat and products	297	91	4	7.7	
Eggs and milk	135	100	5.3	2.3	
Fish	54	50	2.3	1.4	

Other aquatic products	54	100	2.5	0.2	
Cereal products	76	3	0.05-0.24	0.2-2.8	Yau et al., 2016
Legumes, nuts and sedes	24	19	1.34-1.50		
Fats and oils	4	44	0.41-0.52		
Sweet corns	71	3	0.87	0.058-0.182	Cano-Sancho et al., 2012
Peanuts	72	11	2.66		
Pistachios	70	20	8.85		
Peppers	72	57	2.22		
Ethnics food	35	9	8.19		
Breads and toasts	45	-	0.242-0.275	0.51-0.52	Raad et al., 2014
Cakes and pastries	45	-	0.105-0.115	0.037-0.038	
Pizza and pies	45	-	0.043-0.048	0.030-0.031	
Nuts, seeds, olives and dried dates	45	-	0.18-0.26	0.044-0.045	
Chocolate	10	-	0.03-0.07	0.001-0.005	Sirot et al., 2013
Cereals, seeds	39	21	0.20-1.5	0.23-10.6	FSAI, 2016
Peanuts	9	-	6-38	0.42-2.66	FSANZ, 2001
Aflatoxin M1					
Dairy products	-	-	0.025	0.16	Urieta et al., 1996
Milk	72	94	0.0097	0.036-0.043	Cano-Sancho et al., 2012
Yoghurt	72	3	0.0038		
Milk and milk based beverages	30	-	0.11-0.18	0.21-0.22	Raad et al., 2014

3.4.4. Zearalenone

ZEA occurs in many agricultural products, including cereals, mixed feeds, rice, and corn silage. The most frequently contaminated crop was corn, followed by wheat (Stanciu et al., 2015). The toxicity of ZEA is associated with reproductive problems in specific animal species and possibility in humans (Marin et al., 2013; Zatecka et al., 2014). EU had established limits for ZEA in several foodstuffs, in the ranges of 20-200 μg kg^{-1} (EC, 1881/2006) (Table 6).

In cereal products, the ZEA prevalence of 44% were reported by Cano-Sancho (2012) in whole bread while the maximum concentrations were reported in breakfast cereal samples with mean levels of 22.7 μg kg^{-1} (Leblanc et al., 2005). ZEA was also detected in 9% of ethnic food samples at mean concentrations of 6.2 μg kg^{-1} (Cano-Sancho et al., 2012).

The occurrence of ZEA in fats and oils has been monitored in TDS from Hong Kong; 33% of the sample resulted contaminated by ZEA in ranging from 48.75 to 53.75 μg kg^{-1} (Yau et al., 2016). ZEA also was investigated in nuts and oil seed, 5% of the analyzed samples resulted positive at mean levels of 5.7 μg kg^{-1} (Leblanc et al., 2005).

The prevalence of ZEA in sugar was about 50% at levels between 15.03 to 18.15 μg kg^{-1} (Yau et al., 2016) while ZEA quantified in chocolate ranged between 1.55 and 4.1 μg kg^{-1} (Sirot et al., (2013) .

Alcoholic beverages also resulted contaminated by ZEA; 11% of beer samples were positive at mean levels of 3.1 μg kg^{-1} (Cano-Sancho et al., 2012). ZEA reported values in cereal products did not exceeded the EU maximum limit of 200 μg kg^{-1} (EC, 1881/2006).

Table 6 : ***Zearalenone:*** Occurrence and dietary exposure assessment

Matrix	n	Incidence (%)	Mean (μg kg^{-1})	EDI (ng kg^{-1} bw day^{-1})	TDI (%)	Reference
Pasta	70	14	3.8	1.82 – 2.40	0.72-0.96	Cano-Sancho et al., 2012
Wheat flakes	29	14	6.3			
Corn snacks	72	24	5.9			
Sweet corns	72	18	4.9			
Whole wheat breads	71	44	3.7			
Baby food	30	23	4.1			
Ethnics food	35	9	6.2			

Beer	71	11	3.1			
Cereal products	76	2	0.19-7.56	6.1-101.5	1.2-20.3	Yau et al., 2016
Fats and oils	4	33	48.75-53.75			
Sugars	4	50	15.03-18.15			
Condiments	12	11	1.67-8.33			
Vegetarians food	35	3	8.7	-	-	Leblanc et al., 2005
Breakfast cereals	12	17	22.7	4.5	2.25	
Vegetables	4	25	17	2.97	1.48	
Nuts and oilseeds	22	5	5.7	3.28	1.64	
Breads	14	-	1.5-5	2.41-8.03	1.205-4.015	Sirot et al., 2013
Croissants	6	-	1.5-5	0.22-0.74	0.11-0.37	
Biscuits	8	-	3-5.8	0.29-0.64	0.145-0.32	
Pizzas, quiches	4	-	1.5-5	0.39-1.29	0.195-0.645	
Chocolates	10	-	1.55-4.1	0.14-0.31	0.07-0.155	

3.4.5.Fumonisins

FBs occur naturally at significant levels in maize and a variety of maize-based human foodstuffs and animal feeds (Silva et al., 2009; Oldenburg et al., 2017; James & Zikankuba, 2018), but in low concentrations in other cereal products (Piacentini et al., 2015; Stanciu et al., 2015). FB_1 is the most prevalent FBs in human food and also is classified in Group 2B as probably carcinogenic (IARC, 2012). For instance, EU has established limits for sum FBs in several foodstuffs, in the ranges of 200-2000 $\mu g\ kg^{-1}$ (EC, 1881/2006).

The incidence of FBs ranged from 4% to 100% (Table 7). A high incidence up to 60% was found in breakfast cereal samples (Beltrán et al., 2013). Similar values were reported by Cano-Sancho et al., (2012) who detected FBs in 51% of ethnic food samples. Leblanc et al., (2005) quantified also FBs in 42% of the breakfast cereal samples. However, only 4% of cereal products were reported FBs contaminated in by Yau et al. (2016).

A mean concentration of 202.9 μg kg^{-1} was observed in ethnic food samples (Cano-Sancho et al., 2012). FBs were also detected in biscuits samples ranging from 36.5 to 75 μg kg^{-1} (Sirot et al., 2013). Similar values were reported by Leblanc et al., (2005) who quantified FBs in breakfast cereals samples at mean concentrations of 41.3 μg kg^{-1}.

These reported values of FBs in cereal products did not exceed the EU maximum limits of 400 μg kg^{-1} for corn-based food for direct human consumption (EC, 1881/2006).

High incidence of FBs was detected in beer alcoholic beverages; Cano-Sancho et al., (2012) and Beltrán et al., (2013) detected FBs in 90% and 100% of beer samples respectively, ranging from 1.3 to 36.9 μg L^{-1} while, Leblanc et al., (2005) quantified FBs in 25% of the alcoholic beverages. 50% of soft drink and vegetables samples resulted also contaminated by FBs at mean concentrations of 8.2 μg L^{-1} and 47.5 μg kg^{-1}, respectively (Leblanc et al., 2005).

Table 7: ***Fumonisins:*** Occurrence and dietary exposure assessment

Matrix	**n**	**Incidence (%)**	**Mean (μg kg^{-1})**	**EDI (ng kg^{-1} bw day^{-1})**	**Risk assessment (%)**	**Reference**
Cereal products	76	4	2.58-9.76	1.6-97.3	0.08-4.9	Yau et al., 2016
Corn flakes	72	39	78.9	100-103	5-5.15	Cano-Sancho et al., 2012
Corns snacks	72	61	119.1			
Baby food	30	23	36.4			
Gluten-free food	18	33	10.4			
Ethnics food	35	51	202.9			
Beer	71	90	36.9			
Breakfast cereal	10	60	1.5-3.5	6.21-14.5	0.310-0.725	Beltrán et al., 2013
Beer	10	100	1.3-13	3.79-37.14	0.189-1.857	
Offal	6	50	52.5	0.42	0.021	Leblanc et al., 2005
Breakfast cereals	12	42	41.3	4.88	0.244	

Vegetables	4	50	47.5	3.11	0.1555	
Alcoholic beverages	4	25	7.4	50.1	2.505	
Soft drinks	2	50	8.2	1.17	0.0585	
Breakfast cereals	-	-	18	46.9	2.34	López et al., 2016; Sprong et al., 2016
Dried fruits	-	-	10	1.17	0.0585	
Breakfast cereals	6	-	8-13.3	0.32-0.64	0.016-0.032	Sirot et al., 2013
Biscuits	8	-	36.5-75	2.63-2.79	0.131-0.14	

3.4.6. Patulin

PAT occurs mainly in fruits, in particular in apple based products like juice and cooked apples (Iqbal et al., 2018; Abrunhosa et al., 2016). PAT exposure is related to acute human toxicity effects including nausea, vomiting, ulceration and haemorrhage (Alshannaq and Yu 2017). EU has established limits for PAT in several foodstuffs, in the ranges of 10 to 50 μg kg^{-1} for infantile food and fruit juices respectively (EC, 1881/2006).

PAT was detected mainly in fruit based products (Table 8). In the first French TDS, 50% of alcohol and soft drink samples were contaminated with PAT (Leblanc et al., 2005). Cano-Sancho et al., (2012) also reported presence of PAT in 42% and 34% of apple juice samples and baby food respectively, from Spain. Concerning concentrations, the highest levels were reported in non-alcoholic beverages and cakes with contents of 31.9 μg L^{-1} and 31.7 μg kg^{-1} respectively (FSAI, 2016; Leblanc et al., 2005), while the smallest concentrations were detected in fruits ranging from 0.04μg kg^{-1} to 6.09 (Sirot et al., 2013). None findings exceed the EU maximum limits (EC, 1881/2006).

Table 8: ***Patulin:*** Occurrence and dietary exposure assessment

Matrix	n	Incidence (%)	Mean (μg kg^{-1})	EDI (ng kg^{-1} bw day^{-1})	Risk assessment (%)	Reference
Jams	77	5	13.5			
Baby food	124	34	7.1	4.28	1.07	Cano-Sancho et al., 2012
Apple juice	71	42	8.1			
Fruits	39	-	0.04-6.09	0.28-10.59	0.07-2.6	Sirot et al., 2013
Compotes and cooked fruits	6	-	1-8.33	0.24-1.58	0.06-0.39	
Non-alcoholic beverages	25	-	0.11-3.65	0.08-5.66	0.02-1.41	
Fruits	11	-	21.3			
Alcohol beverages	4	-	17	10.0-12.0	2.5-3	FSAI, 2016
Non-alcoholic beverages	13	-	31.9			
Cakes	6	33	31.7	5.45	1.362	
Soft drinks	2	50	20.5	0.78	0.195	Leblanc et al., 2005
Alcohol beverages	2	50	19.5	3.54	0.885	

3.4.7. Others mycotoxins

López et al., (2016) detected emerging mycotoxins, sterigmatocystin, Alternaria toxins and ergo alkaloids in TDS performed in the Netherland. Enniatins, mainly ENN B and ENN B_1, were quantified in apple sauce and cereal-based composites at 6.6 and 58 μg kg^{-1} respectively. BEA was quantified in nuts, dried fruits and tomato products by 2 μg kg^{-1}. Sterigmatocystin was also present in chocolate composite at LOQ level (0.5 μg kg^{-1}). Alternaria toxins (AME, AOH) were detected in a large variety of 88 composite samples analyzed; seven composite samples were positive for AOH, which varied from wine (1.0 μg kg^{-1}) to tomato products (8.9 μg kg^{-1}), and nine composite were contaminated with AME, which varied from pasta and breakfast cereals (3.2 μg kg^{-1}) to dried

fruit (8.1 μg kg^{-1}). Ergot alkaloids only occurred in grain-based composites in concentrations ranging from 15 to 47 μg kg^{-1}.

In other TDS conducted in China, Qiu et al., (2017) analyzed seven mycotoxins in 240 mixed dietary samples. Cyclopiazonic acid was detected in bean samples ranging from 0.47 to 1.57 μg kg^{-1} and in alcohol samples at levels between 0.19 to 2.26 μg kg^{-1}. Moreover the contents of citrinin in fruit were 5.31 μg kg^{-1} and the content of moniliformin in milk of 3.60 μg L^{-1}.

3.5. Dietary exposure assessment

TDS are performed to assess dietary exposure to chemical substances in food to assess the effectiveness of risk management strategies to anticipate public health problems (EFSA, 2010; Petersen, 2013). The most commonly used methodology for estimating intake is by combining levels of contamination and food consumption data (Join FAO/WHO Expert Committee on Food Additives, 2001). Although, the estimated exposure in a TDS is rather complicated because of the diversity of food and variations in dietary (Kim et al., 2015).

The health risk characterization of each mycotoxin is performed then by comparing the estimate dietary intake (EDI) previously calculated with the tolerable daily intake (TDI) established (ng kg^{-1} bw day^{-1}) of the respective mycotoxins as reported in Tables 3 to 8.

Most studies reviewed had considered lower and upper bound scenarios for assessment of dietary exposure. In the lower bound approach, the undetected values were replaced by zero and the un-quantified values were replaced by LOD. While, in the upper bound approach, the undetected values were

substituted by LOD and the un-quantified values were replaced by LOQ (Sirot et al., 2013; Raad et al., 2014; Yau et al., 2016; Sprong et al., 2016b; Cano-Sancho et al., 2012; FSAI, 2016). However, Huong et al., considered medium bound as additional scenario. In this case, the undetected values were replaced by LOD/2 to estimate dietary exposure (Huong et al., 2016). Others authors used the mean levels of mycotoxins for dietary intake calculation (Leblanc et al., 2005; Tam et al., 2011; Beltrán et al., 2013; Urieta et al., 1996; FSANZ, 2001).

3.5.1. Ochratoxin A

The EDIs reported ranged between 0.003 and 5 ng kg bw^{-1} (Leblanc eta al., 2005; Sirot et al., 2013; Huong et al., 2016; Raad et al., 2014; Cano-Sancho et al., 2012; Tam et al., 2011; FSAI, 2016 and Sprong et al., 2016b) (Table 3). OTA Tolerable Weekly Intake (TWI) set is 120 ng bw^{-1} (JECFA, 2001). Risk assessment values (%TDI) were ranged from 0.01 to 29.41% (Tam et al., 2011; Huong et al., 2016). The main contributor to OTA dietary exposure was meat, with 29.41% of the TDI from Viet Nam TDS followed eggs, milk and fish with 8.2% (Huong et al., 2016). However, lowest values were reported in TDS performed in France by breakfast cereals consumption contributing from 0.01 to 1% of TDI (Sirot et al., 2013). Caffeinated beverages, contributed to OTA risk assessment from 0.47% reported in the first French TDS to 8.71% reported in Lebanon TDS (Leblanc et al., 2005; Raad et al., 2014). The findings suggested that caffeinated beverages did not represent a health risk for the average adult consumer since the estimated EDI of OTA was lower than the TDI established by JECFA (2001).

3.5.2. Trichothecenes

Cereals products were the main contributors to DON dietary exposure (Table 4). The EDI values were between 0.5 to 1052 ng kg^{-1} bw day^{-1} (Beltrán et al., 2013; Raad et al., 2014). TDS performed in France and Spain reported EDIs ranging from 0.83 to 560 ng kg^{-1} bw day^{-1} (Leblanc et al., 2005; Cano-Sancho et al., 2012), while the lowest values were observed in The Netherlands, with daily intake percentile 50 between 95.8 to 107 ng kg^{-1} bw day^{-1} (Sprong et al., 2016b). The risk assessment was assessed comparing the previously calculated EDI with the tolerable daily intake (TDI) (1000 ng kg^{-1} bw day^{-1}) (JECFA, 2001). TDI values for DON were between 0.05% to 105.2% (Sirot et al., 2013; Raad et al., 2014). These results highlighted that DON exposure might be important, exceeding sometimes the toxicological reference values for average consumers. The main contributors to DON dietary exposure were bread and toast reaching a 105.2% of the TDI (Raad et al., 2014). However, breakfast cereals consumption was associated with low contribution to DON dietary exposure with 0.05% of TDI (Sirot et al., 2013). TDS performed in France, China and Spain, notified also comparable scenarios concerning DON dietary exposure through consumption of different foodstuffs commonly consumed in those countries (Leblanc et al., 2005; Sirot et al., 2013; Cano-Sancho et al., 2012; Yau et al., 2016).

NIV dietary exposure was also assessed by cereal products, nuts, oilseed and mixed dishes consumption in first and second French TDS. Data ranged from 0.79 to 27.7 ng kg^{-1} bw day^{-1} by (Sirot et al., 2013; Leblanc et al., 2005) (Table 4). Tolerable daily intake (TDI) of NIV was 1200 ng kg^{-1} bw day^{-1} (Scientific Committee on Food, 2002). Bread was the main contributor to NIV

dietary exposure with 2.30% of the TDI (Leblanc et al., 2005), while rice products contributed up to 0.23% of the TDI (Sirot et al., 2013).
EDI for the sum of T-2 and HT-2 toxins were also investigated and data obtained ranged between 2.6 and 38 ng kg^{-1} bw day^{-1} mainly by cereal products, dried fruit and apple juice consumption (Cano-Sancho et al., 2012; Sirot et al., 2013; Sprong et al., 2016b).

Regarding the risk assessment for T-2 and HT-2 toxins, TDI of 100 ng kg^{-1} bw day^{-1} by was used by Cano-Sancho et al., (2012) as a benchmark dose analysis that EFSA established and TDI of 60 ng kg^{-1} bw day^{-1} was used for French TDS as JECFA proposed as a Provisional Maximum Tolerable Daily Intake (PMTDI) for these toxins (EFSA, 2011; Sirot et al., 2013). The main contributors to T-2 and HT-2 toxin dietary exposure were pasta and cereal products with 2.78% to 38 % of TDI (Cano-Sancho et al., 2012; Sirot et al., 2013) (Table 4).

3.5.3. Aflatoxins

AFs are classified as carcinogenic and genotoxic compounds and even exposure levels as low as 1 ng kg^{-1} bw day^{-1} may induce liver cancer cases (American Cancer Society, 2011). Concentrations of AFs in food should be reduced As Low As Reasonable Achievable (ALARA) (EFSA, 2007).

The AFs exposure ranged from 0.001 to 22.2 ng kg^{-1} bw day^{-1} through dietary consumption in different countries: New Zealand, China, France, Lebanon, Spain, and Viet Nam (FSANZ, 2001; Leblanc et al., 2005; (Sirot et al., 2013; Cano-Sancho et al., 2012; Raad et al., 2014; Yau et al., 2016) (Table 5).
Rice products were pointed out to be the main source of AFB_1 exposure reaching 22.2 ng kg^{-1} bw day^{-1} and the association between high consumption of

the AFs contaminated products and liver cancer for adult Viet Nam population was mentioned (Huong et al., 2016). EDI values of AFs in adult Ireland population were between 0.23-10.6 ng kg^{-1} bw day^{-1} through cereal products and seeds consumption (FSAI, 2016). AFB_1 EDI values up to 0.52 ng kg^{-1} bw day^{-1} for the adult Lebanese population were reported for bread and toast consumption (Raad et al., 2014).

AFM_1 exposure through milk and milk based beverages consumption obtained was ranged from 0.036 ng kg^{-1} bw day^{-1} to 0.22 ng kg^{-1} bw day^{-1} for adult Spanish and Lebanese population (Urieta et al., 1996; Cano-Sancho et al., 2012; Raad et al., 2014;).

3.5.4. Zearalenone

The main contributors to ZEA dietary exposure reported in the TDS from China were cereal products, fats, oil, sugar and condiments reaching 20.3% of the TDI. Authors used PMTDI of 500 ng kg^{-1} bw day^{-1} for risk assessment (Yau et al., 2016). Nevertheless, values up 4% of the TDI were notified in the first and second French TDS for bread consumption, assuming TDI of 200 ng kg^{-1} bw day^{-1} (SCF, 2000a) (Sirot et al., 2013; Leblanc et al., 2005) (Table 6).

3.5.5. Fumonisins

FBs EDI ranged from 0.32 to 103 ng kg^{-1} bw day^{-1} through consumption of cereal products, gluten-free food, vegetable, offal, alcoholic beverages and soft drink, in TDS from France, Spain and China (Leblanc et al., 2005; Sirot et al., 2013; Cano-Sancho et al., 2012; Yau et al., 2016). Sprong et al., (2016b) reported EDIs in upper-bound scenario of 46.9 ng kg^{-1} bw day^{-1} through consumption of breakfast cereals and dried fruits.

The risk assessment (%TDI) was calculated by comparing the EDI previously estimated and TDI of 2000 ng kg^{-1} bw day^{-1} of TDI (JECFA, 2001). The main contributor to the dietary exposure to FBs were cereal products contributing up to 5% of TDI for mean consumer for Catalonia region population (Cano-Sancho et al., 2012), even lower values were notified in the French TDS (0.016 to 0.14% of the TDI) (Sirot et al., 2013).

3.5.6. Patulin

The risk assessment for PAT was calculated by comparing the EDI estimated and TDI of 400 ng kg^{-1} bw day^{-1} (SCF, 2000b; Leblanc et al., 2005; Sirot et al., 2013; Cano-Sancho et al., 2012; FSAI, 2016). The main contributor to PAT dietary exposure were fruits, alcoholic and non-alcoholic beverages up to 3% of the TDI for mean consumer for Ireland population (FSAI, 2016; Leblanc et al., 2005; Sirot et al., 2013).

Summary of estimate dietary exposure to mycotoxins performed in the different TDS is provided in the Table 9.

EDI for all mycotoxins were ranged between 0.001 ng kg^{-1} bw day^{-1} for AFs to 1052 ng kg^{-1} bw day^{-1} for DON; being cereal products the main contributor to DON the dietary exposure in Lebanese TDS reaching 105.2% of TDI (Sirot et al., 2013; Raad et al., 2014). Lower values were notified for OTA and FBs 0.01% each one reported in French TDS through breakfast cereals consumption (Sirot et al., 2013).

Table 9: Summary of estimated dietary exposure to mycotoxins presented in the different TDS.

Mycotoxin	TDI (ng kg bw day)	EDI (ng kg bw day)	TDI (%)	Reference
OTA	17	0.003 – 5	0.01 – 29.41	Huong et al., 2016; Cano-Sancho et., 2012; Raad et al., 2014; Yau et al., 2016; Tam et al., 2011; Beltrán et al., 2013; Leblanc et al., 2005; Sirot et al., 2013; FSAI, 2016; Sprong et al., 2016
DON	1000	0.5 – 1052	0.05 – 105.2	Cano-Sancho et al., 2012; Yau et al., 2016; Beltrán et al., 2013; Raad et al., 2014; Leblanc et al., 2005, Sirot et al., 2013; Sprong et al., 2016
NIV	1200	0.79-27.7	0.065-2.30	Leblanc et al., 2005; Sirot et al., 2013
T-2	60; 100	2.6-38	2.6 – 38	Cano-Sancho et al., 2012; Sprong et al., 2016
HT-2	60; 100	0.04-38	0.06 – 38	Cano-Sancho et al., 2012
Afs	-	0.0019 – 22.2	-	Huong et al., 2016; Yau et al., 2016; Cano-Sancho et al., 2012; Raad et al., 2014; Sirot et al., 2013; FSAI, 2016; FSANZ, 2001
AFM1	-	0.036 – 0.22	-	Urieta et al., 1996; Cano-Sancho et al., 2012; Raad et al., 2014

ZEA	250	0.14 - 101.5	0.07 - 20.3	Cano-Sancho et al., 2012; Yau et al., 2016; Leblanc et al., 2005; Sirot et al., 2013
FBs	2000	0.32 – 103	0.016 - 5.15	Yau et al., 2016; Cano-Sancho et al., 2012; Beltrán et al., 2013; Leblanc et al., 2005; Sprong et al., 2016; Sirot et al., 2013
PAT	400	0.08 - 12.0	0.02 - 3.0	Cano-Sancho et al., 2012; Sirot et al., 2013; FSAI, 2016; Leblanc et al., 2005

4. Conclusions

This review on mycotoxins total diet summarized about eighteen studies performed in several countries highlighting the increasing interest for mycotoxin exposure through diet. HPLC-FD was the most widely used and OTA the most analyzed mycotoxin. Mycotoxin levels reported were relatively low in most of analyzed food except for milk and wine beverages which could approach the maximum limits. Despite the relatively high frequency of mycotoxins detected in the different food of habitual consumption, calculated EDIs were generally below the established TDIs, except for DON and OTA who amounted in some TDSs, appreciable ratio of their respective TDI through cereal and meat products.

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Conflict of interest

The authors declare no conflict of interest and informed consent was obtained from all individual participants included in the study.

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3.2. Evaluation of mycotoxin residues on ready-to-eat food by chromatographic methods coupled to mass spectrometry in tandem

Toxins (2018)

Evaluation of mycotoxin residues on ready-to-eat food by chromatographic methods coupled to mass spectrometry in tandem

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Abstract

Simultaneous determination of twenty-seven mycotoxins in ready-to-eat food samples using "QuEChERS extraction and chromatographic methods coupled to mass spectrometry in tandem is described in this study. Mycotoxins included in this survey were aflatoxins (B_1, B_2, G_1, G_2), enniatins (A, A_1, B, B_1), beauvericin (BEA), fumonisins (FB_1, FB_2), sterigmatocystin (STG), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), nivalenol (NIV), neosolaniol (NEO), diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), zearalenone (ZEA), α-zearalanol (αZAL), β-zearalenone (βZAL), α-zearalenol (αZOL), β-zearalenol (βzol), T2 and HT-2 toxin. The method showed satisfactory extraction results with recoveries ranging from 63 to 119%, for the different food matrix samples. Limits of detection (LOD_S) and quantification (LOQs) were between 0.15-1.5 μg/kg and 0.5-5 μg/kg respectively. The method was successfully applied to the analysis of 25 ready-to-eat food samples. Results showed presence of deoxynivalenol at 36% of samples (2.61-21.59 μg/kg), enniatin B at 20% of samples (9.83-86.32 μg/kg), HT-2 toxin at 16% of samples (9.06-34.43 μg/kg) and aflatoxin G_2 at 4% of samples (2.84 μg/kg). Mycotoxins were detected mainly in ready-to-eat food samples prepared with cereals, vegetables and legumes, even at levels below those often obtained from raw food.

1. Introduction

Mycotoxins are a group of toxic compounds produced as secondary metabolites by certain fungi of the genus *Aspergillus, Penicillium, Fusarium, Alternaria* and *Claviceps* that grow under different climate conditions and have been reported in several food matrices like cereals, peanuts, meat, eggs, milk and

fruits [1,2]. Chronic exposition to some mycotoxins can produce carcinogenic, mutagenic or teratogenic effects. Aflatoxins (AFs) are indicated as carcinogenic and hepatotoxic and fumonisins (FBs) and ochratoxin A (OTA) are possibly teratogenic, hepatotoxic, and nephrotoxic while patulin (PAT), zearalenone (ZEA), deoxynivalenol (DON), nivalenol (NIV), T-2 and HT-2 toxins are related to toxicological effects mainly on gastrointestinal tract, immune and endocrine system [3]. Emerging mycotoxins, such as ENs and beauvericin are cytotoxic [4] and their potent cytotoxic activity was demonstrated in several mammalian cell lines [5] turning it in a topic for human health [6]. The European Union through Regulation (EC) 1881/2006 set maximum limits in foodstuffs susceptible to mycotoxins contamination to control their quality and safety [7]. (Table 1).

Table 1: Summary of maximum levels of some mycotoxin in foodstuffs .

Mycotoxin	Food product	Maximum levels (μg/kg)
AFB_1	Processed cereal-based foods and baby foods for infants and young children, dietetic food destined for physical treatment, cereals and cereal products, dried fruit, groundnuts and nuts and spices	0,1-8,0
Sum of AFs	Cereal and cereal-based products, dried fruit, coffee, groundnuts and nuts	4,0-15,0
OTA	Processed cereal-based foods and baby foods for infants and young children, dietetic food destined for physical treatment, cereals and	0,5-10

	cereal products, dried vine fruit, wine, coffee, grape juice	
DON	Processed cereal-based products and baby foods for infants and young children, cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption, pasta dry, bread, pastries, biscuits, cereal snacks and breakfast cereals	200-750
ZEA	Processed maize-based products and baby foods for infants and young children, maize and maize-based products, bread and other unprocessed cereal	20-200
Sum of FBs	Processed maize-based products and baby foods for infants and young children, maize-based products and unprocessed maize	200-2000

AFB1: Aflatoxin B_1, AFs: Aflatoxins; OTA: ochratoxin A; DON:

The determination of mycotoxins has been highly reported in different food matrices such as cereal products [8], coffee [9], tomatoes and tomato products [10], fruits, vegetables and legumes [11]. There are several food processing that may modify mycotoxins stability [12]. Visconti et al. [13] and Serrano et al. [14] reported DON and ENs gradual reduction contents in pasta and AFs and OTA decreased in cooked rice through thermal treatments [15].

However, there is limited information on the presence of mycotoxins in ready-to-eat food. Recently, the EFSA (European Food Safety Authority) Panel on Contaminants in the food Chain (CONTAM) emitted a Scientific Opinion recommending that further studies should be conducted on the fate of

mycotoxins during the preparation of grain-based products, specially focused in bread, pasta and fine bakery wares [16].

Chromatographic methods coupled to mass spectrometry are the techniques often used for the determination of mycotoxins in food matrices [17, 18]. One of the main objectives pursued in most laboratories is the application of multiple analyte determination with minimal sample treatment. To gain increase throughput over mycotoxin traditional extraction methods, novel techniques raised out by using appropriate mixture of extraction and disperser solvent in dispersive liquid-liquid microextraction (DLLME), and also by adding inorganic salt into a mixture of water and organic solvent in salting-out liquid-liquid extraction (SALLE) .

The "Quick Easy Cheap Rough and Safe" (QuEChERS), originally developed by Anastassiades et al. [19], based on acetonitrile extraction followed by a salting-out and quick dispersive solid-phase extraction (d-SPE), takes profit of electric charge of target compounds to allow their extraction with small amounts of non-chlorinated organic solvents. It is also pointed out as a fast and economical mycotoxins extraction from different processed cereal- based food [20].

To the best of our knowledge, scarce data exist in the literature concerning mycotoxins level on ready-to-eat food, and in this sense the development of simple and efficient method to assess human mycotoxins exposure along ready to eat food analysis is necessary.

In the present investigation an analytical method based on QuEChERS extraction was used to evaluate the presence of twenty-seven mycotoxins AFB_1,

AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ENA, ENA_1, ENB, ENB_1, BEA, STG, ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, T-2 and HT-2 in ready-to-eat food by LC-MS/MS and GC-MS/MS.

2. Results

2.1. Analytical method validation

The parameters considered for validation purpose were instrumental linearity, matrix effect, sensitivity (LOD and LOQ), accuracy according to the EU Commission Decision 2002/657 EC [21]. The criteria for confirmation of positive findings was to fulfil, retention time agreement and peak areas ratio obtained from reference standard quantification (Q) and qualification (q) transitions (Table 2).

All mycotoxins exhibited good linearity over the working range (LOQ and 100× LOQ).The regression coefficients of all calibration curves were (r^2) higher than 0.996 demonstrating a good linearity.

Satisfactory results in terms of recoveries were found for all mycotoxins studied at three fortification levels (25, 50, 100 μg/kg). The range of recoveries values for the three concentrations tested in the five food groups studied was between 63 and 119 % .

The LODs and LOQs obtained were from 0.15 to 5 μg/kg for all mycotoxins analyzed (Table 2). As can be observed ENs reached very good sensitivity by LC-MS/MS and DON by GC-MS/MS, achieving LOQs 0.15-0.5 μg/kg, respectively.

The matrix effect (signal enhancement or suppression) was investigated by calculating the percentage ratio between the slopes of the matrix-matched calibration curve and the curve in solvent.

The matrix effect parameter (ME) behaviour in the different studied matrix is shown in table 2. The most of the analytes showed a signal enhancement, especially trichothecenes (ZON, 3-ADON and NEO) in fish composite with ME of 158% 173% and 175% respectively. On the other hand signal suppression was registered for AFG1, AFG2 and FB2 in legumes composite with ME values of 53%, 58% and 59% respectively. Similar value was obtained for BEA in meat composite with ME of 58%. The matrix-matched standards were used for effective qualifications taking into account results of suppression and enhancement of the signal.

Table 2: Analytical performance of the proposed method: Mass spectrometry transitions, LOD, LOQ, ME, and recovery range for the different food matrices studied.

Mycotoxin	Transitions		Cereals		Legumes		Fish		Vegetables		Meats		ME	Recovery
	Quantitative **Q**	Qualitative **q**	LOD **µg/kg**	LOQ **µg/kg**	LOD **µg/kg**	LOQ **µg/kg**	LOD **µg/kg**	LOQ **µg/kg**	LOD **µg/kg**	LOQ **µg/kg**	LOD **µg/kg**	LOQ **µg/kg**	**(%)**	**(%)**
OTA	404>102[a]	404> 239	1.5	5	1.5	5	1.5	5	1.5	5	1.5	5	73-121	71-91
AFB_1	313>241a	313>289	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	0.3	1	65-106	67-98
AFB2	315>286[a]	315>259	0.3	1	0.3	1	0.3	1	0.3	1	0.3	1	63-77	67-116
AFG_1	329>243[a]	329>311	0.3	1	1.5	5	0.3	1	0.3	1	0.15	0.5	53-117	79-102
AFG_2	331>313[a]	331>245	0.15	0.5	0.3	1	0.3	1	0.3	1	0.3	1	58-97	67-90
FB1	722>334[a]	722>352	1.5	5	1.5	5	1.5	5	0.3	1	1.5	5	61-141	69-114
FB2	706>336[a]	706>318	1.5	5	1.5	5	1.5	5	0.3	1	1.5	5	59-102	65-116
ENA	699>228[a]	699>210	0.15	0.5	0.3	0.5	0.3	1	0.15	0.5	0.15	0.5	65-117	74-114
ENA_1	685>214[a]	685>210	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	91-125	62-104
ENB	657>196[a]	657>214	0.15	0.5	0.3	1	0.15	0.5	0.15	0.5	0.3	1	68-101	69-111
ENB_1	671>214[a]	671>228	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	74-96	69-119
BEA	801>784[a]	801>244	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	0.3	1	58-103	78-99
STG	325>281[a]	325>310	1.5	5	1.5	5	0.3	1	1.5	5	1.5	5	70-116	71-102
DON	392>259[b]	407>197	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	82-114	75-107
3ADON	392>287[b]	467>147	0.75	2.5	0.15	0.5	0.15	0.5	1.5	5	0.15	0.5	78-173	89-117
15ADON	392>217[b]	392>184	0.75	2.5	0.15	0.5	0.15	0.5	0.15	0.5	0.15	0.5	77-148	73-98

NIV	289>73[b]	379>73	1.5	5	0.75	2.5	1.5	5	1.5	5	0.75	2.5	82-153	60-79
NEO	252>195[b]	252>167	0.15	0.5	0.15	0.5	0.75	2.5	0.15	0.5	0.15	0.5	77-175	91-114
DAS	350>229[b]	378>124	0.75	2.5	0.75	2.5	0.75	2.5	0.75	2.5	0.15	0.5	60-144	60-102
FUS-X	450>260[b]	450>245	0.75	2.5	0.75	2.5	0.75	2.5	1.5	5	0.15	0.5	78-151	68-106
T-2	350>244[b]	350>229	0.75	2.5	0.75	2.5	0.75	2.5	0.75	2.5	0.75	2.5	96-151	73-107
HT-2	347>157[b]	347>185	0.15	0.5	0.15	0.5	0.15	0.5	0.75	2.5	0.15	0.5	73-137	73-113
ZON	462>151[b]	462>333	0.15	0.5	0.15	0.5	1.5	5	0.15	0.5	1.5	5	65-158	67-104
α-ZAL	433>309[b]	433>295	0.15	0.5	0.15	0.5	0.75	2.5	0.15	0.5	0.15	0.5	73-155	66-117
β-ZAL	307>292[b]	307>277	0.75	2.5	1.5	5	1.5	5	1.5	5	1.5	5	62-132	70-89
α-ZOL	305>289[b]	305>73	1.5	5	1.5	5	1.5	5	1.5	5	0.75	2.5	58-145	63-77
β-ZOL	536>446[b]	536>333	0.15	0.5	1.5	5	1.5	5	0.75	2.5	1.5	5	71-128	63-79

[a]LC-MS/MS determination [b]GC-MS/MS determination

2.2. Samples analysis

Once validated, the method proposed was applied to evaluate the presence of mycotoxins in 25 ready-to-eat food samples. The results obtained are summarized in Table 3.

Table 3: Detected concentrations of DON, ENB, HT-2, AFG_2 in ready-to-eat food samples.

Samples	Concentration µg/kg RSD (%)			
	DON	**ENB**	**HT-2**	**AFG_2**
Cereals				
Lasagne	2.84±2.2	12.25±3.2	34.43±0.6	n.d.
Macaroni	5.27±4.2	9.83±1.0	n.d.	n.d.
Spaghetti pesto	21.59±1.0	11.51±6.2	7.23±4.5	n.d.
Noodles	4.75±10.5	86.32±12.2	18.85±1.6	n.d.
Rice (Paella)	n.d.	n.d.	n.d.	2.84
Rice salad	2.61±0.2	n.d.	n.d.	n.d.
Pizza	3.39±4.8	n.d.	9.06	n.d.
Quiche	4.1±1.0	14.68±4.2	n.d.	n.d.
Vegetable				
Vegetable soup	n.d.	n.d.	n.d.	n.d.
Garlic soup	6.19±0.2	n.d.	n.d.	n.d.
Cream of leek	n.d.	n.d.	n.d.	n.d.
Gratin broccoli	n.d.	n.d.	n.d.	n.d.
Scrambled spinach	n.d.	n.d.	n.d.	n.d.
Sauteed artichoke	n.d.	n.d.	n.d.	n.d.
Fish				
Grilled salmon	n.d.	n.d.	n.d.	n.d.
Grilled tuna	n.d.	n.d.	n.d.	n.d.
Baked perch fillets	n.d.	n.d.	n.d.	n.d.
Hake in white wine	n.d.	n.d.	n.d.	n.d.
Grilled sole	n.d.	n.d.	n.d.	n.d.
Legume				
Lentils	n.d.	n.d.	n.d.	n.d.
Broad beans	6.98±0.2	n.d.	n.d.	n.d.
Red beans	n.d.	n.d.	n.d.	n.d.
Cream of chickpeas	n.d.	n.d.	n.d.	n.d.

Meats				
Grilled chicken breast	n.d.	n.d.	n.d.	n.d.
Grilled pork loin	n.d.	n.d.	n.d.	n.d.

n.d.: not detected.

Mycotoxins have been detected mainly in food prepared with cereals, vegetables and legumes. The most prevalent mycotoxin was DON with total incidence of 36% at concentrations of 2.61–21.59 μg/kg being present in pasta, quiche, pizza, garlic soup and broad beans. ENB was detected in pasta and quiche samples with total incidence of 20% at concentrations of 9.83– 86.32 μg/kg. HT-2 toxin was found in pasta and pizza sample with total incidence of 16% at concentrations of 9.06–34.43 μg/kg. Finally, AFG_2 was evidenced at concentration of 2.84 μg/kg in one sample of rice for paella. Figure 1 shows chromatogram from pasta sample (n=3) naturally contaminated by ENB (86.32±12.2 μg/kg) and DON (21.59±6.2 μg/kg).

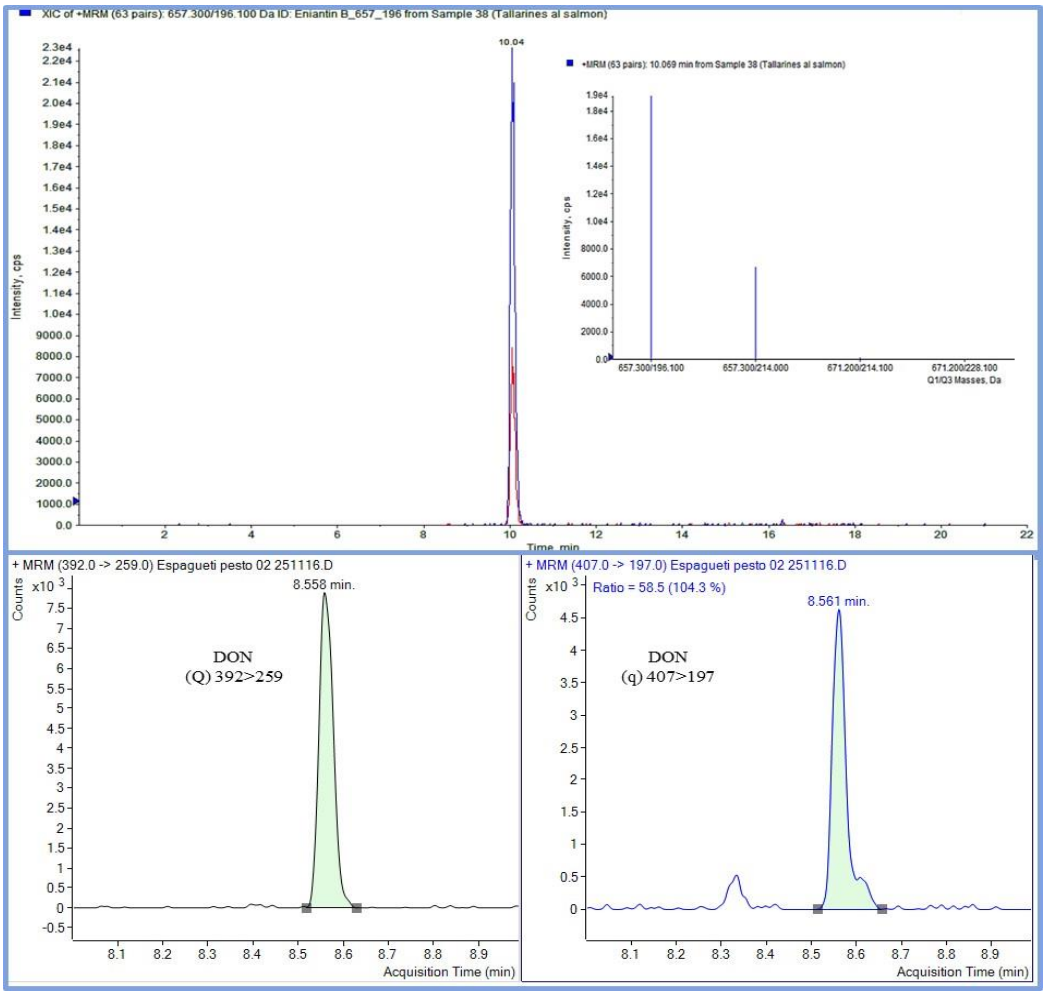

Figure 1: Chromatogram obtained from a sample of ready-to-eat food mainly prepared with cereals and naturally contaminated with (A) ENB content 86.32 µg/kg (LC-MS/MS) and (B) DON content 21.59 µg/kg at Multiple Reaction Monitoring (MRM) mode by (GC-MS/MS).

2.3. Mycotoxins occurrence in ready-to-eat samples

Occurrence of analyzed mycotoxins in main food group studied is represented at Table 4. The samples mainly prepared with cereals showed contamination by DON (88%), ENB (63%), HT-2 (50%) and AFG_2 (13%). The samples prepared mainly with legumes and vegetables showed DON contamination on 17% and 25% respectively.

Table 4: Occurrence, mean, and range (minimum and maximum) of mycotoxins detected in ready-to-eat food samples.

Mycotoxin	Parameter	Cereals (*n*=8)	Vegetables (*n*= 6)	Legumes (*n*=4)	Total (*n*= 25)
DON	Occurrence (%)	7 (88)	1 (17)	1 (25)	9 (36)
	Mean μg/kg	6.36	6.19	6.98	6.51
	Range (min-max) μg/kg	2.61-21.59	6.19	6.98	2.61-21.59
ENB	Occurrence(%)	5 (63)	-	-	5 (20)
	Mean μg/kg	26.91	n.d.	n.d.	26.91
	Range (min-max) μg/kg	9.83-86.32	n.d.	n.d.	9.83-86.32
HT-2	Occurrence(%)	4 (50)	-	-	4 (16)
	Mean μg/kg	17.39	n.d.	n.d.	17.39
	Range (min-max) μg/kg	9.06-34.43	n.d.	n.d.	9.06-34.43
AFG_2	Occurrence(%)	1 (13)	-	-	1 (4)
	Mean μg/kg	2.84	n.d.	n.d.	2.84
	Range (min-max) μg/kg	2.84	n.d.	n.d.	2.84

n.d.: not detected.

3. Discusión

Regarding mycotoxin presence, the contents determined here were comparable with previous researches performed in total diet studies (TDS). López et al. [22] reported DON and ENB contents in pasta at concentrations of 8.7 and 35 μg/kg respectively. Yau et al. [23] also detected DON in a range 29.95-33.11 μg/kg in pasta while in vegetables, legumes and meat samples the amounts were lower than 5.0 μg/kg. Raad et al. [24] detected DON contents at levels of 62.50 μg/kg in pasta and cereal products, 121.16 μg/kg in pizza and 31.25 μg/kg in legumes. Sirot et al. [25] detected also DON average of 132 μg/kg in pasta. The results obtained by Beltrán et al. [26] showed occurrence of DON in 100% of pasta and cereal products samples with a maximum value of 203 μg/kg. The concentration of DON in cereals samples never exceed the EU maximum limits of 750 μg/kg for dry pasta [7].

Concerning HT-2, the maximum concentration detected in pasta reached 34.43 μg/kg. Similar amounts were reported by Sirot et al. [25] who quantified HT-2 at levels of 3-10 μg/kg. Leblanc et al. [27] also quantified HT-2 in 238 composite samples with an average level of 270 μg/kg. There are many factors that predispose to mycotoxin production as temperature and storage-processing conditions [28].

In this study mycotoxins were not found in ready-to-eat meal based on meat and fish. However, Tolosa et al. [29] reported the presence ENs in fish products samples at levels ranging from 1.3 to 103 μg/kg. Sun et al. [30] also suggested that dried seafood could be invaded by mycotoxigenic fungi under improper storage conditions, and reported high levels of ZEA in seafood samples (317.3

μg/kg) and OTA (1.9 μg/kg) which were kept for three months at room temperature.

Several studies were carried out on raw food; Cano-Sancho et al. [31] analyzed the presence of mycotoxins in 479 cereal-based food samples and the percentage of DON positive samples in pasta was 73.4% with mean concentration of 226 μg/kg while HT-2 toxin was present in 10% of samples with mean concentration of 51 μg/kg. Tolosa et al. [32] analyzed 58 samples of different conventional pasta products, the most prevalent mycotoxin was DON (100%) with mean content of 96.93 μg/kg while HT-2 toxin and ENB were detected in 90% of samples at concentrations from 12.46 to 326.17 μg/kg respectively. Other mycotoxins were detected with high incidence like NIV, ZEA, ENA_1, while BEA was present in 10% of analyzed samples.

A recent study conducted by Stanciu et al. [33] did not quantify BEA in 40 pasta samples, while ENB was detected in 11% of the samples at average levels of 10.4 μg/kg and ENB in 9% of the samples at average level 1.9 μg/kg.

Regarding the presence of emerging mycotoxins in rice, Sifou et al. [34] reported the presence of BEA in 75.5% of samples between 3.8 and 26.3 mg/kg and ENA in 5,7% of analyzed samples with maximum concentration of 448 mg/kg. Makun et al. [35] detected AFs (B_1,B_2, G_1, G_2) in 100% of rice samples at concentration levels ranged between 28 and 372 μg/kg and OTA in 66.7% of the samples at average level of 141 μg/kg. ZEA was also quantified at 53.4% of the rice samples at average level 10.6 μg/kg, DON in 28% of the samples at average level of 18.9 μg/kg and FB_1 and FB_2 were found in 14.3% and 4.8% at concentrations of 0.2 and 6 μg/kg respectively. Sun et al. [36] reported contents

of AFB_1 in rice, the contents were less than 5 μg/kg while 7% samples were detected as exceeding the national maximum residue limits 10 μg/kg.

On the other hand, some authors have investigated the reduction of mycotoxins during food processing (Table 5); Visconti et al. [13] reported the reduction of DON (until 80%) in pasta by cooking. Cano-Sancho et al. [37] analyzed the transfer of DON from pasta to boiling water, reaching levels of reduction of DON in pasta cooked until 75%. Similar values have been reported by Rodriguez-Carrasco et al. [38] who obtained reduction of DON in pasta cooked from 13% to 58%. Nijs et al. [39] evidenced also reduction of DON (40%) and ENNs (17-19%) in pasta samples after cooking. Comparable results on ENB reduction levels after the cooking of pasta were reported in another study conducted by Serrano et al. [40].

Table 5: Mycotoxin's reduction by cooking methods .

Mycotoxin	Food	Reduction (%)	Reference
DON	Pasta	67-80	[13]
DON	Pasta	25-75	[37]
DON	Pasta	13-58	[38]
DON	Pasta	40-50	[39]
ENB	Pasta	17-19	[39]
ENB	Pasta	14-65	[40]
AFs	Rice	51-95	[41]
AFs	Rice	24.8	[42]
AFB1	Rice	78-88	[43]

Castells et al. [41] investigated AFs reduction in rice samples by extrusion-cooking and reported reduction of aflatoxin contents, which ranged from 51%

to 95%. Furthermore, Park et al. [42] also reported AFB_1 loss (78% - 88%) after pressure cooking. Two different cooking methods were used to evaluate AFs reduction and the authors concluded that rice sample steam based cooking method leaded to highest aflatoxin reduction (24,8%) [43]. Neither of the methods described reached 100% of aflatoxin reduction which highlighted mycotoxins stability and resistance to the different applied processes.

Nevertheless, data obtained show that mycotoxins levels found on ready to eat samples are below those often reported for raw food and support that ready-to-eat meal analysis is suitable alternative to perform for an accurate mycotoxin exposure assessment.

4. Conclusions

Chromatographic methods coupled to mass spectrometry in tandem were used for evaluation of twenty-seven mycotoxins in ready-to-eat food samples achieving very low limits of quantification. DON was quantified in 36%, ENB in 20%, HT-2 in 16% and AFG_2 in 4% of samples, respectively. No mycotoxins were detected in meat and fish diches. The occurrence of mycotoxins in ready-to-eat food samples was lower than reported on cereals, vegetables and legumes no processed. Exposure assessment from ready to eat meals show lowers levels than those obtained from raw food. The evaluation of mycotoxins in ready-to-eat meals offers a reliable tool for risk assessment, since the culinary processes are taken account.

5. Materials and methods

5.1. Chemicals and reagents

Solvents (acetonitrile, hexane and methanol) were supplied by Merck (Darmstadt, Germany). Deionized water (<18, 2MOhm cm resistivity) was obtained in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Beadford, MA, USA).

Ammonium formate (99%) and formic acid (≥98%), anhydrous magnesium sulphate and sodium chloride were supplied by Sigma Aldrich (Madrid, Spain). Syringe nylon filter (13mm diameter 0.22 μm pore size) were obtained from Analysis Vinicos S.L. (Tomelloso Spain). The derivatization reagent composed of BSA (N,O-bis(trimethylsily) + TMCS (trimthylcholorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

5.2. Standards and solutions

The standards of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ENA, ENA_1, ENB, ENB_1, BEA, STG, ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, T-2 and HT-2 toxins were purchased from Sigma Aldrich. Individual stock of all analytes were prepared to obtain 20 mg/L in methanol and multianalyte working solutions of 2 mg/L were also used by diluting the individual stock solutions. The multianalyte working standard solution was used for standard calibration curves, matrix-

matched calibration curves and recovery assays. All standards were stored in darkness and kept at-20°C.

5.3. Procedures

5.3.1. Samples

The collection of sample criteria was based on the consumption patterns of the Mediterranean diet, which is characterized by a greater consumption of cereals, vegetables, legumes and fish and less significate amount of meat products CIEAM/FAO [44].

A total of 25 ready-to-eat food samples mainly prepared with cereals (n=8), fish (n=6), legume (n=5), vegetables (n=4) and meat (n=2) were collected from Valencia University restaurant. For validation step, 50 g of each black beans, chickpeas and lentils were cooked and triturated and 2 g of this legume composite were used for QuEChERS extraction. The same pattern was used for all the studied composites. 2 g of cereals composite was taken from 50 g of each bread, pasta and rice mixture while vegetables composite resulted from 50 g of each of red pepper, green pepper, onions, potatoes, zucchini, tomato and eggplant. The meat composite was prepared blending 50 g of each of beef, pork, chicken, and finally fish composite resulted from mixing 50 g of each of salmon, perch and tuna.

Only the edible parts of each food were considered. Seeds and skins were removed from fruit. The inedible part of meat and fish such as bones, thorns and skin were also removed according to the Commission Regulation EC/401/2006 [45], before analysis performance. All samples were milled with a

knife mill (Oster Classic grinder, Valencia, Spain) and obtained mixture was stored at -18°C until analysis.

5.3.2. Extraction procedure

All samples were triturated and homogenized before their analysis. Briefly, 2 g of homogeneous sample were weighed and placed into 50 mL PTFE centrifugal tubes, and then 10mL of water containing 2% of formic acid were added. The tubes were stirred for 30 min at 250 rpm using a horizontal shaking device (IKA KS260 basic Stirrer, Staufen, Germany). 10 mL of acetonitrile were added into of tube containing soaked sample and vigorously stirred for 30 min at 250 rpm. In the next step, 4g $MgSO_4$ and 1g NaCl were added and shaken for 30s in vortex and then centrifuged for 10 min at 5°C and 5000 rpm using Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany). Then, 2 mL of acetonitrile extract wer e added to 0.1 g of C18 silica sorbent and 0.3 g of $MgSO_4$ for purification before centrifugation (5000rpm) for 10 min. The purified extract was filtered through a syringe nylon filter and transferred into a vial for the LC-MS/MS analysis and for GC-MS/MS analysis the supernatant was collected and evaporated to dryness under a gentle nitrogen flow.

5.4. GC–MS/MS analysis

Before GC-MS/MS analysis, 50 μL of BSA + TMCS + TMSI (3:2:3) was added to the dry extract and left 30 min at room temperature. Then 200 μL of hexane was added, mixed thoroughly on vortex for 30 s and washed with 1 ml of phosphate buffer (60 mM, pH7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to auto sampler vial.

Gas chromatographic determination was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadruple mass spectrometer with inter electron-impact ion source (EI, 70Ev) and Agilent 7693 auto sampler (Agilent Technologies, Palo Alto, USA). Quantitation data were acquired at selection reaction monitoring (SRM). The transfer line and source temperatures were 280° and 230°, 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). Analytes were separated on a HP-5MS 30m x 0.25mm x 0.25µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode in program able temperature vaporization (PTV) inlet at 250°C employing helium as the carried gas at fixed pressure of 20.3 psi. The oven temperature started at 80°C, and increased to 245°C at 60 ºC/min, hold their time for 3 min and increased to 260 ºC progressively at 3 ºC/min and finally to 270 ºC at 10 ºC/min and then held for 10 min. Data were acquired and processed using Agilent Masshunter version B.04.00 software.

2.5. LC–MS/MS analysis

LC-MS/MS analyses were conducted on a system consisting of an Agilent 1200 chromatographic system (Agilent Technologies, Palo Alto CA, USA) coupled to a 3200 QTRAP® mass spectrometer (AB SCIEX, Foster City, CA) equipped with a turbo electrospray ionization (ESI) interface. Separation of analyte was performed using a Gemini-NX LC-column (Phenomenex Aschaffenburg, Germany) (150mm ×4.6 mm, 5 µm of particle size) preceded by a guard column. The flow rate was set to 0.8 mL min^{-1}, and the oven temperature was 40°C. The two elution mobile phases were made up of the water slightly

acidified with 5 mM ammonium formate and 0,1% formic acid (mobile phase A) and methanol acidified with 5 mM ammonium formate 0,1% formic acid (mobile phase B). The elution gradient started with 0% of eluent B, increased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 70% in 2 min. The column was, readjusted to the initial conditions and equilibrated for 7 min. The volume of the injections was 20 μL.

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

2.6. Method validation

The method was validated for linearity, accuracy, repeatability (intraday and interday), following the EU Commission Decision 2002/657/EC [21], with food mixture of cereals, fish, legume, vegetables and meats samples. In order to determine the linearity, calibration curves for each studied mycotoxin were constructed from standards and from extract of blank samples of cereals, fish, legume, vegetables and meat previous analyzed and did not contain any studied compound. Linear range was tested at eight concentration levels from 0.15 to 200 μg/kg. Matrix-matched calibration curves were built by spiking blank samples with select mycotoxins after extraction process. Both external calibration curves and matrix-matched calibration curves were constructed by plotting peak areas against concentration and linear were applied to the calibration curves. Matrix effect (ME) was assessed for each analyte by

comparing the slope of the standard calibration curve ($slope_{with\ standard}$) with that of the matrix-matched calibration curve ($slope_{with\ matrix}$), for the same concentration levels.

Sensitivity was evaluated by limit of detection (LD) and limit quantification (LQ), which were estimated for a signal-to-noise ratio (S/N) ≥ 3 and ≥ 10, respectively, from chromatograms of samples spiked at the lowest level validated. LDs were estimated using an extract of a blank of the different matrix (previous analyzed and negative for the mycotoxin included in this study), fortified with decreasing concentrations of the analytes, where the response of the qualifier ion was at least 3 times the response of the blank extract (n = 9). The LOQs were estimated in the same way as the LDs, but using criterion of S/N ≥ 10 for the qualifier ion.

Accuracy was evaluated through recovery studies and was determined calculating the ratio of the peak areas for each mycotoxin by analysing the samples spiked before and after extraction at three addition levels 25, 50 and 100 μg/kg for all mycotoxins analyzed (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ENA, ENA_1, ENB, ENB_1, BEA, STG, ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO, T-2 and HT-2 toxins). Intra-day precision and inter-day precision (repeatability) were also investigated.

The precision of the method was determined by repeatability (n=3) and reproducibility (n=9) studies, and expressed as the relative standard deviation (RSD, %). The intra-day precision was expressed as the standard deviation of the recovery values of the spiked samples measured during the same day (n=3). The inter-day precision was determined by analysing the spiked samples for three different day (n=9).

Conflict of interest: The authors declare no conflict of interest and informed consent was obtained from all individual participants included in the study.

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3.3. Presence of mycotoxins in ready-to-eat food and subsequent risk assessment

Food and Chemical Toxicology (2018)

Presence of mycotoxins in ready-to-eat food and subsequent risk assessment

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Abstract

A study on a set of ready-to-eat meals (n=328) based on cereals, legumes, vegetables, fish and meat was carried out to determine the natural presence of twenty-seven mycotoxins by both liquid chromatography and gas chromatography coupled mass spectrometry in tandem (MS/MS) after QuEChERS extraction. The occurrence of mycotoxins was headed by cereal samples with 35% of samples contaminated by at least one mycotoxin followed by vegetables (32%), legumes (15%) and lastly, 9% of fish and meat samples were contaminated. DON was the most detected mycotoxin in vegetables, meat, fish and cereals with an incidence of 13% 18% 19% and 60%, respectively, and the highest mean levels were found in fish (1.19 μg/kg) and vegetable (1.53 μg/kg), respectively. The highest levels means were for HT-2 toxin ranging from 4.03 to 7.79 μg/kg, in cereal and legume samples respectively. In this last, HT-2 toxin was also the most prevalent (54%). In meat samples, OTA resulted with highest value with 8.09 μg/kg. Likewise, PCA analysis revealed a correlation between the mycotoxins and the food groups analyzed. The findings indicate that there is no toxicological concern associated with exposure to mycotoxins for consumers as all levels were in accordance with the legislation.

1. Introduction

Ready-to-eat foods are becoming very popular worldwide as they give a consumer the convenience of use without any further preparation. Ready-to-eat foods may also contain organisms or dangerous substances due to the intentional addition of substances such as pesticides, veterinary drugs and other products

used in primary production or toxic substances naturally present in foods that have been generated during the processed or storage, such as mycotoxins.

The Rapid Alert System for Food and Feed of the European Union places mycotoxins in the second position according to the total number of hazard notifications (RASFF, 2017).

Mycotoxins are secondary metabolites produced by fungi species belonging to various genera such as *Aspergillus, Penicillium and Fusarium*. There is proven relationship of mycotoxins consumption with teratogenesis, carcinogenesis, and mutagenesis. teratogenesis, carcinogenesis, and mutagenesis. Aflatoxin B_1 have been listed as carcinogen (Group I) by the International Agency for Research on Cancer (IARC, 2012), while Ochratoxin A (OTA) is classified by IARC in the Group 2B as possible human carcinogen (Marin et al., 2013). Reproductive issues were reported in farm animals exposed to zearalenone (ZEA) through the feed (Abrunhosa et al., 2016; Escrivá et al., 2015). Fumonisins are possibly teratogenic, hepatotoxic, and nephrotoxic (Alshannaq and Yu, 2017). Chronic exposure to deoxynivalenol (DON) is associated with delayed growth as well as immunotoxic and hematotoxic effects (Sirot et al., 2013). Nivalenol (NIV), T-2 and HT-2 toxins are related to toxicological effects mainly on gastrointestinal tract, immune and endocrine system (Juan et al., 2013). Emerging mycotoxins, such as enniatins (ENNs) and beauvericin (BEA) are cytotoxic and their potent cytotoxic activity was demonstrated in several mammalian cell lines even their toxicity in vivo is still to be proven (Prosperini et al., 2017).

Food consumption of food is the main route of human exposure to mycotoxins (Sirot et al., 2013). Mycotoxins have been investigated in several raw

food stuffs (Stanciu et al., 2017; Juan et al., 2017; Danezis et al., 2016; Abrunhosa et al., 2016; Yang et al., 2014).

To determine the intake of mycotoxins on individual foods, which combine food consumption patterns and contamination level are often carried out (Peltonen et al., 2011). Some published studies focused on multi-mycotoxin presence applying total diet studies approach in order to obtain reliable information about the exposure of human population to these toxic compounds (Leblanc et al., 2005; López et al., 2016; Sirot et al., 2013; Raad et al., 2014; Yau et al., 2016; Huong et al., 2016; Tam et al., 2011). Nowadays, very few studies have been carried out on mycotoxins occurrence in cooked food (Sakuma et al., 2013; Carballo et al., 2018). In this sense, the risk assessment purpose of measuring the presence of contaminants is to characterise the distribution of one or more substances in the population or of a country (EFSA, 2010). Food alerts in Europe have generated great interest and concern in consumers, and it is increasingly necessary to establish adequate control measures to ensure safe consumption of food.

The Mediterranean diet is abundant in fruits, vegetables, whole grains, legumes and olive oil, and is considered as the world's healthiest diet (CIHEAM/FAO 2015). The Mediterranean diet has been suggested as a key element for the prevention of age-related chronic diseases (cardiovascular diseases and type 2 diabetes) (Assmann et al., 2017; Galilea-Zalbalza et al., 2018). Low environmental impacts and high biodiversity, high sociocultural food values and positive local economic return are also some of its advantages (Dermini et al., 2016).

With the purpose to carry out a closer exposure assessment approach, the presence of twenty-seven different mycotoxins; deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), nivalenol (NIV), neosolaniol (NEO), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), T-2 and HT-2 toxins, sterigmatocystin (STG), ochratoxin-A (OTA), four aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2), zearalenone (ZEA), α-zearalanol (αZAL), β-zearalenone (βZAL), α-zearalenol (αZOL), β-zearalenol (βzol), fumonisins (FB_1, FB_2), as well as five emerging mycotoxins: enniatins (ENNA, $ENNA_1$, ENNB and $ENNB_1$) and beauvericin (BEA) was evaluated in ready-to-eat food by LC-MS/MS and GC-MS/MS. Data obtained were also used to characterize a Valencian population risk through ready-to-eat food intake mainly based on Mediterranean diet.

2. Materials and methods

2.1. Chemicals and reagents

Solvents (acetonitrile, hexane and methanol) were supplied by Merck (Darmstadt, Germany). Deionized water (<18, 2MΩ_{cm} resistivity) was obtained in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Beadford, MA, USA).

Ammonium formate (99%) and formic acid (≥98%), anhydrous magnesium sulphate and sodium chloride were supplied by Sigma Aldrich (Madrid, Spain). Syringe nylon filter (13mm diameter 0.22 μm pore size) were obtained from Analysis Vinicos S.L. (Tomelloso Spain). The derivatization reagent composed of BSA (N,O-bis(trimethylsily) + TMCS (trimthylcholorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium

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

2.2. Standards and solutions

The standards of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, ENNA, $ENNA_1$, ENNB, $ENNB_1$, BEA, STG, ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3-ADON, 15ADON, DAS, NIV, FUS-X, NEO, T-2 and HT-2 toxins were purchased from Sigma Aldrich. Individual stock of all analytes were prepared to obtain 20 mg/L in methanol and multianalyte working solutions of 2 mg/L were also used by diluting the individual stock solutions. The multianalyte working standard solution was used for standard calibration curves, matrix-matched calibration curves and recovery assays. All standards were stored in darkness and kept at -20°C.

2.3. Samples

A total of 328 samples, representative of the Mediterranean diet, were collected from the University restaurant mainly by September 2016 to December 2016. The samples selection was based on the basis that food items formed part of the menu offered at least twice a week and has been associated with fungal contamination. The most consumed meal groups during a week were selected and distinguished according to the major component into the five food groups; cereals, vegetables, fish, legumes and meat. Most foods collected were thermally processed except salads based on vegetable samples as carrots, onions, peppers and tomato, which are often consumed raw.

Only the edible parts of each food were considered. Seeds and skins were removed from fruit. The inedible part of meat and fish such as bones, thorns

and skin were also removed according to the European Commission Regulation (EC/401/2006), before analysis performance. All samples were milled with a food knife mill (Oster Classic grinder, Valencia, Spain) and obtained mixture was stored at -18°C until analysis.

2.4. Extraction procedure

Samples extraction was performed according to the previously validated method by Carballo et al. (2018). Briefly, 2 g of homogeneous sample were weighed and placed into 50 mL PTFE centrifugal tubes, and then 10mL of water containing 2% of formic acid were added. The tubes were stirred for 30 min at 250 rpm using a horizontal shaking device (IKA KS260 basic Stirrer, Staufen, Germany). 10 mL of acetonitrile were added into of tube containing soaked sample and vigorously stirred for 30 min at 250 rpm. In the next step, 4g $MgSO_4$ and 1g NaCl were added and shaken for 30s in vortex and then centrifuged for 10 min at 5°C and 5000 g using Eppendorf Centrifuge 5810R (Eppendorf, Hamburg, Germany). Then, 2 mL of acetonitrile extract were added to 0.1 g of C18 silica sorbent and 0.3 g of $MgSO_4$ for purification before centrifugation (5000 g) for 10 min. The purified extract was filtered through a syringe nylon filter and transferred into a vial for the LC-MS/MS analysis and for GC-MS/MS analysis the supernatant was collected and evaporated to dryness under a gentle nitrogen flow.

2.5. GC–MS/MS analysis

Before GC-MS/MS analysis, 50 μL of BSA + TMCS + TMSI (3:2:3) was added to the dry extract and left 30 min at room temperature. Then 200 μL of hexane was added, mixed thoroughly on vortex for 30 s and washed with 1 ml

of phosphate buffer (60 mM, pH7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to auto sampler vial.

The final extract (1 μL) was injected in splitless mode at 250 ºC in programmable temperature vaporization (PTV) using a Agilent 7890A GC system coupled with an Agilent 7000A triple quadruple mass spectrometer with inter electron-impact ion source (EI, 70Ev). The transfer line and source temperatures were 280° and 230°, 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). Analytes were separated on a HP-5MS 30m x 0.25mm x 0.25μm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode in program able temperature vaporization (PTV) inlet at 250°C employing helium as the carried gas at fixed pressure of 20.3 psi. The oven temperature started at 80°C, and increased to 245°C at 60 ºC/min, hold their time for 3 min and increased to 260 ºC progressively at 3 ºC/min and finally to 270 ºC at 10 ºC/min and then held for 10 min.

2.6. LC–MS/MS analysis

The analysis was performed using LC-MS/MS system consisting of a LC Agilent 1200 using a binary pump and an automatic injector and coupled to a 3200 QTRAP® ABSCIEX equipped with a Turbo-VTM source (ESI) interface. The chromatographic separation of the analytes was conducted at 25ºC with a reverse analytical column Gemini® NX-C18 (3 μM, 150 × 2 mm ID) and guard column C18 (4 × 2 mm, ID; 3 μM). Mobile phases was a time programmed gradient using methanol as phase A (5 mM ammonium formate and 0,1% formic acid) and water and phase B (5 mM ammonium formate 0,1% formic acid). The

elution gradient started with 0% of eluent B, increased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 100% in 10 min, decreased to 80% in 5 min and, finally, decreased to 70% in 2 min. The flow rate was 0.25 mL/min in all steps. Total run time was 21 min. The volume of the injections was 20 μL.

2.7. Statistical analysis

All results were included for statistical analysis, assuming for negative samples a value of 0 (EFSA, 2010). Analysis of variance (ANOVA test error type III) was used to assess the significance of the differences between the determined mycotoxin concentrations. All P-values of <0.05 (statistical significance of 95%) were considered to be statistically significant.

The relationships between mycotoxin concentrations and food group (cereals, legumes, vegetables, fish and meat) were assessed using the analysis of variance (ANOVA) and the pairwise t-test. Furthermore, principal component analysis (PCA) was performed using the mixOmics R package. Factor analysis was established to evaluate possible associations between different studied analytes in each food group.

2.8. Risk assessment and mycotoxins daily intake

For the exposure assessment based on a deterministic approach the probable daily intake (PDI) was obtained combining the mycotoxin contents and the food consumption data for adult population (Rodriguez-Carrasco et al., 2013). Food consumption data were available at the statistical database of the Spanish Ministry of Agriculture, Food and Environment (MAPAMA, 2016). The PDI (probable daily intake) of each mycotoxin was calculated as indicated in the following equation (1):

$PDI = (C_m \times K)/bw$ (1)

Where, C_m is the average concentration of mycotoxins detected in food expressed as (μg/kg), *K:* represents food consumption expressed in kg per day, *bw:* 70 kg were used as the average weight.

The daily consumption was set on 0.06 kg for cereals, 0.008 kg for legumes, 0.14 kg for vegetables, 0.09 kg for fish and 0.08 kg for meat per capita (MAPAMA, 2016).

The health risk characterization of each mycotoxin (% relevant TDI) was performed by comparing the PDI with the tolerable daily intake as indicated equation (2).

$\%TDI = (PDI/TDI) \times 100$ (2)

3. Results and discussion

3.1. Developed mycotoxin analysis

The analytical method was performed for linearity accuracy and sensibility, according to previous studies (Carballo et al., 2018), following de EU Commission Decision (2002/657). The criteria for confirmation of positive findings were set by area ratio obtained from quantification (Q) and confirmation (q) standard transitions, and the agreement with the retention times. For the recovery analysis, cereals, vegetables, legumes, meat and fish samples, were used in the validation procedure. Recoveries were between 63-119% for the levels of addition of 25, 50 and 100 μg/kg. Matrix effect (ME) was assessed for each analyte by comparing the slope of the standard calibration with that of the matrix-matched calibration, for the same concentration levels. The matrix-matched standards were used for effective qualifications taking into

account results of suppression and enhancement of the signal obtained. Linear range studied was from LOQ to 100 LOQ. A good linearity ($R^2 > 0.996$) within tested the tested range was obtained in all food matrices evaluated. 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 signal-to-noise ratio (S/N) 10:1. Ranges from 0.04-1.5 μg/kg and from 0.13-5 μg/kg were obtained for LOD and LOQ, respectively. The validation parameters as instrumental linearity, matrix effect, sensitivity (LOD and LOQ), and accuracy considered in the EU Commission Decision 2002/657 EC were reached.

3.2. Mycotoxin occurrence in different food group

Of the five analyzed food group (cereals, legumes, vegetables, fish and meat), the mycotoxins detected were DON, 3ADON, 15ADON, T-2, HT-2, NEO, NIV, ZEA, ENNB, $ENNB_1$ ENNA, $ENNA_1$, BEA, AFG2, OTA, DAS and βZAL. However the mycotoxins FB_1, FB_2, AFB_1, AFB_2, AFG_1, STG, αZAL, αZOL, βZOL and FUS-X were not detected.

Cereal food presented a greater occurrence of mycotoxins (35%), followed by vegetables (32%), legumes (15%), meat (9%) and fish (9%), which in all cases showed levels lower than the maximum limit (ML) established by EU for legislated mycotoxins (European Commission 1881/2006).
DON was the most common mycotoxin in the five food groups. Mycotoxins T-2, ZEA and $ENNA_1$ were only present in cereals, while, ENNA and DAS were only detected in fish.

The contents of the studied mycotoxins in the five food groups are very variable for each food group. The higher mean contents occurred in legumes for

βZAL and HT-2 toxin, followed by OTA and BEA in the meat group, and DON and HT-2 toxin in cereals group.

A two-way ANOVA analysis has been used to find out the relationship between the quantitative dependent variable (mean concentrations of each mycotoxin) and the two qualitative independent variables (food group and mycotoxins).

The food groups have different behaviours according to the mycotoxin and contents. Therefore they show no normality and no homogeneity of the variances. Consequently, the ANOVA model used is non-parametric, in which measures must be taken to compensate for unequal sample sizes in order to maintain the validity of the analysis. The results obtained point that individually the mycotoxin and food group variables relation is not significant but the interaction between them is significant. That is, there is a relationship between the food groups and the mycotoxins.

The principal component analysis (PCA) revealed the existence of correlation between mycotoxins and the different food groups ranging from 60% to 100% (Fig. 1). In cereal group, DON, 15ADON, T-2, NEO, NIV and AFG_2 present high correlation. The same behaviour was registered for NEO and AFG_2 in legumes group, and ENNB, $ENNB_1$ and BEA in vegetable group. In fish group, 3ADON, NEO, ENNA, DAS and 15ADON showed good correlation and also NIV, AFG_2 and βZAL in meat group. These results highlight a high correlation between the studied mycotoxins and the food group.

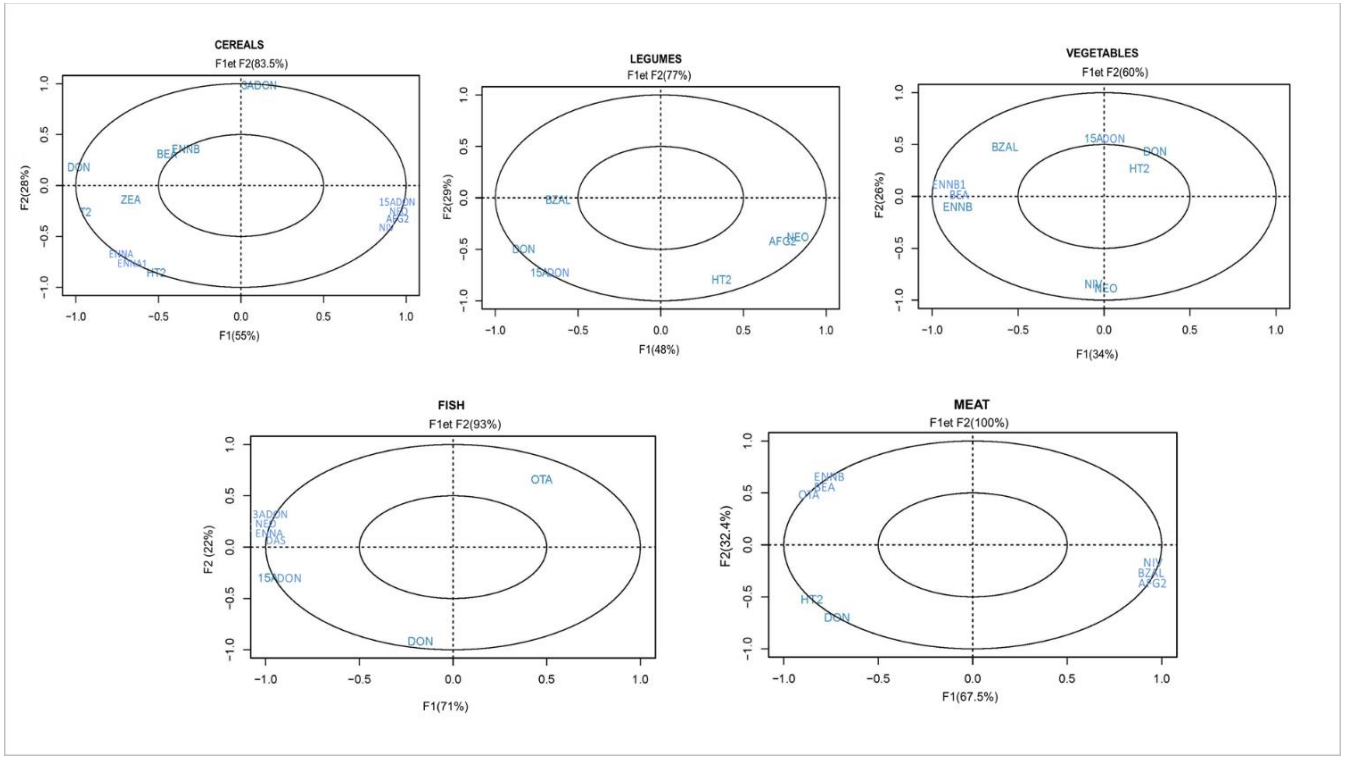

Fig. 1. Principal component analysis (PCA) for different food group (cereals legumes, vegetables, fish and meat

Interactions between mycotoxins showing enhancement of their toxic activity have been reported in several investigations *in vitro* in different cell lines. Combinations of mycotoxins as BEA+T-2 and BEA+DON+T-2 displayed synergistic interactions after 24 and 48 h of exposure (Ruiz et al., 2011). Binary combinations of 3ADON+15ADON and ENB+DON at 24h exhibited also a boost of cytotoxic effect (Juan-García et al., 2015; Gómez et al., 2015).

3.2.1. Cereal samples

The results obtained in pasta, rice, bread, and mixed dishes are presented in the Table 1. The overall incidence were ranged from 3% for 3ADON to 60 % for DON.

DON was the most prevalent mycotoxin in pasta, bread and mixed diches with an incidence from 85% to 100% and levels from 3.61 to 5.17 μg/kg. In rice samples, NEO presented a high incidence (79%). Different authors, reported higher values of DON: in bread samples ranging between 28 to 524 μg/kg (Leblanc et al., 2005; Beltrán et al., 2013; Sirot et al., 2013; López et al., 2016; Cano-Sancho et al., 2012; Raad et al., 2014), in cooked pasta sample from 2.84 to 60.1 μg/kg (López et al., 2016; Carballo et al., 2018; Sirot et al., 2013; Rodríguez-Carrasco et al., 2015) and in mixed dishes samples from 10.57 to 23.4 μg/kg (Yau et al., 2016; Leblanc et al., 2005; Sirot et al., 2013).

The HT-2 toxin was also detected in pasta rice and mixed dishes with incidence from 24% to 69% and at concentrations between 2.06 to 9.85 and μg/kg, respectively. Our results found in cooked pasta are similar (3-10 μg/kg) to those reported by Sirot et al. (2013) and slightly lower (34.43 μg/kg) to those reported by Carballo et al. (2018).

ENNB incidence was pretty high in pasta and bread samples (64% to 93%) with concentrations from 3 to 7.11 μg/kg. These results are in concordance with those reported in pasta and bread samples at levels from 8.7 to 16 μg/kg (López et al., 2016; Saladino et al., 2017).

AFG_2 was also found in rice samples at mean contents of 0.17 μg/kg. Comparable results were found in cereal samples from 0.05 to 2.6 μg/kg (Raad et al., 2014; Huong et al., 2016; Yau et al., 2016; Carballo et al., 2018).

3ADON, 15ADON, T-2, NIV, $ENNA_1$ BEA and ZEA were detected with an incidence between 3% to 18% of samples in concentrations ranged from 0.08 to 1.96 μg/kg.

Table 1: Incidence and mean contents of mycotoxins in ready-to-eat food prepared with cereals products.

Food products	DON[a]		3ADON		15ADON		HT-2		T-2		NEO		NIV		ZEA[b]		ENN B		$ENNA_1$		BEA		AFG_2[c]	
Cereals n=88	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)
Pasta n=22	5.17	100	-	-	-	-	9.85	64	3.58	18	0.42	18	0.13	5	0.69	14	3.00	64	0.65	23	0.25	9	-	-
Rice n=38	0.29	13	0.05	2	0.71	29	3.47	24	0.52	8	2.09	79	2.16	34	0.13	3	0.08	5	-	-	0.04	5	0.17	10
Bread n=15	3.61	93	0.14	2	0.14	7	0.74	20	1.99	7	0.50	20	-	-			7.11	93	-	-	-	-	-	-
Mixed dishes n=13	4.48	85	0.12	2	0.12	2	2.06	69	1.74	8	0.56	23	-	-	0.78	15	-	-	-	-	0.71	13	-	-
Overall mean (µg/kg) and incidence	*3.39*	*60*	*0.08*	*3*	*0.21*	*14*	*4.03*	*40*	*1.96*	*10*	*0.89*	*45*	*0.57*	*18*	*0.4*	*7*	*2.72*	*39*	*0.16*	*6*	*0.25*	*7*	*0.04*	*5*

[a] Maximum levels established (MLs) of 500 -750 µg/kg for DON in bread and cereals intended for direct human consumption, cereal flour, bran and germ as end product marketed for direct human consumption (European Commission, 1881/2006).
[b] MLs of 20 - 75 µg/kg for ZEA in processed cereal-based foods, bread (including small bakery wares), pastries, biscuits, cereal snacks and breakfast cereals, excluding maize snacks and maize based breakfast cereals, cereals intended for direct human consumption, cereal flour, bran as end product marketed for direct human consumption and germ (European Commission, 1881/2006).
[c] MLs of <4.0 µg/kg for AFs in processed cereal-based foods (European Commission, 1881/2006).

3.2.2. Legume samples

The results obtained in lentils, chickpeas and beans are presented in Table 2. The mycotoxins detected were DON, 15ADON, HT-2 toxin, NEO, AFG_2 and βZAL. The overall incidence ranged from 8% for 15ADON and AFG_2 to 54% for HT-2 toxin, which was the most prevalent mycotoxin in legumes samples with mean contents of 7.8 μg/kg.

βZAL was found in 29% of legume samples mainly in red beans and lentils samples at concentrations of 16.08 and 25.10 μg/kg, respectively.
DON was also detected in 21% of lentils, green and red beans samples at levels from 1.69 to 6.90 μg/kg. These contents are slightly lower to those reported in the literature in legume samples with mean level of 31.25 μg/kg (Raad et al., 2014).

AFG_2 was detected with an incidence of 33% of flat beans at mean level of 3.4 μg/kg and similar AFs content were reported in beans samples at levels from 1.35 to 2.9 μg/kg (Yau et al., 2016; Huong et al., 2016).

3.2.3. Vegetable products

The results obtained in zucchini, eggplant, carrot, onions, peppers, sweet corn, mushrooms, tomato, potato and vegetable soup are presented in the Table 2. The mycotoxins detected were: DON, 15ADON, HT-2 toxin, NEO, NIV, ENNB, $ENNB_1$, BEA, and βZAL. The overall incidence ranged from 2% for 15ADON, $ENNB_1$ and BEA to 13% for DON.

DON was the most prevalent mycotoxin, with incidence ranging from 4% for potatoes to 50% for mushrooms, and levels from 0.25 to 3.43 μg/kg.

DON was also reported on other studies on vegetable samples and sweet at levels from 1.5 to 114 μg/kg (Sirot et al., 2013; Cano-Sancho et al., 2012).

HT-2 toxin was found in 4% of potatoes to 20% in carrots analysed at levels from 0.39 to 2.35 μg/kg. Higher HT-2 toxin contents were previously detected in sweet corn sample at mean levels of 31.6 μg/kg (Cano-Sancho et al., 2012).

βZAL was determined in potato and soup samples at concentrations from 4 and 6.40 μg/kg. In the literature, comparable values than those reported in this survey were reported for ZEA in sweet corn and vegetable samples (4.9 to 17 μg/kg) (Leblanc et al., 2005; Cano-Sancho et al., 2012). While, in other studies ZEA and metabolites were not detected in vegetable products (Yau et al., 2016; Sirot et al., 2013).

BEA was detected in 6% of potato samples at 0.26 μg/kg. This data are similar than those found by López et al., (2016), who detected BEA in tomato products at mean concentration of 2.4 μg/kg.

15ADON, NEO, NIV, ENNB and $ENNB_1$ were detected in 2% to 33% of the positive vegetable products at levels lower than 2.34 μg/kg.

Table 2: Incidence and mean contents of mycotoxins in ready-to-eat food prepared with legumes products

Food products	DON		15ADON		HT-2		NEO		AFG_2		βZAL		ENNB		ENNB1		BEA	
Legumes n=24	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)	Mean µg/kg	I (%)
Red beans n=2	6.90	100	-	-	11.68	100	-	-	-	-	16.08	50	-	-	-	-	-	-
Green beans n=7	1.93	29	6.92	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Lentils n=4	1.69	25	-	-	-	-	-	-	-	-	25.10	100	-	-	-	-	-	-
Flat beans n=6	-	-	-	-	12.53	100	5.20	50	3.46	33	2.62	17	-	-	-	-	-	-
White beans n=3	-	-	-	-	11.03	100	3.28	33	-	-	8.70	33	-	-	-	-	-	-
Chickpeas n=2	-	-	-	-	11.51	100	-	-	-	-	-	-	-	-	-	-	-	-
Overall mean (µg/kg) and incidence (%)	*1.75*	*21*	*1.15*	*8*	*7.79*	*54*	*1.43*	*17*	*0.58*	*8*	*8.75*	*29*	-	-	-	-	-	-
Vegetables n=141																		
Zucchini n=8	0.92	13	0.88	13	1.70	13	-	-	-	-	-	-	-	-	-	-	-	-
Eggplant n=9	0.82	11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carrot n=5	1.56	20	-	-	1.90	20	-	-	-	-	-	-	-	-	-	-	-	-
Onions n=11	0.64	9	-	-	0.65	9	0.89	9	-	-	-	-	-	-	-	-	-	-
Peppers	1.06	15	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Sweet corn n=6	1.11	17	-	-	2.35	17	1.74	17	-	-	-	-	-	-	-	-	-	-
Mushrooms n=6	3.43	50	-	-	1.43	17	-	-	-	-	-	-	-	-	-	-	-	-
Tomato n=16	0.43	6	-	-	-	-	1.26	13	-	-	-	-	-	-	-	-	-	-
Potato n=54	0.25	4	-	-	0.39	4	0.18	2	-	-	4.00	20	0.14	13	-	-	-	-
Vegetable soup n=6	-	-	2.34	33	1.33	17	-	-	-	-	6.40	17	0.17	4	0.18	4	0.19	6
Overall mean (µg/kg) and incidence (%)	*1.53*	*13*	*0.27*	*2*	*1.27*	*6*	*0.52*	*4*	*-*	*-*	*0.87*	*9*	*0.03*	*3*	*0.03*	*2*	*0.87*	*9*

3.2.4. Fish and fish aquatic products

The results obtained in fish, squid and prawns are presented in the Table 3. The mycotoxins detected were: DON, 3ADON, 15ADON, NEO, ENNA, OTA and DAS.

DON was determined in 17% of fish meals and 36% of squid meals at mean concentrations of 1.19 to 3.58 μg/kg. OTA was detected in prawn samples with a prevalence of 20% at mean level of 1.08 μg/kg. OTA contents were also previously reported in fish and aquatic products at levels from 2.24 to 4.85 μg/kg (Huong et al., 2016). 3ADON, 15ADON, NEO, ENNA and DAS were also determined even at levels lower than 1μg/kg.

3.2.5. Meat samples

The results obtained in pork, beef, and chicken meat are presented in the Table 3. The overall incidence were between 5% for BEA and 18% for DON, NIV and OTA at levels from 0.1 μg/kg for ENNB to 8.09 μg/kg for OTA.
OTA was detected in 44% of pork meals at mean contents of 24.27 μg/kg. Lower values were reported for OTA in meat products with levels from 0.01 to 2.68 μg/kg (Leblanc et al., 2005; Huong et al., 2016; Sirot et al., 2013; Yau et al., 2016).

AFG_2 was also detected in 22% of chicken meat at mean contents of 0.63 μg/kg. Our results are similar to those reported of AFB_1 in meat products at mean concentration of 4 μg/kg (Huong et al., 2016). DON, HT-2 toxin, NIV, ENNB, BEA and AFG_2, were between determined at 11% to 50% of meat samples, at concentrations of 0.31 to 3.40 μg/kg. Trichothecenes and ENNs occur frequently in grains and grain-based products and, to the best of our

knowledge, very rarely in meat products. Therefore, cross-contamination during manufacturing or by mixing ingredients may be the main reason of the contamination.

Food processing reduce mycotoxin concentrations significantly, but do not eliminate them completely (Milani and Maleki, 2014). For instance, several studies reported slightly higher contents of mycotoxins in raw food. DON and NEO were reported with contents from 4.1 to 91.8 μg/kg in rice samples (Rodriguez-Carrasco et al., 2013). In pasta samples, DON, NIV, T-2 and HT-2 toxin were reported at 1.46-36.45 μg/kg and also, ENNA, $ENNA_1$, ENNB and BEA at 4.25-53.96 μg/kg (Tolosa et al., 2017). In pepper samples BEA (15-57 μg/kg) and ENNA (4.1 μg/kg) have been detected (Zhao et al., 2018). While, in fish sample ENNA (4.2 μg/kg), ENNB (13.5 μg/kg) and $ENNB_1$ (10.9 μg/kg) have been detected (Tolosa et al., 2014). In meat products, OTA levels from 56-158 μg/kg were reported, higher than those found in this survey (Sorensen et al., 2010).

There are many factors that predispose to mycotoxin production and temperature and storage-processing conditions are the most reported (Covarelli et al., 2015). The trichothecenes are in general very stable compounds, both during storage (milling) and processing (cooking) of food, and are hardly degraded by elevated temperatures (EFSA, 2011).

Table 3: Incidence and mean contents of mycotoxins in ready-to-eat food prepared with fish and aquatic products

Food products	DON		3ADON		15ADON		HT-2		NIV		NEO		ENNA		ENNB		BEA		AFG2		OTA		DAS	
Fish and aquatic products n=53	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %	Mean µg/kg	I %
Fish n=29	1.19	17	0.58	7	0.58	7	-	-	-	-	0.86	7	0.89	7	-	-	-	-	-	-	-	-	0.55	7
Squid n=14	3.58	36	-	-	0.48	7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Prawns n=10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.08	20	-	-
Overall mean (µg/kg) and incidence (%)	*1.19*	*19*	*0.15*	*9*	*0.35*	*9*	-	-	-	-	*0.21*	*4*	*0.23*	*4*	-	-	-	-	-	-	*0.27*	*4*	*0.14*	*4*
Meat n=22																								
Pork n=9	1.51	22	-	-	-	-	1.48	11	-	-	-	-	-	-	0.31	22	18	11	-	-	24.27	44	-	-
Beef n=4	3.40	50	-	-	-	-	2.33	25	-	-	-	-	-	-	-	-	-	-	-	-			-	-
Chicken n=9	-	-	-	-	-	-	-	-	4.58	44	-	-	-	-	-	-	-	-	0.63	22			-	-
Overall mean (µg/kg) and incidence (%)	*1.64*	*18*	-	-	-	-	*1.27*	*9*	*1.53*	*18*	-	-	-	-	*0.1*	*9*	*6.21*	*5*	*0.21*	*9*	*8.09*	*18*	-	-

3.3. Estimation of mycotoxin intake through ready-to-eat meals.

The Joint FAO/WHO Expert Committee and Food Additives (JECFA) and Scientific Committee on Food (SCF) has established tolerable weekly intake (TWI) of 120 ng/kg bw for OTA and Tolerable daily intake (TDI) of 250 ng/kg bw for ZEA, 100 ng/kg bw for the sum of T-2 and HT-2 toxins and 1200 ng/kg bw for NIV and 1000 ng/kg bw for DON and they acetyls (JECFA, 2001; SCF, 2002). Since aflatoxins are human carcinogens, no tolerable intakes have been set for this group of mycotoxins.

Table 4 summarizes the risk characterization of mycotoxins evaluated in the five food groups based on probable daily intake.

The main contributor to mycotoxin daily intake through cereal meals was HT-2 toxin with 3.45 ng/kg bw followed by DON with 2.90 ng/kg bw. The risk assessment ratios (TDI %) through cereal consumption were between 0.004% for βZAL to 3.45 % for HT-2 toxin. Other studies have already estimated the mycotoxin intake in cooked food, for instance data of HT-2 toxin intake through processed cereal-based products were reported up to 14.93 ng/kg bw/day for adult French population (Sirot et al., 2013). In this same country, the study conducted by Leblanc et al., (2005), reported DON daily intake through cereal products between 0.21 to 188 ng/kg bw. However, Raad et al., (2014) reported daily dietary exposure to DON up to 1560 ng/kg mainly by consumption of cereals products predisposing the excessive consumer of exceeding the toxicological reference value of DON. On the other hand, daily intake through cereal-based products consumption reported for OTA and for AFB_1 were up to 22.2 ng/kg bw. and 7.9 ng/kg bw respectively (Huong et al., 2016).

In legume samples the main contributor to the dietary exposure was βZAL with 1.00 ng/kg bw/day, while the lowest values were found for 3ADON, T-2, NIV, ZEA, ENNA, $ENNA_1$, ENNB, $ENNB_1$, BEA, OTA and DAS with 0.002 ng/kg bw/day for each one. The risk assessment ratios were between 0.0002% for 3ADON, NIV and T-2 toxin to 0.89% for HT-2 toxin, respectively. The average intake of ZEA and DON estimated by Leblanc et al., (2005) through legume consumption were between 0.02 and 0.08 ng/kg bw/day. Huong et al., (2016) estimated daily intake of OTA and AFB_1 by legume consumption were 0.1 to 0.2 ng/kg bw.

The dietary exposure through vegetables consumption was from 0.04 ng/kg bw/day for not detected mycotoxins to 3.06 ng/kg bw/day for DON. The risk assessment was between 0.004% for 3ADON to 2.54% for HT-2 toxin contributing in major amount to the mycotoxin dietary exposure through vegetable intake. In other study Leblanc et al., (2005) estimated daily intake for OTA, ZEA and DON of 0.06, 0.32 and 0.37 ng/kg bw/day. Similar exposure data for ZEA of 0.08-0.36 ng/kg bw/day and DON 0.2-0.8 ng/kg bw/day respectively, were previously published (Sirot et al., 2013). Huong et al., (2016) also estimated OTA and AFB_1 daily intake from cooked vegetable consumption at 0.6 to 1.6 ng/kg bw/day, respectively.

In fish samples the main contributor to daily mycotoxin exposure was DON with 1.53 ng/kg bw, while the risk assessment ratio was up to 2.04 % for OTA. Similar average OTA daily intakes were estimated by Huong et al., (2016); 1.4 ng/kg bw through fish and 0.3 ng/kg bw through other aquatic products assayed.

In meat meals the main contributor to the dietary exposure was OTA with 9.24 ng/kg bw which has been materialized in risk assessment ratio of 54.3%. Various studies have already estimated mycotoxin intake in meat cooked. For instance, Leblanc et al., (2005) estimated OTA daily intake through meat consumption of 0.08 ng/kg bw/day. However, Huong et al., (2016) estimated dietary exposure to OTA 5 ng/kg bw/day for OTA by meat consumption while Sirot et al., (2013) estimated it at of 0.017-0.099 ng/kg bw/day for average consumer.

HT-2 toxin and OTA were the mycotoxins that contributed in major amount to dietary exposure and 3ADON and 15ADON in minor degree but, in general these finding do not reveal a health risk for the average as the estimated PDIs were lower than the TDIs set by FAO/WHO expert JECFA committees.

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

Mycotoxin	TDI (ng/kg bw/day)	Cereals Mean (µg/kg)	Cereals PDI (ng/kg bw/day)	Cereals TDI (%)	Legumes Mean (µg/kg)	Legumes PDI (ng/kg bw/day)	Legumes TDI (%)	Vegetables Mean (µg/kg)	Vegetables PDI (ng/kg bw/day)	Vegetables TDI (%)	Fish Mean (µg/kg)	Fish PDI (ng/kg bw/day)	Fish TDI (%)	Meat Mean (µg/kg)	Meat PDI (ng/kg bw/day)	Meat TDI (%)
DON	1000	3.39	2.90	0.29	1.75	0.2	0.02	1.53	3.06	0.30	1.19	1.53	0.15	1.64	1.87	0,18
3AcDON	1000	0.08	0.06	0.006	<LOD	*0.002*	*0.0002*	<LOD	*0.04*	*0.004*	0.15	0.19	0,01	<LOD	*0,02*	*0.002*
15AcDON	1000	0.21	0.18	0.01	1.15	0.13	0.01	0.27	0.54	0.05	0.35	0.45	0.04	<LOD	*0.02*	*0.002*
T-2	100	1.96	1.68	1.68	<LOD	*0.002*	*0.0002*	<LOD	*0.04*	*0.04*	<LOD	*0.02*	*0.002*	<LOD	*0.02*	*0.02*
HT-2	100	4.03	3.45	3.45	7.79	0.89	0.89	1.27	2.54	2.54	<LOD	*0.02*	*0.002*	1.27	1.45	1.45
NEO	-	0.89	0.76	-	1.43	0.16	-	0.52	1.04	-	0.21	0.27	-	<LOD	*0.02*	-
NIV	1200	0.57	0.48	0.04	<LOD	*0.002*	*0.0002*	0.52	1.04	0.08	<LOD	*0.02*	*0.001*	1.53	1.74	0.14
ZEA	250	0.4	0.34	1.37	<LOD	*0.002*	*0.0008*	<LOD	*0.04*	0.01	<LOD	*0.02*	*0.008*	<LOD	*0.02*	*0.008*
ENNA	-	<LOD	0.01	-	<LOD	*0.002*	-	<LOD	*0.04*	-	0.23	*0.29*	-	<LOD	*0.02*	-
ENNA1	-	0.16	0.13	-	<LOD	*0.002*	-	<LOD	*0.04*	-	<LOD	*0.02*	-	<LOD	*0.02*	-
ENNB	-	2.72	2.33	-	<LOD	*0.002*	-	0.03	0.06	-	<LOD	*0.02*	-	0.1	0.11	-
ENNB1	-	<LOD	0.01	-	<LOD	*0.002*	-	0.03	0.06	-	<LOD	*0.02*	-	<LOD	0.02	-
BEA	-	0.25	0.21	-	<LOD	*0.002*	-	0.03	0.06	-	<LOD	*0.02*	-	6.21	7.09	-
AFG2	-	0.04	0.03	-	0.58	0.06	-	<LOD	*0.04*	-	<LOD	*0.02*	-	0.21	0.24	-
βZAL	250	<LOD	*0.01*	*0.004*	8.75	1	0.4	0.87	1,74	0.69	<LOD	*0.02*	*0.008*	<LOD	*0,02*	*0.008*
OTA	17	<LOD	*0,01*	*0.1*	<LOD	*0.002*	*0.01*	<LOD	*0.04*	*0.23*	0.27	0.34	2.04	8.09	9.24	54.3
DAS	-	<LOD	*0,01*	-	<LOD	*0.002*	-	<LOD	*0.04*	-	0.14	0.18	-	<LOD	*0.02*	-

Italic data show the calculated value in non-detected samples assuming LOD/2.

4. Conclusion

The presence of twenty-seven mycotoxins was evaluated in 328 samples of ready-to-eat foods that are commonly consumed in Spain, mainly by the adult population. Our results show that contamination levels in all food groups were lower than those often obtained from raw foods and even in cooked food. A high prevalence of mycotoxins in the five food groups was for DON. A relationship between the food group and mycotoxins was observed. The exposure assessment carried out in several food commodities showed that the probable daily intakes of the studied mycotoxins were below the tolerable daily intakes. Although there is no toxicological concern for the Spanish population through the ready-to-eat food consumption, continuous monitoring of mycotoxins levels in ready-to-eat food is necessary to ensure food security consumers

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Conflict of interest

The authors declare no conflict of interest and informed consent was obtained from all individual participants included in the study.

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3.4. Dietary exposure assessment to mycotoxin through fruits juice

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Dietary exposure to mycotoxins through fruits juice consumption

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Resumen

Se presenta un estudio sobre zumos de frutas a base de manzana, piña, albaricoque, naranja y pera para determinar la presencia natural de quince micotoxinas mediante cromatografía de gases acoplada a espectrometría de masas en tándem (EM/EM). El procedimiento desarrollado de multi-micotoxinas se llevó a cabo mediante micro-extracción líquida-líquida dispersiva (DLLME). El 36% de las muestras analizadas presentaron contaminación con micotoxinas y una muestra de jugo de naranja, superó el nivel máximo (ML) establecido por la UE para PAT (50 μg/L). Se detectó PAT en naranja, manzana, frutas mezcladas y jugos de piña con una prevalencia de 86%, 60%, 29%, 14% a concentraciones promedio de 34.57 μg/L, 33.41 μg/L, 8.59 μg/L, 8.02 μg/L, respectivamente. La toxina HT-2 estaba presente en el jugo mixto (43%) a un nivel medio de 22.38 μg/L. En general, ninguna preocupación toxicológica se asoció con la exposición a micotoxinas en la población de niños y adultos, los resultados ponen de relieve la necesidad de estudios rigurosos de monitoreo de HT-2 en el zumo de fruta.

Abstract

A study on fruit juice products (apple, pineapple, apricot, orange and pear) was carried out to determine the natural occurrence of fifteen mycotoxins by gas chromatography coupled to tandem mass spectrometry (MS/MS). A developed multi-mycotoxin procedure was carried out by dispersive liquid-liquid microextraction (DLLME). 36% of the analyzed samples presented mycotoxin contamination. PAT was detected in orange, apple, mixed fruits and pineapple juices with prevalence of 86%, 60%, 29%, 14% at mean concentrations of 34.57 μg/L, 33.41 μg/L, 8.59 μg/L, 8.02 μg/L, respectively. One orange juice sample,

exceeded the maximum level (ML) established by EU for PAT (50 μg/L). HT-2 toxin was found in mixed juice (43%) at mean level of 22.38 μg/L. Overall no toxicological concern was associated to mycotoxins exposure for children and adult population and the results obtained highlight the necessity for rigorous monitoring studies on HT-2 in fruit juice.

1. Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi as *Aspergillus, Penicillium, Fusarium and Claviceps*. More than 400 mycotoxins are known and only some of them represent a real threat to food security. The most relevant are aflatoxins (AFs), ochratoxin A (OTA), Patulin (PAT) fumonisins (FBs), zearalenone (ZEA) and trichothecenes (TCs) [1]. Chronic exposition to some mycotoxins can produce carcinogenic, mutagenic, teratogenic, cytotoxic, neurotoxic, nephrotoxic, immunosuppressive and estrogenic effect. Their seriousness effects depend largely on the ingested amounts and duration of exposure that may result from simultaneous ingestion of various mycotoxins [2]. Mycotoxins can be present along the entire process of food production; in field, before and after the harvest, during processing, storage and also in a finished product [3].

Various factors affect the levels of contamination of mycotoxins in fruit and fruit products such as type and variety of fruit, climate conditions, geographical location, year production treatments before and after the harvest, use and pesticides, damage to the surface of the fruit, and storage conditions [4]. European Food Safety Authority (EFSA) have established maximum permitted levels for certain mycotoxins as aflatoxins B_1, B_2, G_1, G_2 y M_1, OTA,

PAT, ZEA, DON and fumonisins B_1 and B_2, T-2 and HT-2 toxins [5]. In fruits and fruit juices, only PAT and OTA are legislated. A maximum up to 50 µg/kg is set for Patulin in reconstituted concentrated fruit juices and fruit nectar (Table1). While for Ochratoxin A a maximum level of 2 µg/kg is set up in grape juices, reconstituted concentrated grape juice, grape nectar, grape must and reconstituted concentrated grape must, intended for direct human consumption [5].

Table 1: Summary of maximum levels specified for patulin in fruit and fruit juice

Food products	**Maximum level µg/kg**
Fruit juices, concentrated fruit juices as reconstituted and fruit, nectars, spirit drinks, cider and other fermented drinks derived from apples or containing apple juice	50
Apple juice and solid apple products	10

The presence of mycotoxins has been highly investigated in different fruit juices such as apple juice [6], orange juice [7], pear juice [8] apricot and peach juice [9] and berry juice [10]. The most of the method used for extraction mycotoxins in fruit juice are QuEChERS extraction [11], liquid-liquid extraction [12], and dispersive liquid-liquid microextraction [13]. DLLME offers some advantages over traditional technique being simple, fast and low cost technique extraction [14].

Regarding analytical method for mycotoxins determination, liquid chromatography mass spectrometry in tandem (LC-MS/MS) and gas chromatography coupled to mass spectrometry detector (GC-MS/MS) have become the most extensively technique used for determination of mycotoxins

in biological and food samples [15, 16]. The gas chromatographic techniques offers some advantages as lower detection limits and greater selectivity [17].

In this sense the aim of the present study was to evaluate the presence of fifteen mycotoxins DON, 3-AcDON, 15-AcDON, NEO, DAS, NIV, ZON, α-ZOL, β-ZOL,α-ZAL, β-ZAL, FUS X, T-2, HT-2 and PAT and to carry a risk exposure of the population to these mycotoxins through the fruit juice consumption.

2. Material and methods

2.1. Chemicals and reagents

Solvents (acetonitrile, hexane, chloroform and methanol) were supplied by Merck (Darmstadt, Germany). Deionized water (<18.2 MΩ cm resistivity) was obtained in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Beadford, MA, USA). Ammonium formate (99%) and formic acid (≥98%), sodium chloride were supplied by Sigma Aldrich (Madrid, Spain). Syringe nylon filter (13mm diameter 0.22 μm pore size) were obtained from Analysis Vinicos S.L. (Tomelloso Spain). The derivatization reagent composed of BSA (N,O-bis(trimethylsily) + TMCS (trimthylcholorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

2.1.1. Standards and solutions

The standards of DON, 3-ADON, 15-ADON, DAS, NIV, FUS-X, NEO ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, T-2 and HT-2 toxins were purchased from Sigma Aldrich. Individual stock of all analytes were prepared to obtain 20 mg/L in methanol and multianalyte working solutions of 2 mg/L were also used by diluting the individual stock solutions. The multianalyte working standard solution was used for standard calibration curves, matrix-matched calibration curves and recovery assays. All standards were stored in darkness and kept at-20°C.

2.2. Procedures

2.2.1. Commercial samples

A total of 42 samples were purchased from different supermarkets in Valencia, Spain. The samples were classified in as 10 samples of apple juice, 7 samples of each of pineapple, apricot, mixed fruits and orange juices and 4 samples of pear juice.

2.2.2. Dispersive liquid-liquid microextraction

Samples extraction was performed according to the previously validated method by Pallarés et al. [18]. Briefly, 5 mL aliquot of fruit juice sample were placed in a 10 mL conical tube, a mixture of dispersion solvent (950 μL of ACN) and the extraction solvent (620 μL of EtOAc) were added; the resulting mixture was shaken for 1 min, forming a cloudy solution of the three components. The mixture was centrifuged 4000 rpm for 5 min, and the organic phase at the top of the tube was recovered and placed in second conical tube. Next, a mixture of dispersion solvent (950 μL of MeOH) and extraction solvent (620 μL of

CHCl3) was added to the remaining residue, and after agitation and centrifugation, the separated organic phase was recovered and added to the first organic phase. The solvent in the conical tube containing the two recovered phases was evaporated to near dryness under a nitrogen stream using turvovap LV Evaporator (Zimark, Hopkinton, MA). The residue was reconstituted with 1 mL of 20 mM ammonium formate (MeOH/ACN) (50/50 v/v) and filtrated through a 13-mm/0.22 μm nylon filter (Membrane Solutions, Plano, TX). Finally, reconstituted extract was evaporated to dryness under a gentle nitrogen flow.

2.2.3. Derivatization

Before GC-MS/MS analysis, 50 μL of BSA + TMCS + TMSI (3:2:3) was added to the dry extract and left 30 min at room temperature. Then 200 μL of hexane was added, mixed thoroughly on vortex for 30 s, washed with 1 ml of phosphate buffer (60 mM, pH7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to an auto sampler vial.

2.3. GC-MS/MS analysis

Gas chromatographic determination was carried out using a GC system Agilent 7890A coupled with an Agilent 7000A triple quadruple mass spectrometer with inter electron-impact ion source (EI, 70Ev) and Agilent 7693 auto sampler (Agilent Technologies, Palo Alto, USA). Quantitation data were acquired at selection reaction monitoring (SRM). The transfer line and source temperatures were 280° and 230°, 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). Analytes were separated on a HP-5MS 30m x 0.25mm x 0.25μm capillary column. A total

one microliter of the final clean extract of mycotoxins was injected in splitless mode in programmable temperature vaporization (PTV) inlet at 250°C employing helium as the carried gas at fixed pressure of 20.3 psi. The oven temperature started at 80°C, and increased to 245°C at 60 ºC/min, hold their time for 3 min and increased to 260 ºC progressively at to 3 ºC/min and finally to 270 ºC at 10 ºC/min and then held for 10 min. Data were acquired and processed using Agilent Masshunter version B.04.00 software.

The criteria established in Document No. SANCO 11813/2017 [19] was achieved for quantification purposes. For each analyte, two transitions of SRM were required for each compound and compliance of the SRM ratio, defined as the relative intensities of ions between the area of both the quantitation (Q) and the confirmation transition (q). The most intense SRM transition was selected for quantification purposes. The specific parameters of MS/MS for each mycotoxin are detailed in Table 2.

Table 2: MS/MS parameters of the selected mycotoxins

Mycotoxin	Retention time (min)	Quantitative Transition (Q)			Qualitative transition (q)		
		Q1	Q3	CE, eV (Dt, ms)	Q1	Q3	CE, eV (Dt, ms)
DON	8.4	392	259	10 (25)	407	197	10 (25)
3-ADON	9.68	392	287	5 (35)	467	147	10 (25)
15-ADON	9.65	392	217	20(35)	392	184	20(35)
NIV	10.15	289	73	15 (35)	379	73	15 (35)
NEO	11.68	252	195	10 (25)	252	167	15 (35)
DAS	9.85	350	229	15 (35)	378	124	10 (25)
HT-2	14.39	347	157	10 (25)	347	185	10 (25)
T-2	14.8	350	259	10 (25)	350	229	15 (35)
FUS-X	9.73	450	260	10 (35)	450	245	20 (35)
PAT	4.3	226	73	10 (50)	183	75	15 (50)
ZON	15.95	462	151	10 (25)	462	333	10 (25)
α-ZAL	15.45	433	309	20 (35)	433	295	20 (35)
ß-ZAL	15.68	433	295	15 (35)	307	73	10 (35)
α-ZOL	16.45	305	73	15 (25)	305	289	15 (20)
ß-ZOL	16.82	536	333	10 (35)	536	446	15 (20)

2.4. Exposure assessment

The probable daily intake (PDI) is considered one of the most important aspect for the measure and characterization of risk assessment of contaminants in food. With the aim of estimating exposure to the substance in the population [20], the probable daily intakes (PDIs) was calculated and expressed in μg/L bw day as indicated by the following equation:

PDI= (C*K)/bw

Where "C" is the mean concentration of mycotoxin detected in food expressed as μg/L. "K" represents fruit juice consumption expressed in L per day and "bw" is the average weight of the age groups studied. Fruit juice consumption data were available in the statistical data base of the Spanish Ministry of Agricultural and Environment (MAPAMA), considering a consumption of 10 L/annual [21]. As this fruit juice by general population including children, two different body weights were considered (25 kg and 70 kg for children and adults, respectively).

The health risk characterization of mycotoxin (%) percentage of relevant TDI) was performed by dividing the calculated PDI by the tolerable daily intake (TDI) (μg/kg bw day) of the respective mycotoxins.

%TDI= (PDI/TDI)*100

The established provisional maximum tolerable daily intake (PMTDI) for PAT of 400 ng/kg bw was established [22] and 60 ng/kg bw for the sum T-2 and HT-2 toxins [23].

3. Results and discussion

3.1. Method validation and analytical parameters

The analytical method was validated for fruit juice samples (Table 3). Matrix effects were corrected by matrix-assisted calibration curve a mycotoxin-free sample. For the evaluation of the linearity, calibration curves were constructed at eight concentration levels (250 to 1.97 μg/L for all studied analytes).

The results showed good correlation coefficients (r^2>0.9986). Limits of detection (LODs) were calculated using a signal-to-noise of 3 (from 0.58 to 2.34 μg/L). Limit of quantification (LOQ) were calculated using a signal-to-noise of 10 (from 1.95 to 7.81 μg/L). The accuracy was evaluated through essays recoveries at three concentrations (50, 100, 200 μg/L). Intraday and interday precision of the method were carried out by spiking fruit juice at the three levels previously indicated was assessed based on three determination on the same day, and interday precision was assessed based on three determinations on consecutive. Method precision was estimated by calculating the relative standard deviation (RSD) using the results obtained during the same day (intraday) and on three different days (interday) by the repeated analysis three times at the three spiked levels. Recoveries values at three spiked levels were ranged from 61 to 114 %.

Table 3: Analytical parameters for the used method: limits of detection (LOD) and quantification (LOQ), recovery, interday relative standard deviation (RSD_R) (n=9), absolute matrix effect, and linearity expressed as correlation coefficient (r^2)

Analyte	LOD µg/L	LOQ µg/L	Recovery (RSD_R) (%) 50 µg/L		100 µg/L		200 µg/L		Matrix effect (%)	Linearity
DON	0.58	1.95	68	(3)	73	(4)	62	(3)	103	0.996
3ADON	1.17	3.90	103	(2)	103	(7)	105	(11)	88	0.991
15ADON	0.58	1.95	95	(7)	98	(1)	96	(7)	114	0.993
DAS	1.17	3.90	103	(1)	91	(7)	84	(9)	96	0.991
FUS-X	2.34	7.81	84	(9)	106	(9)	98	(4)	106	0.995
T-2	2.34	7.81	98	(3)	96	(12)	78	(12)	71	0.991
HT-2	0.58	1.95	106	(11)	107	(5)	97	(5)	78	0.991
NIV	1.17	3.90	61	(3)	88	(9)	97	(7)	87	0.995
NEO	0.58	1.95	105	(6)	114	(1)	90	(6)	95	0.991
PAT	2.34	7.81	61	(9)	96	(6)	92	(13)	78	0.998
ZON	2.34	7.81	98	(2)	113	(9)	103	(9)	115	0.991
AZAL	0.58	1.95	101	(10)	97	(4)	99	(3)	88	0.992
BZAL	2.34	7.81	97	(5)	105	(8)	104	(4)	91	0.994
AZOL	0.58	1.95	80	(1)	113	(1)	95	(11)	62	0.992
BZOL	1.17	3.90	98	(4)	93	(3)	102	(6)	69	0.992

3.2. Natural occurrence of mycotoxin in fruit juice

A total de 42 different samples were evaluated. The results of the natural occurrence are shown in the Table 3.

3.2.1. Occurrence of Patulin

Six apple juice samples (60%) presented patulin contamination at mean levels of 33.41 µg/L, which are above 10 µg/kg of ML established for apple

juice. Orange juice samples also resulted contaminated by PAT in 86% of the sample with mean content of 31.29 μg/L, 29% of mixed juiced sample resulted contaminated by PAT, but only one sample of pineapple juice contained PAT at mean level of 8.05 μg/L.

Table 4: Occurrence of patulin and HT-2 in fruit juice analyzed

Mycotoxin	Parameters	Apple	Pineapple	Orange	Mixed fruits	Apricot	Pear
PAT	Incidence	6/10	1/7	6/7	2/7	0/7	0/4
	Mean (μg/L)	33.41	8.05	34.57	8.59	-	-
	Range (μg/L)	28.07-47.82	8.05	14.78-50.95	6.3-10.89	n.d	n.d.
HT-2	Incidence	-	-	-	(3/7)	-	-
	Mean (μg/L)	n.d.	n.d.	n.d.	22.38	n.d	n.d
	Range (μg/L)	n.d.	n.d.	n.d.	21.38-24.15	n.d	n.d

Figure 1**.** MRM chromatogram of a) standard matrix matched with fruit juice at 125 μg/L of PAT and HT-2 toxin. b) positive orange juice sample containing 14.78 μg/L and positive mixed juice containing HT-2 toxin 21.38 μg/L.

Several studies reported presence of PAT in different types of fruit juice at highest concentrations to those found in this survey. In a recent study Li et al. [6] reported high concentration of PAT until 1234 μg/L in apple juice brand. The presence of PAT in orange juice also was reported in other study conducted by Cho et al. [24] who reported natural occurrence of PAT in orange juice in 8% of sample ranged between 9.9 to 30.9 μg/L. Regarding pineapple juice, similar incidence to those found in this study were reported by Lee et al. [25] who confirmed the presence of PAT in only one sample at concentration of 33.7

μg/L. Drusch and Ragab [26] confirmed in a published review presence of PAT in fruit juice sample that contain pineapple with a maximum concentration of 60 μg/L.

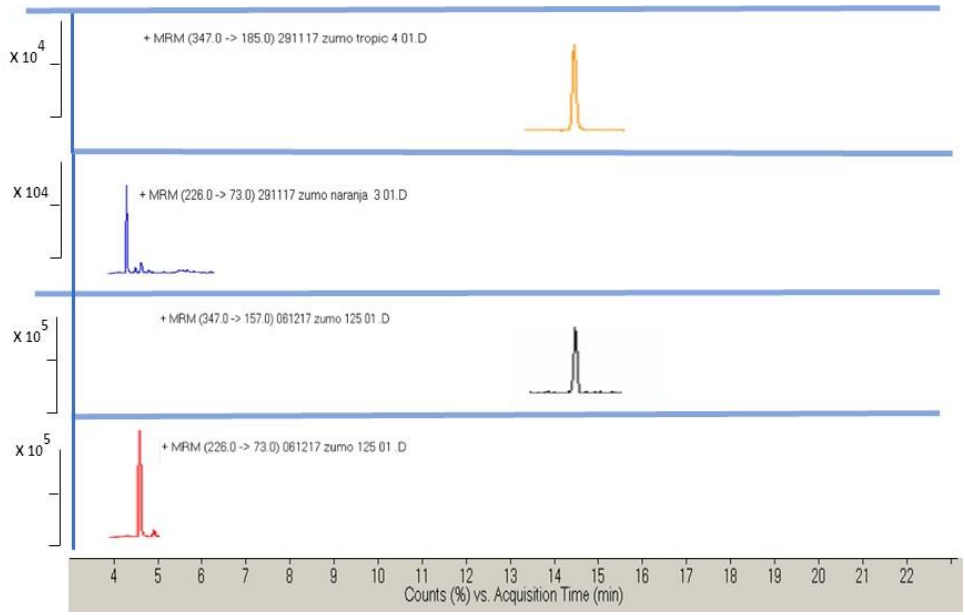

Figure 1. MRM chromatogram of a) standard matrix matched with fruit juice at 125 μg/L of PAT and HT-2 toxin. b) positive orange juice sample containing 14.78 μg/L and positive mixed juice containing HT-2 toxin 21.38 μg/L.

3.2.2. Occurrence of HT-2 toxins

HT-2 toxins were present in 43% of the mixed juice (apple, mango, pineapple) in ranging from 21.38 to 24.15 μg/L. The mycotoxins of the trichothecene group are generally present in grains and grain-based products and, as far as we know, very rarely in juices. However, López et al. [27] reported presence of T-2 toxins in apple juice composite at 14 μg/L and HT-2 toxins only was detected below LOQ (<20 μg/L). Those authors mentioned that cross

contamination during manufacturing the preparation of compound may have caused the presence of T-2 in apple juice composite samples.

In this study mycotoxins were not found in apricot and pear juice samples. However Poapolathep et al. [28] has investigated PAT in 40 apricot juice sample, 10% of samples contained PAT at mean levels of 4.51 μg/L. Similar content were found by Sparado et al. [9] who quantified PAT in 29% of sample at mean concentration of 3.6 μg/L. Moukas et al., et al. [29] also reported PAT in apricot juice samples at mean level of 13.70 μg/L.

Concerning presence of mycotoxin in pear juice, Rahimi et al. [30] analyzed 15 pear juice samples, but only two samples resulted contaminated by PAT at mean concentrations of 22.9 μg/L. Similar results were detected by Drush and Ragab [26] who also reported presence of PAT at maximal concentration of 20 μg/L. Highest content were reported by Zouaoui et al. [8] who detected PAT in in 47% of pear juice samples at average concentration of 62.5 μg/L. This value exceed ML established of PAT in fruit juice which it is 50 μg/kg.

3.2.3. Calculation PAT and HT-2 toxins intake

The PDI calculated values and comparison with the PMTDI for the risk assessment in children and adults population are reported in the Table 5. The PDI obtained for patulin in apple juice was 26.72 and 9.54 ng/kg bw day. The PDI calculated in orange juice were 27.65 and 8.94 ng/kg bw day. In pineapple juice was obtained PDI of 2.44 and 0.87 ng/kg bw day. PDI ranging from were 6.87 and 2.45 ng/kg bw day through the consumption mixed juice. The results indicated a low exposure to PAT through fruit juice consumption.

In others studies, estimate daily intake value calculated for PAT through the consumption of apple juice consumed in Spain ranged between 155 and 55 ng kg/bw day for children and adult, respectively [12]. EDI of PAT obtained by Poapolathep et al. [28] for the children Thailand population through the consumption of apple juice were 0.96 μg/kg bw day.

Table 5 shows the exposure estimates for HT-2 toxins. The PDI calculated were between 17.90 and 6.39 ng/kg bw day. The %PMTDI were 29.84% and 10.65% for children and adults population.

Table 5 : Patulin exposure calculated for children and adults through fruit juice consumption

Commodity	Population group	PDI ng/kg bw	PMTDI (ng/kg bw)	%PMTDI
Apple juice	Children	26.72	400	6.68
	Adults	9.54	400	2.38
Orange juice	Children	27.67	400	6.91
	Adults	8.94	400	2.35
Pineapple juice	Children	6.44	400	1.61
	Adults	2.3	400	0.57
Mixed juice	Children	6.87	400	1.71
	Adults	2.45	400	0.61

Table 6: HT-2 toxin exposure calculated for children and adults through fruit juice consumption

Commodity	Population group	PDI ng/kg bw	PMTDI (ng/kg bw)	%PMTDI
Mixed juice	Children	17.90	60	29.84
	Adults	6.39	60	10.65

4. Conclusions

The herein used analytical procedure was suitable to quantify fifteen mycotoxins in fruit juice products. 36% of the analyzed samples were contaminated by PAT and 7% resulted positive for HT-2 toxins. One orange juice sample exceeded the maximum limit of PAT (50 μg/L).

The risk assessment shows that the intake of patulin through the consumption of fruit juice does not represent a risk for the population. Nevertheless, a risk dietary exposure for HT-2 toxins by mixed juice samples reached 29.84% of PMTDI. The results highlight the necessity for rigorous monitoring studies.

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Conflict of interest

The authors declare no conflict of interest and informed consent was obtained from all individual participants included in the study.

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3.5. Dietary exposure assessment to mycotoxin through alcoholic and non-alcoholic beverages

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Dietary exposure assessment to mycotoxins through alcoholic and non-alcoholic beverages

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Abstract

In the present study, the occurrence of thirty mycotoxins in 110 beverages samples of beer, wine, cava and cider purchased in Valencia-Spain was assessed. The validated method based on Dispersive Liquid-liquid microextraction and chromatographic methods coupled to tandem mass spectrometry was applied. The method showed satisfactory recoveries ranging from 67 to 113% for the different beverages investigated. Limits of detection and quantification were between 0.03–1.17 μg/L and 0.1–7.81 μg/L, respectively. The results showed that beer samples were the most contaminated samples, even at concentrations ranging from 0.24 to 54.76 μg/L. A significant incidence of AOH was found in wine reaching concentration levels up to 43.48 μg/L. PAT and OTA were the most detected mycotoxins in cava and cider samples with incidences of 26% and 40%, respectively. OTA was found in one wine sample exceeding the maximum level established by EU. The results were statistically checked and combined exposure based on the sum of mycotoxins concentrations contaminating the same samples was carried out to provide insight in the magnitude of mycotoxins dietary exposure through the studied beverages. No significant health risk for Valencian population was associated with mycotoxins levels revealed in the analysed beverages.

1. Introduction

Mycotoxins are secondary metabolites produced by a wide variety of filamentous fungi, like *Aspergillus, Fusarium, Penicillium and Alternaria* that might grow under different climatic conditions on agricultural commodities. Fungal pathogenic toxins have been detected along the entire process of food production; in field, before and after harvest, during processing, storage and also

in a finished product (Reddy et al., 2010; Milani & Maleki 2014). Some mycotoxins have been associated with human and animal diseases; classified as carcinogens, hepatotoxins, nephrotoxins or neurotoxins (International Agency for Research on Cancer, 2012). Alcoholic beverages are widely consumed; being beer and wine the most consumed beverages in the European Union (WHO, 2018). Mycotoxins are commonly reported in fruit (grapes and other fruits) as well as cereals (barley wheat and maize) used in wine and beer production (Logrieco et al., 2012; Puangkham et al., 2017).

European legislation has established maximum levels of ochratoxin A (OTA), recommending a tolerance level lower than 2.0 μg/L for all types of wine. However, there is no regulation for other mycotoxins levels in alcoholic beverages (European Commission 1881/2006).

The determination of mycotoxins has been investigated in different alcoholic beverages like wine (Tamura et al., 2011; Mariño-Repizo et al., 2017), beer (Rodriguez-Carrasco et al., 2015; Pascari et al., 2018) and cider (Tagni et al., 2010; Leblanc et al., 2005). However, scarce data exist in literature concerning mycotoxin level in alcoholic beverages such as cava and beverage mixtures.

Cava is a Spanish sparkling wine with a Protected Geographical Status, made from several varieties of grapes with fermentation method similar to the champagne (Real and Baez, 2012).

Cider is a fermented beverage obtained from apple fruits. In Spain, cider is mainly produced in Asturias located in Atlantic coast. Regarding the raw material, a set of Asturian cider apple varieties belonging to the Protected Designation of Origin (EC 2154/2005) has been highly investigated through the assessment of their contents in sugars, acids and aromas (Lobo et al., 2018).

Dispersive liquid-liquid microextraction (DLLME) combined appropriate mixture of extraction and disperser solvents for mycotoxin extraction from liquid matrix and offered several advantages with respect to traditional techniques including simplicity, low cost and ease of method development (Zgoła-Grześkowiak & Grześkowiak, 2011). Moreover, this technique has already been set by our group for multimycotoxin analysis in several food and biological samples (Rodriguez-Carrasco et al., 2016; Serrano et al., 2016; Escrivá et al., 2017).

The most used technique for mycotoxins determination in food samples are based on liquid or gas chromatography coupled to mass spectrometry detectors (Juan et al., 2017). Both techniques have enabled the development of highly selective, sensitive and accurate methods (Bouafifssa et al., 2018).

The aim of the present study was to evaluate the presence of thirty different mycotoxins mainly alternariol (AOH), alternariol methyl eter (AME), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), nivalenol (NIV), neosolaniol (NEO), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), T-2 and HT-2 toxins, sterigmatocystin (STG), ochratoxin-A (OTA), four aflatoxins (AFB_1, AFB_2, AFG_1, and AFG_2), patulin (PAT), zearalenone (ZON), α-zearalanol (αZAL), β-zearalanol (βZAL), α-zearalenol (αZOL), β-zearalenol (βzol), fumonisins (FB_1, FB_2), as well as five emerging mycotoxins: enniatins (ENNA, $ENNA_1$, ENNB and $ENNB_1$) and beauvericin (BEA) to estimate of the potential contribution of alcoholic and non-alcoholic beverages on mycotoxins dietary exposure.

2. Material and methods

2.1. Chemicals and reagents

Solvents (acetonitrile, hexane, chloroform and methanol) were supplied by Merck (Darmstadt, Germany). Deionized water (<18.2 MΩ cm resistivity) was obtained in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Beadford, MA, USA).

Ammonium formate (99%) and formic acid (≥98%), sodium chloride were supplied by Sigma Aldrich (Madrid, Spain). Syringe nylon filter (13mm diameter 0.22 μm pore size) were obtained from Analysis Vinicos S.L. (Tomelloso Spain). The derivatization reagent composed of BSA (N,O-bis(trimethylsily) + TMCS (trimthylcholorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

2.2. Standards and solutions

The mycotoxins standards were purchased from Sigma Aldrich. Individual stock of all analytes were prepared to obtain 20 mg/L in methanol and multianalyte working solutions. The multianalyte working standard solution of 2 mg/L was used for standard calibration curves, matrix-matched calibration curves and recovery assays. All standards were stored in darkness and kept at-20°C.

2.3 Procedures

2.3.1 Samples

A total of 110 samples of beer (n=40), wine (n=40), cava (n=10) and cider (n=20) were purchased from different retail outlets located in Valencia from September 2017 to October 2018. Samples of beer were divided in beer with amount of alcohol up to 5.4% Vol (n=20); beer with lemonade (n=10) that contained alcohol of 2% Vol, alcohol free (A.F.) with alcohol content <1% vol (n=10). Wine samples were classified in wine with alcohol 12% Vol (n=20); wine with lemonade containing alcohol up to 4.5% Vol (n=10) and alcohol free (A.F.) with alcohol content <1% vol (n=10). Cider samples were separated in alcohol free (A.F.) cider with alcohol content <1% vol (n=10) and normal cider (n=10) while all cava samples contained alcohol 12% vol (n=10).

2.3.2 Dispersive liquid-liquid microextraction

Samples extraction was performed according to the previously in house validated method for tea beverages (Pallarés et al., 2017). Briefly, before extraction each bottle of beer, cava, and cider samples, was gently shaken and 100 mL was degassed by sonication for 15 min. Then, an aliquot of 5 mL were placed in a 10 mL conical tube, a mixture (950 μL of ACN) of dispersion solvent and (620 μL of EtOAc) of the extraction solvent was added; and the resulting mixture was shaken for 1 min. The mixture was centrifuged at 4000 rpm for 5 min, and the organic phase at the top of the tube was placed in second conical tube. Next, a mixture (950 μL of MeOH) of dispersion solvent and (620 μL of CHCl3) of extraction solvent was added to the remaining residue, and after agitation and centrifugation, the separated organic phase was added to the first organic phase. The solvent in the conical tube containing the two recovered

phases was evaporated to near dryness under a nitrogen stream using turbovap LV Evaporator (Zimark, Hopkinton, MA). The dry residue was reconstituted with 1 mL of 20 mM ammonium formate (MeOH/ACN) (50/50 v/v) and filtrated through a 13-mm/0.22 μm nylon filter (Membrane Solutions, Plano, TX).

2.4. GC–MS/MS analysis

The Gas Chromatographic analysis was carried out using an Agilent 7890A GC system coupled with an Agilent 7000A triple quadruple mass spectrometer with inter electron-impact ion source (EI, 70Ev). Quantitative data were acquired at selection reaction monitoring mode. The transfer line and source temperatures were 280° and 230°, respectively. Details on derivatization and gas chromatographic separation were described in a previous published study (Rodriguez-Carrasco et al., 2015).

For quantification of each analyte, two selected reaction monitoring (SRM) transitions were required. The most intense SRM transition was selected for quantification purposes as is indicated in the requirements for mass spectrometry (SANTE, 2017). The specific MS/MS parameters for each mycotoxin were 392/259 407/197 for DON, m/z 392/287–467/147 for 3-ADON, m/z 392/217–392/184 for 15-ADON, m/z 350/229-378/124 for DAS, m/z 347/157–347/185 for HT-2, m/z 350/259–350/229 for T-2, m/z 289/73–379/73 m/z for NIV, m/z 252/195-252/167 for NEO, m/z 450/260–450/245 for FUS-X, m/z 226/73-183/75 for PAT, m/z 462/151–462/333 for ZON, m/z 433/309-433/295 for αZAL, m/z 307/292-307/277 for βZAL, m/z 305/289-305/73 for αZOL, m/z 536/446-536/333 for βZAL.

2.5. LC–MS/MS analysis

HPLC-MS/MS analysis was performed using an Agilent 1200 liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® ABSCIEX (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo-VTM source (ESI) interface. Details on chromatographic separation of the analytes and gradient used were according to the previous published study (Juan et al., 2017).

The precursor-to-product ion transitions were m/z 313/285-313241 for AFB1, m/z 315/287-315259 for AFB2, m/z 329/243-329/311 for AFG1, m/z 331/313-331/249 for AFG2, m/z 259/128-259/184 for AOH, m/z 273/128-273/228 for AME, m/z 722/334-722/235 for FB1, m/z 706/336-706/318 for FB2, m/z 699/210-699/228 for ENNA, m/z 685/210-685/214 for ENNA1, m/z 657/196-657/214 for ENNB, m/z 671/214-671/228 for ENNB1, m/z 801/784-801/244 for BEA, m/z 325/281-325/310 for STG, m/z 404/239-404/102 for OTA.

2.6. Method validation

The analytical method was in-house-validated according to the criteria established in SANTE 11813/2017 Document (SANTE, 2017) respect to the main analytical parameters, as linearity, recovery, LODs, LOQs and matrix effect. Cava and cider were grouped with wine beverages as was performed in a previous study by Ruíz-Delgado et al., (2016) due to their similar elaboration and fermentation process. Both external calibration curves and matrix-matched calibration curves were were performed in triplicate between 0.1 to 250 μg/L and linearity was expressed by square correlation coefficient (r^2). Accuracy of the method was evaluated by measuring the recoveries from blank samples spiked

at 50, 100 and 200 μg/L. Precision studies were determined in fortified beer and wine including similar beverages at the same 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 on three different days (inter-day precision) by prepared analysis (n=9) at three spiked levels. Limits of detection (LODs) and quantification (LOQs) were determined as the concentrations for which signal-to-noise ratio (S/N) ≥ 3 and ≥ 10, respectively from chromatograms of samples spiked at the lowest level validated.

2.7. Statistical analysis

Component analysis (PCA) was performed using the mixOmics R package. Factor analysis was established to evaluate possible associations between studied analytes and beverages groups.

2.8. Mycotoxin dietary intake calculation

A deterministic approach was performed for risk assessment. The exposure was estimated by the probable daily intake (PDI) which combined the average amount of mycotoxins found in the different analyzed samples with the beverage consumption estimation in Spanish adult population. According to Spanish Ministry of Agricultural and Environment, annual consumption of different beverages were as following; beer (15.39 L), alcohol free beer (3.11L), wine (3.02 L), other beverages mixed with wine (1.25 L), cava (0.58 L) and cider (0.25 L) (MAPAMA, 2017). The PDI [μg L^{-1} per body weight (bw)/day] of each mycotoxin was calculated as indicated (Rodriguez-Carrasco et al., 2013). The health risk characterization of mycotoxin (% of relevant TDI) was performed comparing the PDI with the tolerable daily intake (TDI).

According to the safety guidelines of the Joint FAO/WHO Expert Committee and Food Additives and Scientific Committee on Food TDIs in ng/kg bw of 250 for ZON, 100 for the sum of T-2 and HT-2, toxins 1200 for NIV, 1000 for DON and their acetyl forms (like 3ADON and 15ADON) and 400 for PAT, were established respectively. For OTA tolerable weekly intake of 120 ng/kg bw was established (JECFA, 2001; SCF, 2002). Aflatoxins are carcinogenic and its intake should be reduced as Low As Reasonably Achievable.

3. Results and discussion

3.1. Analytical method validation

Recovery results were within the range of 67% and 113% and intra- and inter-day data ensured repeatability and reproducibility (Table 1). Matrix effects ranged from 71% to 114%, and matrix-matched calibration curves were used for quantification purposes. LODs and LOQs, ranged between 0.03-1.17 μg/L and 0.1-7.81 μg/L, respectively.

Table 1: Limits of detection and quantification (LODs, LOQs), matrix effects (SSE%), recovery at different spiked concentrations using the chromatographic methods tandem mass spectrometry

	Beer						Wine and similar beverages					
				Recovery (%) Spiked level µg/L						Recovery (%)		
	LOD	LOQ	SSE				LOD	LOQ	SSE	Spiked level µg/L		
Mycotoxin	µg/L	µg/L	(%)	50	100	200	µg/L	µg/L	(%)	50	100	200
DON[a]	0.58	1.95	95	68	69	71	0.58	1.95	96	79	77	71
3ADON[a]	1.17	3.90	78	69	100	106	1.17	3.90	94	100	99	104
15ADON[a]	0.58	1.95	92	87	97	91	0.58	1.95	92	103	101	102
DAS[a]	0.58	1.95	96	67	78	99	0.58	1.95	96	116	94	98
NEO[a]	0.58	1.95	92	67	93	83	0.58	1.95	96	96	98	101
NIV[a]	2.34	7.81	94	71	75	88	2.34	7.81	94	114	114	101
T-2[a]	2.34	7.81	72	69	91	102	2.34	7.81	74	107	100	99
HT-2[a]	0.58	1.95	78	71	113	107	1.17	3.90	94	86	101	101
PAT[a]	2.34	7.81	81	74	81	92	1.17	3.90	84	61	96	91
FUS-X[a]	2.34	7.81	93	89	84	80	1.17	3.90	100	87	97	96
ZON[a]	2.34	7.81	111	67	77	97	1.17	3.90	90	108	103	91
αZAL[a]	1.17	3.90	101	89	99	107	0.58	1.95	114	98	99	109
βZAL[a]	2.34	7.81	72	72	67	106	2.34	7.81	101	95	66	87
αZOL[a]	1.17	3.90	87	75	71	93	2.34	7.81	104	77	100	96
βZOL[a]	1.17	3.90	93	66	73	108	2.34	7.81	71	106	104	106
AFB_{1b}	0.06	0.2	85	83	86	81	0.3	1	79	71	79	98
AFB_2[b]	0.3	1	95	85	97	85	1.5	5	81	78	89	83
AFG_1[b]	0.06	0.2	77	81	92	82	0.3	1	91	111	98	70
AFG_2[b]	0.3	1	81	70	108	101	1.5	5	79	86	85	109
AOH[b]	0.3	1	111	89	111	91	0.03	0.1	92	101	90	107
AME[b]	1.5	5	78	76	98	103	0.3	1	84	99	76	89
FB_1[b]	1.5	5	71	83	71	87	1.5	5	76	69	71	79
FB_2[b]	1.5	5	87	76	69	82	1.5	5	86	71	65	69
ENN A[b]	0.03	0.1	110	69	85	82	0.15	0.5	71	72	85	87
ENN A_1[b]	0.15	0.5	106	85	94	93	0.03	0.1	86	69	82	102
ENN B[b]	0.15	0.5	92	91	103	98	0.15	0.5	111	73	85	86
ENN B_1[b]	0.03	0.1	91	72	114	102	0.15	0.5	88	73	85	86
BEA[b]	0.3	1	75	98	94	96	1.5	5	87	75	83	88
STG[b]	1.5	5	96	83	93	81	1.5	5	95	85	81	89
OTA[b]	0.06	0.2	99	79	89	87	0.15	0.5	104	107	84	85

[a]GC-MS/MS determination, [b]LC-MS/MS determination

3.2. Mycotoxin occurrence in beer samples

100% of the 40 beer samples were found contaminated by at least one mycotoxin. AOH was the most prevalent mycotoxin and the highest mean concentration was registered for PAT. While, the lowest incidence was for T2 and the lowest mean concentration was observed for AFG_1 with 1.16 μg/L (Table 2 and Figure 1). Bauer et al., (2016), also detected AOH in 100% of the beer samples at 0.56 μg/L and Prellé et al., (2013) also monitored AOH in 30% of beer samples in levels between 6.04-23.2 μg/L.

AOH and DON were the most detected mycotoxins in A.F. beer and 43.19 μg/L the highest concentration reached for βZAL. The lowest incidence was detected for T-2 (10%) and the lowest mean concentration was 0.85 μg/L for AFG_1.

Up to 87% of beer samples have previously been reported contaminated with DON at levels between 4-5.7 μg/L (Papadopoulou-Bouraoui et al., 2004). Other studies performed in Spain, Italy and Estonia reported slightly lowest DON incidence in beer (56% to 68%) and concentrations ranging from 2.1 to 73.2 μg/L (Rodríguez-Carrasco et al. 2015; Juan et al., 2017; Bertuzzi et al. 2011).

In beer with lemonade, DON and OTA were the most prevalent mycotoxins while the highest concentration was found for βZAL with 42.97 μg/L. The lowest incidence was detected for ZON (10%) and the lowest contents for OTA with 1.83 μg/L. 15-ADON was only detected in A.F. beer samples with an incidence of 40% at 12.08 μg/L. Juan et al., (2017) also quantified 15-ADON in 6% of beer samples at similar mean levels.

NIV was present in 30% of beer samples at mean concentration of 10.01 μg/L. Tamura et al., (2011) quantified NIV in 21% of beer samples too at level

under LOQ (<5ng/mL) and Bryla et al., (2018) also reported NIV in 39% of beer samples at mean concentrations of 2.7 μg/L.

AFG_1 and AFB_1 were detected in 35% and 60% of beer and A.F. beer samples at mean levels of 1.16 to 1.88 μg/L respectively. Burdaspal and Legarda (2013) reported presence of AFs in 64.3% of beer samples ranged from 0.07-4.94 ng/L. AFB_1 was also detected in beer samples at low concentrations 0.37 to 10.60 ng/L (Mabli et al., 2007; Benesova et al., 2012). However, higher contents in beer reaching concentrations of 35.5 μg/L were already reported (Molina-García et al., 2011; Matumba et al., 2011).

ZON and βZAL were found in 8% and 25% of the A.F. and lemonade beer samples at mean levels of 14.17 and 43.08 μg/L, respectively. Bauer et al., (2016) also detected ZON in 100% of beer samples at mean concentration of 0.96 μg/L.

OTA was only detected in 20% of beer with lemonade at 1.83 μg/L. However, several studies notified highest OTA incidence in beer samples. Coronel et al., (2012) reported OTA incidence of 89% of the beer samples at mean concentration of 0.02 μg/L, Czerwiecki et al., (2002) reported OTA in 79% of beer samples with mean contents of 25.7 μg/L and Lasram et al., (2012), reported OTA in 48% of beer samples with mean content of 0.12 μg/L. However, low incidences were reported too by Rubert et el., (2013) detecting OTA in 10% of beer samples at mean level of 3.2 μg/L. Finally, PAT was detected in only 5% AF beer at mean levels of 43.18 μg/L. Different technological process applied in the beer brewing may influence mycotoxin contents such as steeping, kilning, mashing, fermentation and clarification (Pascari et al., 2018b). A longer fermentation process could contribute to

increase mycotoxin level transfer from cereal to malt and them to beer due to high thermal stability (Bertuzzi et al., 2011; Rodriguez-Carrasco et al., 2015; Pascari et al., 2018b).

Table 2: Incidence, mean concentration and determined mycotoxins range in beer samples

Mycotoxin	**Beer n=20**			**A.F. Beer (n=10)**			**Beer with lemonade n=10**			**TOTAL n=40**		
	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg L
AFB_1	90	1.06±0.15	0.87-1.38	60	2.70±4	1-10.60	-	n.d.	n.d.	60	1.88±1	0.87-1.38
AFG_1	25	1.47±0.9	0.43-2.92	90	0.85±0.6	0.77-1.98	-	n.d.	n.d.	35	1.16±1	0.7-1.98
AOH	95	24.93±10.42	8.83-49.82	100	28.81±9	20.25-48.37	70	4.44±2	2.01-8.32	90	19.39±13	8.32-49.82
15 ADON	-	n.d.	n.d.	40	12.08±1	10.78-12.93	-	n.d.	n.d.	10	12.08±1	10.78-12.93
Bzal	-	n.d.	n.d.	50	43.19±9	31.46-54.76	50	42.97±1.89	40.43-45.25	25	43.08	31.46-54.76
DON	70	8.65±0.12	8.50-8.85	100	9.63±1	8.58-11.82	80	8.76±0.28	8.44-9.35	80	9.01±0.5	8.44-11.82
HT-2	40	16.31±1	14.42-18.59	20	14.43±5	11.20-17.67	20	15.15±0.07	15.10-15.21	30	15.29±0.9	11.20-18.59
NEO	30	14.20±0.25	13.86-14.46	20	15.17±2	13.90-16.44	-	n.d.	n.d.	20	14.67±0.6	13.86-16.44
NIV	30	10.40±1	8.96-14.01	40	10.34±2	8.96-12.52	30	9.34±0.24	9.13-9.60	30	10.01±0.5	8.96-14.01
OTA	-	n.d.	n.d.	-	n.d.	n.d.	80	1.83±1.18	0.24-3.38	20	1.83±1	0.24-3.38
PAT	-	n.d.	n.d.	20	43.18±0.4	42.89-43.48	-	n.d.	n.d.	5	43.18±0.4	42.89-43.48
T-2	-	n.d.	n.d.	10	29.88±8	29.88±8	-	n.d.	n.d.	3	29.88±8	29.88±8
ZON	-	n.d.	n.d.	20	14.95±1	13.80-16.10	10	13.60±0.2	13.60±0.2	8	14.17±0.9	13.60-16.10

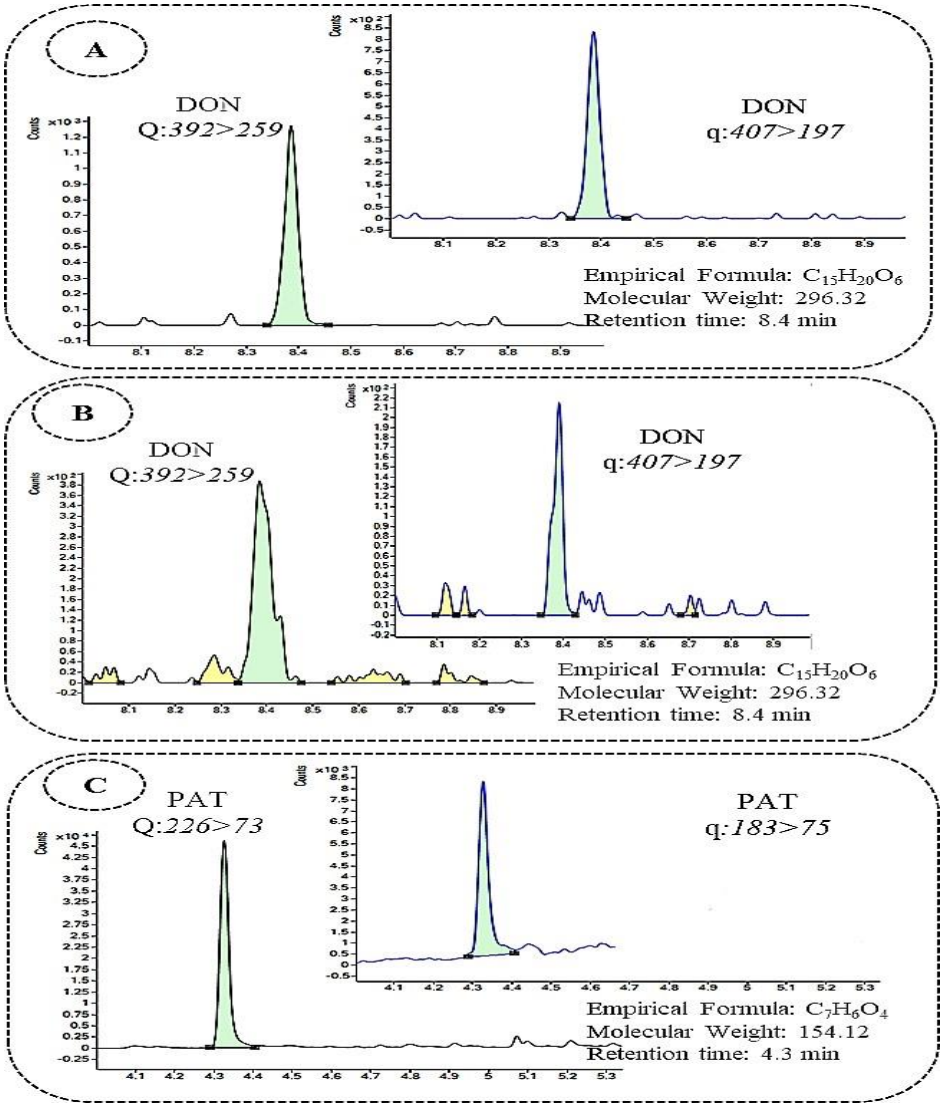

Figure 1: Chromatograms obtained from different beverages samples naturally contaminated with (**A**) DON in beer sample (8.85µg/L), (**B**) DON in wine sample (9.69 µg/L) and (**C**) PAT in cider sample (24.66 µg/L) at Multiple Reaction Monitoring (MRM) by (GC-MS/MS).

3.3. Mycotoxin occurrence in wine samples

88% of wine samples were found contaminated with at least one mycotoxin. The most prevalent mycotoxin was AOH with incidence of 52% while the highest contents were found for PAT and β-ZAL with 24.64 and 25.86 μg/L respectively. The lowest incidence was detected for β-ZAL and HT-2 toxin and the lowest concentration was found for OTA with 1.13 μg/L (Table 3). Highest AME incidence was previously reported in wine samples (up to 93%) and mean values up to 1.0 μg/L (Zwickel et al., 2016; Asam et al., 2010). AOH was also reported in wine samples in more than 60% and in concentrations between 0.03 to 7.7 μg/L (Zwickel et al., 2016; Scott et al., 2006; Asam et al., 2010).

Concerning A.F. wines, OTA was the most frequent mycotoxin even at mean concentration of 1.08 μg/L. The lowest incidence and concentration were detected for PAT (30%) even at mean levels of 17.63 μg/L. Previous studies reported higher OTA incidence in wine ranging from 50% to 100% and concentrations up to 8.6 μg/L (Lasram et al., 2013; De Jesus et al., 2017; Mariño-Repizo et al., 2017). In wine with lemonade, the highest incidences were for AOH and OTA up to 40% while the highest concentration was found for βZAL with 25.86 μg/L.

Limited studies have included Fusarium mycotoxins monitoring in wine. Al-Taher et al., (2013) identified T-2 in 11% of wine samples with mean levels of 0.3 μg/L, Logrieco et al., (2010) reported the occurrence of FB_2 in 17.6% wine commercialized in Italy, in levels ranging from 0.4 to 2.4 μg/L

Table 3: Incidence, mean concentration and determined mycotoxins range in wine samples

Mycotoxin	**Wine n=20**			**A.F. Wine n=10**			**Wine with lemonade n=10**			**TOTAL n=40**		
	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L
AOH	45	7.79±8	1.55-26.86	80	5.35±3	0.83-9.29	40	2.56±2	0.61-4.65	52	5.23±2	0.61-26.86
AME	60	7.55±5	1.36-18.05	50	16.40±4	11.14-23.13	20	12.33±2	10.82-13.85	50	12.09±4	1.36-23.13
15ADON	25	11.28±0.5	10.61-11.91	-	n.d.	n.d.	-	n.d.	n.d.	12	11.28±0.5	10.61-11.91
βZAL	-	n.d.	n.d.	-	n.d.	n.d.	20	25.86±3	23.33-28.40	5	25.86±3	23.33-28.40
DON	60	8.85±0.3	8.48-9.69	-	n.d.	n.d.	-	n.d.	n.d.	30	8.85±0.3	8.48-9.69
HT-2	10	15.65±0.1	15.55-15.75	-	n.d.	n.d.	-	n.d.	n.d.	5	15.65±0.1	15.55-15.75
NEO	45	14.27±0.3	13.89-14.97	-	n.d.	n.d.	-	n.d.	n.d.	22	14.27±0.3	13.89-14.97
NIV	15	21.26±4	18.06-26.58	-	n.d	n.d.	20	10.55±0.6	10.07-11.03	12	16.05±7	10.06-26.58
OTA	45	1.12±0.5	0.66-2.28	90	1.08±1	0.57-1.50	40	1.21±0.4	0.60-1.79	47	1.13±0.06	0.66-2.28
PAT	50	31.66±22	15.35-88.24	30	17.63±4	15.25-22.97	-	n.d.	n.d.	32	24.64±9	15.35-88.24

3.4. Mycotoxin occurrence in cava and cider samples

Despite the OTA high prevalence in cava samples (80%), the concentrations detected were up to of 1.36 μg/L, on the other hand , AOH showed up at 10% even reaching 21.56 μg/L (Table 4). βZAL was the most detected mycotoxin in cider reaching a mean concentration of 61.48 μg/L, PAT was found in 20% of samples and ZON was registered up to 11.53 μg/L. In A.F. cider, PAT was the unique mycotoxin detected with an incidence of 30% and mean concentration of 35.86 μg/L. Harris et al., (2009) reported PAT presence in 19% of cider samples at mean concentrations of 36.9 μg/L and Leblanc et al., (2005) also quantified PAT in 50% of the alcoholic beverages samples including cider at mean levels of 19.50 μg/L.

To interpret the results in terms of incidence and contents, the principal component analysis (PCA) multivariate statistical analysis technique was used. (Figure 2). The distribution map for the first principal components amounted 35% for PAT in wine, cava and A.F. cider, while the second component scored 26% for βZAL in beer with lemonade, A.F. beer and cider. The results revealed similar behaviour for these mycotoxins in those beverages group mentioned. In A.F. beer samples, similar trend was obtained for DON, NIV and AOH. The same trend for AOH and OTA was shown in A.F wine. The highest incidence was observed for OTA and PAT in cava samples.

Table 4: Incidence, mean concentration and determined mycotoxins range in cava and cider samples.

Mycotoxin	**Cava n=10**			**Cider n=10**			**A.F. Cider n=10**			**TOTAL n=30**		
	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L	I (%)	Mean µg/L	Range µg/L
AOH	10	21.56±1	21.56±1	-	n.d	n.d.	-	n.d.	n.d.	3	21.56±1	21.56±1
βZAL	-	n.d.	-	60	61.48±30	25.17-102.96	-	n.d.	n.d.	20	61.48±30	25.17-102.96
OTA	80	1.36±0.6	0.77-2.44	-	n.d.	n.d.	-	n.d.	n.d.	26	1.36±0.6	0.77-2.44
PAT	70	17.81±3	14.73-24.66	20	25.79±5	21.69-29.98	30	35.86±7	26.85-41.93	40	26.48±9	14.73-41.93
ZON	-	n.d.	n.d.	30	11.53±13	2.53-26.41	-	n.d.	n.d.	10	11.53±13	2.53-26.41

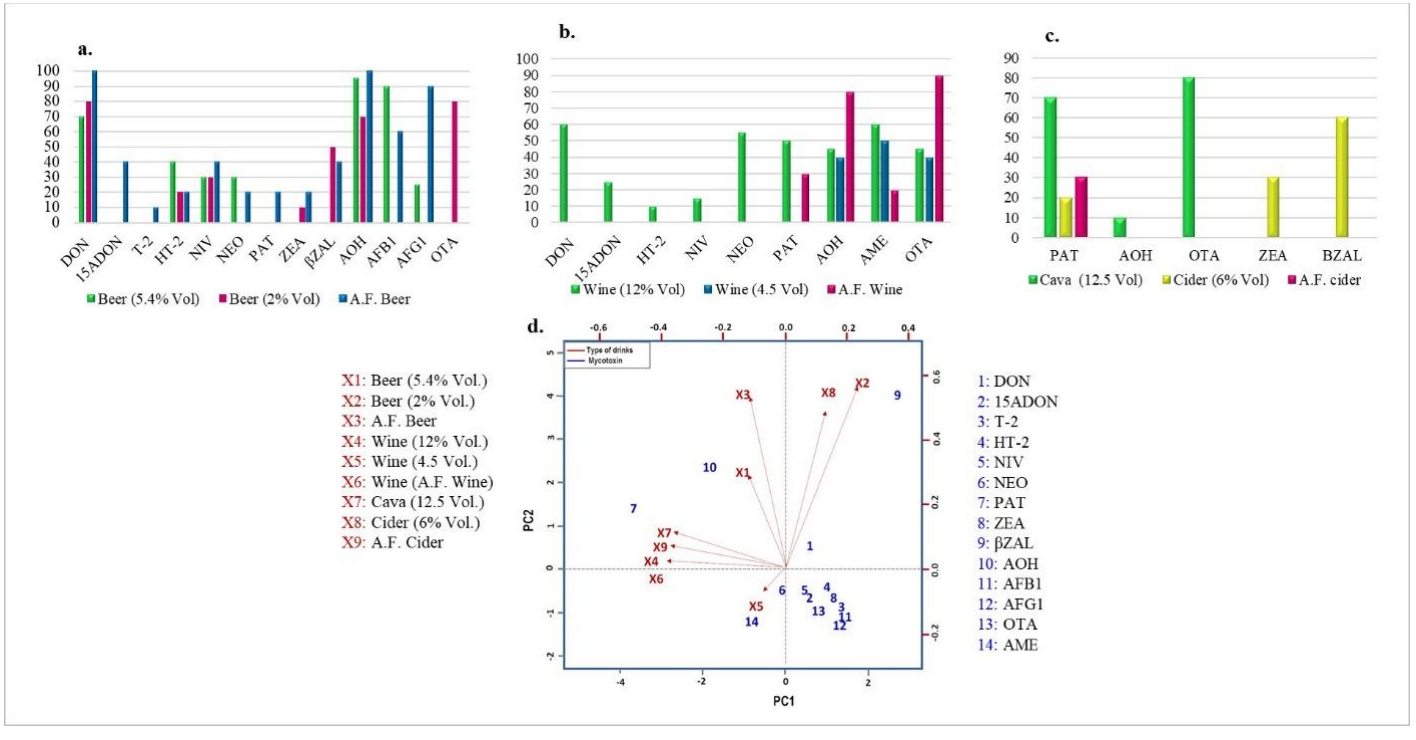

Figure 2: Incidence values (%) (a. beer, b. wine, c. cava and cider) and biplot loadings PCA (d. PCA) of the different beverages.

3.5. Multi-Mycotoxin occurrence in analysed beverages samples

Co-occurrence of mycotoxins in beer samples was found in 53% of beer samples, 13% of beer with lemonade and 23% of A.F. beers. The sum of mycotoxins concentrations simultaneously contaminating the same samples amounted between 10.86 to 185.15 μg/L. (Table 5). Rodriguez-Carrasco et al., (2015) detected simultaneously DON and HT-2 in 9.1% of the analyzed samples. Similar results were reported by Juan et al., (2017) who detected co-occurrence of DON and 15ADON in 9% of the analyzed samples. Bertuzzi et al., (2011) previously notified doublet co-occurrence of OTA-DON and OTA-FB_1 in 41.5% and 42.4% of beer samples. Benesova et al., (2012) reported co-occurrence of AFB_2, AFG_1 and AFG_2 in 5.1% at mean levels of 31 μg/L while in a recent study in beer, Pascari et al., (2018) reported co-occurrence of DON, 3G-DON and FB_1.

Co-occurrence of mycotoxins in in wine samples was found in 35% of wine samples, 8% of wine with lemonade and 18% of A.F. wine. The sum of mycotoxins concentrations simultaneously presents in the same positive samples amounted between 5.93 and 103.92 μg/L. While, 60% of cava samples and 40% of cider samples were found contaminated with at least two mycotoxins concurrently, summing up concentrations from 17.60 to 25.52 μg/L and 50.90 to 129.37 μg/L, respectively.

Rodriguez-Carrasco et al., (2015) detected simultaneously DON and HT-2 in 9.1% of the analyzed samples. Co-occurrence of DON and 15ADON in 9% of beer samples were also reported by Juan et al., (2017). Moreover, Bertuzzi et al., (2011) notified OTA-DON and OTA, DON and FB1 co-occurrence in 41.5% and 42.4% of beer samples. Benesova et al., (2012) reported

too co-occurrence of AFB_2, AFG_1 and AFG_2 in 5.1% of beer samples at mean levels of 31 μg/L and Tamura et al., (2011) reported presence of OTA-FB_1 in 13% of beer samples at contents from 0.2 to 1.0 μg/L.

Table 5: Co-occurrence mycotoxins data on the sum of the concentrations found in the same sample and combined PDI and PMTDI from different beverages.

Co-occurrence	Sample (N)	Sum. C.min. (µg/L)	Sum. C.max. (µg/L)	Sum PDI min. (ng/kg bw/day)	PMTDI (%)	Sum PDI max. (ng/kg bw/day)	PMTDI (%)
Two mycotoxins							
AOH, AFB_1	Beer (1)	22.95	*	13.11	-	-	-
NIV, AOH	Beer (1)	22.84	*	13.05	1.08	-	-
DON, OTA	Beer with lemonade (1)	10.86	*	1.24	0.24	-	-
AOH, AME	A.F. Wine (1)	5.93	*	0.25		-	-
OTA, AOH	A.F. Wine(1)	4.86	5.78	0.20	1.17	0.24	1.41
OTA, AOH	Wine with lemonade (1)	5.58	*	0.23	1.35	-	-
PAT, OTA	Cava (6)	17.60	25.52	0.25	0.11	0.36	0.17
ZON, βZAL	Cider (3)	50.90	129.37	0.43	0.17	1.10	0.44
PAT, βZAL	Cider (2)	46.46	63.28	0.39	0.12	0.54	0.16
Three mycotoxins							
DON, AOH, AFB_1	Beer (2)	30.22	46.30	17.26	1.72	26.45	2.64
DON, AOH, AFB_1	Beer with lemonade (2)	11.05	16.56	1.26	0.12	1.89	0.18
HT-2, AOH, AFB_1	Beer (1)	67.29	*	38.45	38.45	-	-
AOH, AFB_1, AFG_1	Beer (1)	17.80	*	10.17	-	-	-
βZAL, AOH, OTA	Beer with lemonade (1)	52.05	*	5.94	4.44	-	-
DON, OTA, AME	Wine (1)	11.98	*	0.22	0.04	-	-
DON, 15ADON, NEO	Wine (1)	35.64	*	4.07	0.40	-	-
DON, NEO, PAT	Wine (1)	59.51	*	5.77	0.82	-	-
DON, PAT, AME	Wine (1)	103.92	*	11.87	1.69	-	-
PAT, AOH, AME	A.F. Wine (1)	39.17	*	1.67	0.41	-	-
OTA, AOH, AME	A.F. Wine (2)	16.08	28.43	0.68	4	1.21	7.11
PAT, AOH, OTA	A.F. Wine (1)	17.57	*	0.75	0.35	-	-

						-	
Four mycotoxins							
DON, NEO, AOH, AFB_1	Beer (4)	39.54	48.32	22.59	2.2	27.61	2.76
DON, HT-2, AOH, AFB_1	Beer (2)	52.44	54.96	29.96	5.44	31.40	5.70
NIV, βZAL, AOH, OTA	Beer with lemonade (1)	60.46	*	6.90	1.63	-	-
DON, 15ADON, βZAL, AOH	A.F. Beer (1)	68.66	*	7.84	1.25	-	-
DON, βZAL, AOH, AFG_1	A.F. Beer (2)	77.52	79.44	8.85	1.41	9.07	1.45
DON, 15ADON, NEO, PAT	Wine (1)	51.15	*	5.84	0.83	-	-
PAT, OTA, AOH, AME	Wine (2)	44.37	78.92	5.07	2.43	9.01	4.32
PAT, OTA, AOH, AME	A.F. Wine (1)	56.32	*	2.41	1.15	-	-
NIV, OTA, AOH, AME	Wine (1)	42.07	*	4.80	0.78	-	-
NIV, OTA, AOH, βZAL	Wine with lemonade (1)	41.51	*	1.77	0.29	-	-
OTA, AOH, AME, βZAL	Wine with lemonade (1)	39.07	*	1.67	1.25	-	-
Five mycotoxins							
DON, HT2, NIV, AOH, AFB_1	Beer (1)	54.63	*	31.21	4.07	-	-
DON, NIV, AOH, AFB1, AFG_1	Beer (1)	83.20	*	47.54	4.32	-	-
DON, NIV, NEO, AOH, AFB_1	Beer (1)	44.73	*	25.56	2.32	-	-
DON, HT-2, AOH, AFB1, AFG_1	Beer (2)	72.46	*	41.40	7.52	-	-
DON, HT-2, AOH, AFB1, AFG_1	A.F. Beer (1)	67.17	*	7.67	1.39	-	-
DON, NIV, AOH, AFB1, AFG_1	A.F. Beer (1)	53.84	*	6.15	0.55	-	-
DON, 15ADON, βZAL, AOH, AFG_1	A.F. Beer (1)	91.48	*	10.45	1.67	-	-
DON, 15ADON, AOH, AFB1, AFG_1	A.F. Beer (1)	49.88	*	5.70	0.57	-	-
HT-2, NIV, PAT, AOH, AME	Wine (1)	63.14	*	7.21	1.10	-	-
DON, NEO, OTA, AOH, AME	Wine (1)	36.50	44.26	4.17	0.82	5.05	0.99
DON, 15ADON, HT-2, NEO, PAT	Wine (1)	68.92	*	7.87	1.57	-	-
DON, 15ADON, NEO, AOH, AME	Wine (1)	61.65	*	7.04	0.70	-	-
HT2, NIV, PAT, AOH, AME	Wine (1)	63.14	*	7.21	1.27	-	-
Six mycotoxins						-	-
DON, NIV, NEO, AOH, AFB_1, AFG_1	Beer (1)	60.37	*	34.49	3.13	-	-
DON, HT-2, NIV, βZAL, AOH, OTA	Beer (1)	83.15	*	47.51	9.25	-	-
DON, NIV, ZON, βZAL, AOH, OTA	Beer (1)	77.89	*	44.50	7.21	-	-
DON, NIV, PAT, AOH,AFB_1, AFG_1	Beer (1)	84.56	*	48.32	5.57	-	-

				Resultado			
DON, NEO, PAT, OTA, AOH, AME	Wine (1)	51.17	*	5.84	1.23	-	-
DON, 15ADON, NIV, NEO, PAT, AOH	Wine (1)	80.63	*	9.21	1.06	-	-
Eight mycotoxins						**-**	**-**
DON, 15ADON, NIV, NEO, ZON, AOH, AFB_1, AFG_1	A.F. Beer (1)	108.10	*	12.35	1.67	-	-
Ten mycotoxins						**-**	**-**
DON, T-2, HT-2, NIV, NEO, PAT, ZON, AOH, AFB_1, AFG_1	AF. Beer (1)	185.15	*	21.16	3.65	-	-

3.6. Risk assessment

The main contributors to the PMTDI from beer, A.F. beer and beer with lemonade were HT-2 (9.82%), T-2 (3.64%) and βZAL (2.09%), respectively. (Table 6). Regarding the sum PDI values, beer with lemonade could supply up to 1.24 ng/kg bw/day, while common beer might furnish up to 48.32 ng/kg bw/day. The major contributors to PMTDI from wine, A.F. wine and wine with lemonade were HT-2 (1.84%), OTA (0.31%) and βZAL (0.50%), respectively. The sum PDI values through wine consumption ranged from 0.20 ng/kg bw/day for A.F. wine to 11.87 ng/kg bw/day through common wine intake.

Moreover for cava samples, the main contributor was βZAL with 0.18% and the accumulative PDI values ranged from 0.25 to 0.36 ng/kg bw/day, while, in cider and A.F. cider samples, the main contributors to PMTDI were βZAL (0.24%) and PAT (0.08%), respectively. The sum PDI values through consumption of cider ranged from 0.39 to 1.10 ng/kg/bw/ day.

Indeed, the values obtained in this study demonstrate that the intake of these mycotoxins by beverages consumption did not represent a concern from toxicological point of view being the exposure far below the TDIs set by FAO/WHO expert JECFA committees.

Table 6: Mycotoxins exposure calculated for adult population through alcohol and non-alcohol beverages consumption

	Beer		A.F. Beer		Beer with lemonade		Wine		A.F. Wine		Wine with lemonade		Cava		Cider alcohol		A.F. Cider	
Mycotoxin	PDI ng/kg bw/day	% PMTDI	PDI (ng/kg bw/day)	% PMTDI	PDI ng/kg bw/day	% PMTDI	PDI ng/kg bw/day	% PMTDI	PDI ng/kg bw day	% PMTDI	PDI ng/kg bw/day	% PMTDI	PDI ng kg bw/day	% PMTDI	PDI ng/kg bw/day	% PMTDI	PDI ng/kg bw/day	% PMTDI
AFB1	0.63	-	0.32	-	-	-	-	-	-		-	-	-	-	-	-	-	-
AFG1	0.88	-	0.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AME	-	-	-	-	-	-	0.89	-	0.80	-	0.60	-	-	-	-	-	-	-
AOH	15.01	-	-	-	0.54	-	0.92	-	0.26	-	0.12	-	0.04	-	-	-	-	-
15ADON	-	-	1.47	0.14	-	-	1.33	0.13	-	-	-	-	-	-	-	-	-	-
BZAL	-	-	5.25	2.10	5.23	2.09	-	-	-	-	1.26	0.50	-	-	0.60	0.24	-	-
DON	5.21	0.52	1.17	0.11	1.06	0.10	1.04	0.10	-	-	-	-	-	-	-	-	-	-
HT-2	9.82	9.82	1.76	1.76	1.84	1.84	1.84	1.84	-	-	-	-	-	-	-	-	-	-
NEO	8.54	-	1.84	-	-	-	1.69	-	-	-	-	-	-	-	-	-	-	-
NIV	6.24	0.52	1.25	0.10	1.13	0.09	2.51	0.20	-	-	0.51	0.04	-	-	-	-	-	-
OTA	-	-	3.50	-	0.22	1.31	0.13	0.77	0.05	0.31	0.06	0.35	0.03	0.18	-	-	-	-
PAT	-	-	5.25	1.31	-	-	3.74	0.93	0.86	0.21	-	-	0.40	0.10	0.25	0.06	0.25	0.08
T-2	-	-	3.64	3.64	-	-	-		-	-	-	-	-	-	-	-	-	-
ZON	-	-	1.81	0.72	1.66	0.66	-	-	-	-	-	-	-	-	0.11	0.04	-	-

4. Conclusions

Alcoholic and non-alcoholic beverages were evaluated for the presence of thirty mycotoxins in this survey. 95% of the samples were contaminated with at least one mycotoxin. DON and AOH, showed the highest incidences followed by AME, OTA and PAT. The high muti-occurrence of mycotoxins in different beverages samples also was observed, mainly in beer samples, while OTA was found in one wine sample exceeding the maximum level established by EU. The risk assessment carried out did not raise any toxicological concern for all consumers.

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Conflict of interest

The authors declare no conflict of interest and informed consent was obtained from all individual participants included in the study.

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3.6. Mycotoxins dietary exposure assessment of Valencian population by total diet study

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Mycotoxins dietary exposure assessment of Valencian population by total diet study

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Abstract

The assessment of dietary exposure to mycotoxins was carried out through a total diet study mainly based on Mediterranean diet for Valencian population. Thirty one mycotoxins, including alternariol, alternariol methyl eter, deoxynivalenol, 3-acetyl-deoxynivalenol, 15-acetyl-deoxynivalenol, nivalenol, neosolaniol, fusarenon-X, diacetoxyscirpenol, T-2 and HT-2 toxins, sterigmatocystin, ochratoxin-A, aflatoxins, patulin, zearalenone, α-zearalanol, β-zearalanol, α-zearalenol, β-zearalenol, fumonisins, enniatins and beauvericin were determined in 576 ready to eat food samples collected for the total diet study conduction. The results showed that 62.1% of the samples contained at least one mycotoxin with concentrations ranging from 0.03 μg L^{-1} for AFM1 in milk to 212 μg kg^{-1} for NIV in dried fruits. The most frequently detected mycotoxins were DON (25%), NEO (20%), HT-2 (18%) and AOH (13%). A dietary exposure of Valencian population was assessed to perform the health risk characterization considering lower bound (LB) and upper bound (UB) assumption for left-censorship management. PCA statistical procedure was used to investigate how mycotoxins were spread out among food group samples and the major contributors to these food contaminants in Mediterranean diet were found to be dried fruits, meat and beers. The average daily intakes of mycotoxins in the Valencian population ranged from 0.001 ng kg^{-1} bw day^{-1} for STG through dried fruit to 12.75 ng kg^{-1} bw day^{-1} for AOH through beer intake, none exceeded the established tolerable daily intakes.

1. Introduction

It is well recognized that diet composition may affect the health status of the population. Many organizations and public health authorities have

highlighted the importance of a varied, balanced and moderated diet. In this sense, the called Mediterranean diet, mainly followed by Valencian population, is characterized by a nutritional model that incorporate primarily plant-based foods, such as fruits and vegetables, whole grains, legumes and nuts, substitute butter with olive oil, limit red meat to no more than a few times a month, eat fish and poultry at least twice a week and drink red wine in moderation (Nomikos et al., 2018; CIEAM, 2015). Various studies have demonstrated that serving a day antioxidant-rich fruits and vegetables and restringing saturated fats and hydrogenated oils is linked to cardiovascular and cognitive health improvement (Boccardi et al., 2018; Tosti et al., 2018; Davis et al., 2015). Food may also contain organisms or dangerous substances due to the intentional addition of substances such as pesticides, veterinary drugs or other natural contaminants as mycotoxins.

Mycotoxin contamination of food is an ongoing global concern. The Rapid Alert System for Food and Feed of the European Union placed mycotoxins in the second position according to the total number hazard notifications (RASFF, 2017). The toxigenic fungi can infect agricultural crops during crop growth, harvest, storage, or processing and posing a difficult challenge to food safety (Yang et al., 2014).

More than 400 mycotoxins have been reported, even only a few groups that are important from the perspective of safety and economic evaluation received extensive scientific and industrial attentions, such as ochratoxin A (OTA), aflatoxins (AFs), patulin (PAT), *Fusarium* and *Alternaria* toxins (Alshannaq and Yu, 2017; Shi et al., 2018; Escola et al., 2018). Besides, some of these mycotoxins are linked hepatotoxic, genotoxic, nephrotoxic,

immunosuppressive, teratogenic and carcinogenic effects (Ostry et al., 2017; Alshannaq & Yu et al., 2017; Smith et al., 2016).

Several core food commodities are frequently reported as affected by mycotoxins contamination, mainly cereals (Pereira et al., 2014; Stanciu et al., 2015), dried fruit (Azaiez et al., 2015) coffee (García-Moraleja et al., 2015), legumes and some fruits (Danezis et al., 2016; Abrunhosa et al., 2014), milk (Michlig et al., 2016); meat (Montanha et al., 2018), alcoholic beverages (Pascari et al., 2018; De Jesu et al., 2017) and fruit juices (Carballo et al., 2018). Furthermore, many mycotoxins are resistant to food processing because of their stability against different physical, and chemical treatments (Sirot et al., 2013; Milani and Maleki 2014; Alshannaq and Yu 2017).

Considering their heat stability, their diverse chemical and biological properties and their toxic effects, these substances constitute a potential risk for human and animal health and it is widely recognized that they more attention should be addressed to reduce mycotoxin contamination worldwide issues (Marin et al., 2013; Leblanc et al., 2005).

To measure the health impact, determining contaminants levels of in foods to estimate population dietary exposure is highly recommendable. In this sense, various collections provide exposure information like food monitoring studies, duplicate diet and total diet studies (De Nijs et al., 2016). A Total Diet Studies (TDS) consisted mainly on selection, collection and analysis of commonly consumed food and assessment of dietary exposure population groups to both beneficial and harmful chemical substances in order to help the risk manager on public health decisions (EFSA/FAO/WHO, 2011).

The WHO Global Environmental Monitoring System/Food Contamination Monitoring and Assessment Programme (GEMS/Food) had for

many years supported the use of the TDS as one of the most cost-effective methods for monitoring dietary intake of chemicals or nutrients (WHO, 2015; Leblanc et al., 2005).

The TDS approaches have been already employed to assess the dietary exposure to different mycotoxins in several countries mainly; France (Sirot et al., 2013; Leblanc et al., 2005), Ireland (Food Safety Authority of Ireland, 2016), New Zealand (Food Standards Australian New Zealand, 2001; 2003; 2011), Canada (Tam et al., 2011), The Netherlands (Sprong 2015a; 2015b; López et al., 2016), Hong Kong (Yau et al., 2016), Lebanon (Raad et al., 2014), Spain (Beltrán et al., 2013; Cano-Sancho et al., 2012; Urieta 1991; Urieta 1996), and Viet Nam (Huong et al., 2016).

The aim of the present study is to evaluate the presence of thirty-one mycotoxins; alternariol (AOH), alternariol methyl eter (AME), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3ADON), 15-acetyl-deoxynivalenol (15ADON), nivalenol (NIV), neosolaniol (NEO), fusarenon-X (FUS-X), diacetoxyscirpenol (DAS), T-2 and HT-2 toxins, sterigmatocystin (STG), ochratoxin-A (OTA), five aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2, and AFM_1), patulin (PAT), zearalenone (ZEA), α-zearalanol (αZAL), β-zearalanol (βZAL), α-zearalenol (αZOL), β-zearalenol (βZOL), fumonisins (FB_1, FB_2), as well as five emerging mycotoxins: enniatins (ENNA, $ENNA_1$, ENNB and $ENNB_1$) and beauvericin (BEA) to evaluate mycotoxin contamination of Valencia population diet, the third largest city in Spain, by LC-MS/MS and GC-MS/MS and to assess their dietary intake using both low bound scenario (LB) and upper bound scenario (UB).

2. Materials and methods

2.1. Chemicals and reagents

Solvents (acetonitrile, hexane, chloroform and methanol) were supplied by Merck (Darmstadt, Germany). Deionized water (<18, 2MΩ$_{cm}$ resistivity) was obtained in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Beadford, MA, USA). Ammonium formate (99%) and formic acid (≥98%), anhydrous magnesium sulphate and sodium chloride were supplied by Sigma Aldrich (Madrid, Spain). Syringe nylon filter (13mm diameter 0.22 μm pore size) were obtained from Analisis Vinicos S.L. (Tomelloso, Spain). The derivatization reagent composed of BSA (N,O-bis(trimethylsilyl) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilylimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

2.1.2. Standards and solutions

The standards of AOH, AME, AFB$_1$, AFB$_2$, AFG$_1$, AFG$_2$, AFM$_1$, OTA, FB$_1$, FB$_2$, ENNA, ENNA$_1$, ENNB, ENNB$_1$, BEA, STG, ZON, α-ZAL, β-ZAL, α-ZOL, β-ZOL, DON, 3ADON, 15ADON, DAS, NIV, FUS-X, NEO, PAT, T-2 and HT-2 toxins were purchased from Sigma Aldrich. Individual stock of all analytes were prepared to obtain 20 mg/L in methanol and multianalyte working solutions of 2 mg/L were also used by diluting the individual stock solutions. The multianalyte working standard solution was used for standard calibration curves, matrix-matched calibration curves and recovery assays. All standards were stored in darkness and kept at -20°C.

2.3 Sampling

A total of 576 samples, representative of the Mediterranean diet, were collected from Valencia University restaurant mainly by September 2016 to December 2017 (Table 1). The samples selection was based on the basis that food items formed part of the menu offered at least twice a week and which components have been associated with fungal contamination in the literature. The most consumed meal groups during a week including drinks were selected and distinguished according to the major diet components.

Only the edible parts of each food were considered. Seeds and skins were removed from fruit. The inedible part of meat and fish such as bones, thorns and skin were also removed according to the European Commission Regulation (EC/401/2006), before analysis performance. All samples were milled with a food knife mill (Oster Classic grinder, Valencia, Spain) and obtained mixture was stored at -18°C until analysis.

Table 1:Classification of the food group used and their daily intake recommended

Food group	Food item	Analyzed samples (N)	Daily intake (g/day)
Cereals	Rice	38	10.43
	Pasta	22	11.2
	Bread	15	80
	Mixed dishes	13	37.86
Vegetables	Zuchinni	8	9.53
	Eggplant	9	3.94
	Carrot	5	8.87
	Onnions	11	19.15
	Peppers	20	12.70
	Sweet corn	6	1.12
	Mushrooms	6	3.34
	Tomato	16	35
	Potato	54	78
	Vegetable soup	6	2.16
Fruits	Apple	10	26,95
	Orange	10	48,82
	Grapes	10	5,47
	Strawberry	10	6,98
Dried fruit	Dried fruit	30	2.99
Legumes	Beans	18	5.17
	Lentils	4	2.57
	Chickpeas	2	3.42
Meat	Beef	4	14.21
	Pork	9	28.02
	Chicken	9	35.58
Fish and fish aquatic products	Fish	29	28.84
	Squid	14	9.64
	Prawns	10	5.97
Beer	Beer	20	42.16
	Beer AF	10	8.52
	Beer with lemonade	10	8.52
Wine	Wine	20	22.21
Fruit juice	Fruit juice	38	25
Coffee	Coffee	20	4.63
Tea	Tea	20	4.63
Milk	Milk	20	89.31

2.4. Extraction procedure

Samples extraction was performed according QuEChERS to the previously home validated method by Carballo et al. (2018) for matrix cereals, vegetables, legumes, meat and fish matrixes. Several extraction methods were applied for mycotoxins extraction from different beverages samples; Dispersive Liquid-liquid MicroExtraction method previously validated by Pallarés et al., (2017) was used for beers, juices, tea and wine was used, Liquid-liquid Extraction method was used reported by García-Moraleja et al., (2015) was applied for coffee drinks and QuEChERS extraction method reported by Michlig et al., (2016) was tested for milk beverages.

2.5. Analysis of food samples

2.5.1. GC–MS/MS analysis

The analysis was carried out using an Agilent 7890A GC system coupled with an Agilent 7000A triple quadruple mass spectrometer with inter electron-impact ion source (EI, 70Ev). Quantitative data were acquired at selection reaction monitoring mode. The transfer line and source temperatures were 280° and 230°, 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). Analytes were separated on a HP-5MS 30m x 0.25mm x 0.25µm capillary column. One microliter of the final clean extract of mycotoxins was injected in splitless mode in programmable temperature vaporization (PTV) inlet at 250°C employing helium as the carried gas at fixed pressure of 20.3 psi. The oven temperature started at 80°C, and increased to

245°C at 60 ºC/min, hold their time for 3 min and increased to 260 ºC progressively at 3 ºC/min and finally to 270 ºC at 10 ºC/min and then held for 10 min.

Before GC-MS/MS analysis, 50 µL of BSA + TMCS + TMSI (3:2:3) was added to the dry extract and left 30 min at room temperature. Then 200 µL of hexane was added, mixed thoroughly on vortex for 30 s and washed with 1 ml of phosphate buffer (60 mM, pH7) and mixed until the upper layer was clear. Finally, the hexane layer was transferred to auto sampler vial.

2.5.2. LC–MS/MS and LC-ESI-qTOF analysis

HPLC-MS/MS analysis was performed using an Agilent 1200 liquid chromatography (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® ABSCIEX (Applied Biosystems, Foster City, CA, USA) equipped with a Turbo-VTM source (ESI) interface. The chromatographic separation of the analytes was conducted at 25ºC with a reverse analytical column Gemini® NX-C18 (3 µM, 150 × 2 mm ID) and guard column C18 (4 × 2 mm, ID; 3 µM). Mobile phases was a time programmed gradient using methanol as phase A (5 mM ammonium formate and 0,1% formic acid) and water phase B (5 mM ammonium formate 0,1% formic acid). The gradient was program as follows: 0-10 min 100% B; 11-15 min 80% B; 16-18 min 70% B. In the next 6 min, the column was readjusted to initial conditions employed and equilibrated. The volume of the injections was 20 µL and the flow rate used was 0.25 mL/min.

An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, autosampler, and binary pump, was used for the chromatographic determination. The column was Gemini® NX-C18 (3 µM, 150 × 2 mm ID) and guard column C18 (4 × 2 mm, ID; 3 µM) (Phenomenex). The mobile phases consisted of water (A) and acetonitrile (B), both 0.1% in

formic acid. The gradient program was as follows: 0–6 min, 50% B; 7–12 min, 100% B; 13–20 min, 50% B. The injection volume for standards and sample extracts was 5 μL and the flow rate used was 0.2 mL/min. A mass spectrometry (MS) analysis was carried out using a 6540 Agilent Ultra- High-Definition Accurate-Mass q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in positive ionization mode. Integration and data elaboration were performed using Mass Hunter Workstation software (Agilent Technologies).

2.6. Statistical analysis

Analysis of variance (ANOVA test error type III) was used to assess the significance of the differences between the determined mycotoxin concentrations. All P-values of <0.05 (statistical significance of 95%) were considered to be statistically significant. The relationships between mycotoxin concentrations and food group were assessed using the analysis of Bayes VarSel R, taking into account Mediterranean pyramid of food, which classify some food as daily, weekly or occasionally consumed. According to the number of daily or weekly intakes, a value has been assigned to each group of food considered in the study (Table 2).

Table 2: Score dietary recommendation of Mediterranean diet pyramid.

Consumption	**Score**
Daily	
Cereals, fruit	5
Vegetables, milk	4
Weekly	
Dried fruit, fish	3
Legumes, meat	2
Occasional	
Tea, coffee, alcohol beverages	1
Non-alcohol beverages	0

2.7. Exposure assessment

The exposure of the population to mycotoxins was assessed by combining the national consumption data with the contamination data from the analyses. Deterministic method was applied, using the equation:
EDI (ng kg^{-1} bw day^{-1})= C*K/bw where *C* is the mean of each mycotoxin in the samples analyzed (µg kg^{-1}), *K* is the daily average consumption/person for the food commodity included in the study (Kg $capita^{-1}$ day^{-1}) and *bw* is the body weight used in the population group.

Left-censored results (data below LOQ) were processed according to EFSA recommendations (EFSA, 2010). Two scenarios were considered: (i) the lower-bound (LB) approach by replacing the results below LOD by zero (ii) the upper-bound (UB) approach by replacing the results below LOD by LOD.
The health risk characterization of each mycotoxin (% relevant TDI) was performed by comparing the EDI with TDI legislated as indicated equation:
%TDI: (EDI/TDI)*100

2.8. Method validation

To ensure the reliability of the results, blank samples were tested for each of the food groups studied. Recoveries ranged between 60–119% for studied mycotoxins. The criteria for confirmation of positive findings were set by the ratio obtained from the quantification (Q) and confirmation (q) standard transitions, and the agreement with the retention times. The LOD (signal-to-noise, S/N=3) and LOD (S/N=10) were calculated by experiments using spiked samples at the lowest concentration, and the determined LODs and LOQs for 31 mycotoxins showed in Table 3.

Table 3: LODs and LOQs in 13 food group (μg kg^{-1})

Mycotoxin	Cereals		Vegetables		Fruits		Dried fruits		Legumes		Meat		Fish		Beer		Wine		Fruit juice		Coffee		Tea		Milk	
	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ	LOD	LOQ
AFB_1[a]	0.15	0.5	0.15	0.5	0.3	1	0.3	1	0.15	0.5	0.3	1	0.15	0.5	0.06	0.2	0.3	1	0.3	1	0.03	0.1	0.3	1	-	-
AFB_2[a]	0.3	1	0.3	1	1.5	5	0.3	1	0.3	1	0.3	1	0.3	1	0.3	1	1.5	5	0.3	1	1.5	5	1.5	5	-	-
AFG_1[a]	0.3	1	0.3	1	0.3	1	0.3	1	1.5	5	0.15	0.5	0.3	1	0.06	0.2	0.3	1	0.03	0.1	0.3	1	0.3	1	-	-
AFG_2[a]	0.15	0.5	0.3	1			0.3	1	0.3	1	0.3	1	0.3	1	0.3	1	1.5	5	0.3	1	1.5	5	0.3	1	-	-
AFM_1[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.009	0.03
AME[a]	-	-	-	-	1.5	5	3	10	-	-	-	-	-	-	1	5	0.3	1	0.3	1	1.5	5	1.5	5	-	-
AOH[a]	-	-	-	-	3	10	0.3	1	-	-	-	-	-	-	0.3	1	0.03	0.1	0.03	0.1	1.5	5	3	10	-	-
BEA[a]	0.15	0.5	0.15	0.5	0.3	1	1.5	5	0.15	0.5	0.3	1	0.15	0.5	0.3	1	1.5	5	0.3	1	1.5	5	1.5	5	-	-
DAS[c]	0.75	2.5	0.75	2.5	1.17	3.90	2.34	7.81	0.75	2.5	0.15	0.5	0.75	2.5	0.58	1.95	0.58	1.95	1.17	3.90	1.17	3.90	2.34	7.81	-	-
DON[c]	0.15	0.5	0.15	2.5	1.17	3.90	0.58	1.95	0.15	0.5	0.15	0.5	0.15	0.5	0.58	1.95	0.58	1.95	0.58	1.95	0.58	1.95	0.58	1.95	-	-
3ADON[c]	0.75	2.5	1.5	5	2.34	7.81	2.34	3.90	0.15	0.5	0.15	0.5	0.15	0.5	1.17	3.90	2.34	3.90	1.17	3.90	2.34	7.81	2.34	7.81	-	-
15ADON[c]	0.75	2.5	0.15	0.5	1.17	3.90	2.34	3.90	0.15	0.5	0.15	0.5	0.15	0.5	0.58	1.95	0.58	1.95	0.58	1.95	1.17	3.90	0.58	1.95	-	-
ENNA[a]	0.15	0.5	0.15	0.5	0.3	1	0.3	1	0.15	0.5	0.15	0.5	0.3	1	0.03	0.1	0.15	0.5	0.3	1	0.3	1	0.3	1		
$ENNA_1$[a]	0.15	0.5	0.15	0.5	0.03	0.1	0.3	1	0.3	0.5	0.15	0.5	0.15	0.5	0.15	0.5	0.03	0.1	0.03	0.1	0.03	0.1	0.03	0.1	-	-
ENNB[a]	0.15	0.5	0.15	0.5	0.03	0.1	0.3	1	0.3	1	0.3	1	0.15	0.5	0.15	0.5	0.15	0.5	0.03	0.1	0.03	0.1	0.03	0.1	-	-
$ENNB_1$[a]	0.15	0.5	0.15	0.5	0.3	1	0.3	1	0.15	0.5	0.15	0.5	0.15	0.5	0.03	0.1	0.15	0.5	0.03	0.1	0.3	1	-	-
HT-2[c]	0.15	0.5	0.75	2.5	1.17	3.90	1.17	3.90	0.15	0.5	0.15	0.5	0.15	0.5	0.58	1.95	1.17	3.90	0.58	1.95	1.17	3.90	1.17	3.90	-	-
FUS-X[c]	0.75	2.5	1.5	5	1.17	3.90	2.30	7.81	0.75	2.5	0.15	0.5	0.75	2.5	2.34	7.81	1.17	3.90	2.34	7.81	2.34	7.81	2.34	7.81	-	-
FB_1[a]	1.5	5	0.3	1	3	10	1.5	5	1.5	5	1.5	5	1.5	5	1.5	5	1.5	5	1.5	5	3	10	3	10	-	-
FB_2[a]	1.5	5	0.3	1	3	10	3	10	1.5	5	1.5	5	1.5	5	1.5	5	1.5	5	5	5	1.5	5	3	10	-	-
NEO[c]	0.15	0.5	0.15	0.5	0.58	1.95	0.58	1.95	0.15	0.5	0.15	0.5	0.75	2.5	0.58	1.95	0.97	1.95	0.58	1.95	0.58	1.95	0.58	1.95	-	-
NIV[c]	1.5	5	1.5	5	2.34	3.90	2.34	7.81	0.75	2.5	0.75	2.5	1.5	5	2.34	7.81	2.34	7.81	1.17	3.90	2.34	7.81	1.17	3.90	-	-
OTA[a]	1.5	5	1.5	5	0.3	1	0.3	1	1.5	5	1.5	5	1.5	5	0.06	0.2	0.15	0.5	0.03	0.1	0.15	0.5	1.5	5	-	-
PAT[c]	-	-	-	-	1.17	3.90	2.34	7.81	-	-	-	-	-	-	1.17	3.90	2.34	7.81	2.34	7.81	2.34	7.81	1.17	3.90	-	-
STG[a]	1.5	5	1.5	5	1	5	0.3	1	1.5	5	1.5	5	0.3	1	1.5	5	2.34	7.81	1.5	5	1.5	5	0.3	1	-	-
T-2[c]	0.75	2.5	0.75	2.5	2.34	7.81	2.34	7.81	0.75	2.5	0.75	2.5	0.75	2.5	2.34	7.81	2.34	7.81	2.34	7.81	2.34	7.81	2.34	7.81	-	-
ZON[c]	0.15	5	0.15	0.5	2.34	3.90	2.34	7.81	0.15	0.5	1.5	5	1.5	5	2.34	7.81	2.34	7.81	2.34	7.81	1.17	3.90	1.17	3.90	-	-
α-ZAL[c]	0.15	0.5	0.15	0.5	2.34	7.81	1.17	3.90	0.15	0.5	0.15	0.5	0.75	2.5	1.17	3.90	1.17	3.90	0.58	1.95	1.17	3.90	1.17	3.90	-	-
β-ZAL[c]	0.75	2.5	1.5	5	1.17	3.90	1.17	3.90	1.5	5	1.5	5	1.5	5	2.34	7.81	2.34	7.81	2.34	7.81	2.34	7.81	2.34	7.81	-	-
α-ZOL[c]	1.5	5	1.5	5	2.34	7.81	2.34	7.81	1.5	5	1.5	5	1.5	5	1.17	3.90	2.34	7.81	0.58	7.81	2.34	7.81	1.17	3.90		
β-ZOL[c]	0.15	0.5	0.75	2.5	2.34	7.81	2.34	7.81	1.5	5	1.5	5	1.5	5	1.17	3.90	1.17	3.90	1.17	3.90	2.34	7.81	2.34	7.81	-	-

[a]LC-MS/MS determination [b]LC-ESI-qTOF determination [c]GC-MS/MS determination

3. Results and discussion

3.1. Aflatoxins

AFs showed up in 5% to 60% of food groups tested mainly dried fruits, beer and tea beverages (Table 4). The highest AFs contamination was found in tea beverage samples (23.07 μg L^{-1}) followed by legume samples (10.39 μg kg^{-1}), while lowest mean concentration was detected in milk at mean level of 0.03 μg kg^{-1} for AFM_1. The mean concentrations detected in milk and cereals did not exceed the EU maximum limits of 0.05 to 4 μg kg^{-1}, respectively. However, 13% of dried fruit samples exceeded slightly the EU maximum limits of 4 μg kg^{-1} (EC, 1881/2006).

Several previous total diet studies (TDS) reported AFs contents in foodstuffs. Comparable AFs incidence and mean levels in cereals products were reported in Hong Kong TDS (Yau et al., 2016) where AFB1 was detected in legume samples up to 75% at mean concentrations about 2.90 μg kg^{-1} (Huong et al., 2016; Yau et al., 2016). While, highest incidence, about 67% of rice samples contained AFB_1 at mean concentration of 2.6 μg kg^{-1}in Viet Nam TDS (Huong et al., 2016). High AFs contents were reported in New Zealand TDS in roasted salted peanuts samples reaching 38 μg kg^{-1} (FSANZ, 2001) while Cano-Sancho et al., (2012) reported AFs in 20% of dried fruits at concentrations up to 8.85 μg kg^{-1} and contents down to 0.260 μg kg^{-1} were detected in nuts, seed, and dried date in Lebanon (Raad et al., 2014).

Scarce data are available Concerning AFs occurrence in meat samples even 91% of meat samples were reported contaminated by AFs reaching 4 μg kg^{-1} (Huong et al., 2016).

The average daily exposure to AFs was estimated to range between 0.002-0.003 ng kg^{-1} bw day^{-1} by dried fruit and milk to 1.73-1.77 ng kg^{-1} bw day^{-1} through tea consumption considering LB and UB scenarios, respectively for adult local population (Table 5).

Other studies had already estimated the mycotoxins intake through TDS, for instance AFs data through legume consumption in Viet Nam and Chinese population ranged from 0.1 to 2.8 ng kg^{-1} bw day^{-1}, respectively (Huong et al., 2016; Yau et al., 2016) while through cereals products consumption data ranged from 0.52 to 22 ng kg^{-1} bw day^{-1} (Huong et al., 2016; FSAI, 2016; Raad et al., 2014). Cano-Sancho et al., (2012) and Raad et al., (2014) located also daily intake for AFs by dried fruit ranging from 0.045 to 0.182 ng kg^{-1} bw day^{-1} in Spain and Lebanon respectively.

3.2. Ochratoxin A

OTA was detected in several foods mostly in beer, wine and coffee beverages ranging from 4% of fish samples to 45% of coffee and wine samples (Table 4). The highest mean concentration was detected in meat with 54.09 μg kg^{-1}, while the lowest mean contents were found in beer samples at 3.11 μg kg^{-1}. OTA was also previously detected in dried fruit samples in TDSs performed in Spain, Lebanon, Hong Kong and Ireland with occurrence from 3% to 42% (Cano-Sancho et al., 2012; Raad et al., 2014; Yau et al., 2016; FSAI, 2016). TDS studies performed in Viet Nam, France and Hong Kong reported OTA in meat products ranging between 7% to 64% at mean levels of 0.01to 2.68 μg kg^{-1} and in 50% of fish and 100% of other aquatic products up to 4.85 μg kg^{-1} (Leblanc et al., 2005; Huong et al., 2016; Yau et al., 2016).

Similar OTA incidence in coffee beverages was reported in TDS from Spain where 49% of coffee samples contained OTA (Cano-Sancho et al., 2012). The OTA mean concentrations in coffee reported in the literature were between 0.04 to 2.17 μg L^{-1} (Leblanc et al., 2005; Cano-Sancho et al., 2012; Raad et al., 2014; López et al., 2016).

The average daily exposure to OTA was estimated to range between 0.006-0.007 ng kg^{-1} bw day^{-1} by dried fruit to 3.20-3.23 ng kg^{-1} bw day^{-1} through meat consumption considering LB and UB scenarios, respectively, which contributed from 0.03% to 19.0% to the TDI (Table 5 and Table 6).
Other TDS studies performed estimation of OTA intake and the EDI values ranged between 0.003 ng kg^{-1} bw day^{-1} by cereal products to 5 ng kg^{-1} bw day^{-1} through meat consumption subscribing from 0.01% to 29.41% to the TDI (Leblanc eta al., 2005; Sirot et al., 2013; Huong et al., 2016; Raad et al., 2014; Cano-Sancho et al., 2012; Tam et al., 2011; FSAI, 2016 and Sprong et al., 2016).

3.3. Trichothecenes

TCs were detected in the most food analyzed (Table 4). DON was the most prevalent mycotoxin also detected in cereals, beer and wine samples. The highest incidence was registered in beer samples (80%) with mean concentration of 8.98 μg L^{-1}. NEO was detected in cereals, wine and coffee ranging from 4% for vegetables and fish to 55% for wine samples. The highest concentration was detected in coffee beverages with 28.61 μg L^{-1}.

Different previous TDS reported DON presence in cereal samples (Cano-Sancho et al., 2012; Yau et al., 2016; Beltrán et al., 2013; Raad et al., 2014; Leblanc et al., 2005; Sirot et al., 2013; López et al., 2016). DON was also detected in alcoholic beverages at concentrations between 9.1 to 52.08 μg L^{-1} (Raad et al.,

2014; López et al., 2016). DON incidence of 100% was reported in bread samples (Cano-Sancho et al., 2012) and the highest concentration reported was also in bread and toast samples with mean level of 524.17 μg kg^{-1} (Raad et al., 2014).

HT-2 was detected in several foods with incidence ranging from 6% in vegetables to 54% in legume samples at mean concentration levels from 10.34 to 15.65 μg kg^{-1}, while, T-2 was detected in 3% of beer and 10% of cereal samples.

The most T-2 and HT-2 toxins incidence of 54% was reported in corn flakes samples and the highest concentration was quantified in sweet corn up to 144.8 μg kg^{-1} (Cano-Sancho et al., 2012). The TDS study performed in the Netherlands also referred that apple juice composite contained T-2 at mean level of 14 μg L^{-1} (López et al., 2016).

NIV was mainly found in dried fruit, meat and beer samples. The highest incidence was found dried fruit where mean levels was about 212 μg kg^{-1} and the lowest concentration was found in cereals samples with 4.57 μg kg^{-1}.

NIV was also reported in TDS performed in France, at incidences and concentrations lower than 5% and 20 μg kg^{-1} respectively in a variety of foodstuff as rice, bread, nuts and oil seed (Leblanc et al., 2005; Sirot et al., 2013).

The average daily exposure to TCs was estimated to range between 0.02-0.03 ng kg^{-1} bw day^{-1} through coffee for 15ADON to 4.64-4.71 ng kg^{-1} bw day^{-1} through beer consumption for DON considering LB and UB scenarios, respectively, which contributed from 0.002% to 3.47% to the TDI (Table 5 and Table 6).

Other studies have already estimated TCs intake in several TDS, NIV dietary exposure was also related to cereal products, nuts and oilseed in French TDS and data ranged from 0.79 to 27.7 ng kg^{-1} bw day^{-1} being bread the main contributor to NIV dietary exposure with 2.30% of the TDI (Leblanc et al., 2005), while rice products only contributed up to 0.23% of the TDI (Sirot et al., 2013).

EDI values for DON were reported between 0.5 ng kg^{-1} bw day^{-1} through breakfast cereal to 1052 ng kg^{-1} bw day^{-1} through bread consumption resulting in TDI values between 0.05% to 105.2% (Leblanc et al., 2005; Cano-Sancho et al., 2012; Beltrán et al., 2013;Sirot et al., 2013; Raad et al., 2014; Sprong et al., 2016).

EDI for the sum of T-2 and HT-2 toxins were also previously investigated and data obtained ranged between 2.6 ng kg^{-1} bw day^{-1} by cereal products and 38 ng kg^{-1} bw day^{-1} mainly by dried fruit and apple juice consumption and main contributors to T-2 and HT-2 toxin dietary exposure were pasta and cereal products with 2.78% to 38 % of TDI (Cano-Sancho et al., 2012; Sirot et al., 2013).

Table 4: Mycotoxin contents (μg kg^{-1}) in food groups included in the total diet study

	Cereals (n=88)		Vegetables n=(141)		Fruits (n=40)		Dried fruits (n=30)		Legumes (n=24)		Meat (n=22)		Fish (n=53)		Beer (n=40)		Wine (n=40)		Fruit juice (n=38)		Coffee (n=20)		Tea (n=20)		Milk (n=20)	
Mycotoxin	I (%)	Mean μg/kg	I (%)	Mean μg/kg	I (%)	Mean μg/kg	I (%)	Mean μg/kg	I (%)	Mean μg/kg	I (%)	Mean μg/kg	I (%)	Mean μg/L	I (%)	Mean μg/L	I (%)	Mean μg/L	I (%)	Mean μg/L	I (%)	Mean μg/L	I (%)	Mean μg/L	(%)	Mean μg/L
AFB1	-	n.d.	-	n.d.	-	n.d.	10	2.30	-	n.d.	-	n.d.	-	n.d.	60	1.47	-	n.d.	8	9.36	-	n.d.	-	n.d.	-	n.d.
AFB2	-	n.d.	-	n.d.	-	n.d.	13	4.75	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	13	4.49	-	n.d.	35	23.07	-	n.d.
AFG1	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	35	1.17	-	n.d.	-	n.d.	-	n.d.	25	1.33	-	n.d.
AFG2	5	1.64	-	n.d.	-	n.d.	-	n.d.	8	10.39	9	3.13	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	25	4.53	-	n.d.
AFM1	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	20	0.03
AME	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	60	7.55	21	8.54	-	n.d.	-	n.d.	-	n.d.
AOH	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	90	22.01	45	7.79	34	9.96	-	n.d.	25	3.94	-	n.d.
BEA	7	4.03	12	4.76	-	n.d.	17	33.15	-	n.d.	5	18.62	-	n.d.	-	n.d.	-	n.d.	-	n.d.	35	5.95	-	n.d.	-	n.d.
DAS	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	4	7.98	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
DON	60	4.22	13	7.23	8	8.43	-	n.d.	21	6.81	18	6.79	19	8.46	80	8.98	60	8.85	-	n.d.	-	n.d.	-	n.d.	-	n.d.
3ADON	3	1.79	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	9	8.41	-	n.d.	-	n.d.	-	n.d.	10	16.17	10	9.58	-	n.d.
15ADON	14	2.12	2	7.03	-	n.d.	-	n.d.	8	6.92	-	n.d.	9	6.71	10	12.08	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
ENNA	n.d	n.d.	n.d	n.d.	n.d	n.d.	n.d	n.d.	n.d	n.d.	n.d	n.d.			n.d	n.d.	n.d	n.d.	n.d	n.d.	n.d	n.d.	n.d	n.d.	n.d	n.d.
ENNA1	6	2.85	-	n.d.	-	n.d.	10	16.68	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
ENNB	39	4.63	3	7.39	-	n.d.	10	12.06	-	n.d.	9	1.38	-	n.d.	-	n.d.	-	n.d.	-	n.d.	45	2.14	-	n.d.	-	n.d.
ENNB1	-	n.d.	2	6.32	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
HT-2	40	9.20	6	10.34	40	20.90	37	20.93	54	11.62	9	8.54	-	n.d.	30	15.81	10	15.65	16	19.53	-	n.d.	-	n.d.	-	n.d.
NEO	44	2.70	4	10.42	25	14.28	30	17.80	17	10.40	-	n.d.	4	12.43	40	14.43	55	14.27	-	n.d.	50	28.61	-	n.d.	-	n.d.
NIV	16	4.57	-	n.d.	-	n.d.	50	212	-	n.d	18	10.30	-	n.d.	33	10.12	15	21.26	-	n.d.	-	n.d.	-	n.d.	-	n.d.
OTA	-	n.d.	-	n.d.	-	n.d.	13	3.11	-	n.d.	18	54.09	4	4.30	20	1.83	45	1.12	13	6.25	45	3.43	-	n.d.	-	n.d.
PAT	-	n.d.	-	n.d.	10	20.76	23	17.90	-	n.d.	-	n.d.	-	n.d.	5	43.19	40	31.66	37	28.63	-	n.d.	-	n.d.	-	n.d.
STG	-	n.d.	-	n.d.	-	n.d.	7	3.83	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
T-2	10	19.70	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	3	29.80	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
ZEA	7	5.05	-	n.d.	10	15.53	-	n.d.	-	n.d.	-	n.d.	-	n.d.	8	14.50	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
α-ZAL	-	n.d.	-	n.d.	3	22.37	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.
β-ZAL	-	n.d.	9	38.41	8	62.67	17	43.83	29	26.51	-	n.d.	-	n.d.	25	43.08	-	n.d.	5	23.22	-	n.d.	25	35.61	-	n.d.
β-ZOL	-	n.d.	-	n.d.	3	27.28	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.	-	n.d.

3.4. Zearalenone and metabolites

ZEA and metabolites mainly αZAL, βZAL and βZOL were detected in legumes, dried fruits and beer (Table 4). The highest ZEA concentrations were found in fruit samples (15.53 μg kg^{-1}), while, highest βZAL mean contents were found in dried fruit (62.67 μg kg^{-1}).

Different TDS reported presence of ZEA in several foodstuffs (Cano-Sancho et al., 2012; Yau et al., 2016; Leblanc et al., 2005 and Sirot et al., 2013). ZEA was also reported in 11% of beer samples at mean levels of 3.1 μg kg^{-1} (Cano-Sancho et al., 2012).

The average daily exposure to ZEA and metabolites was estimated to range between 0.009-0.05 ng kg^{-1} bw day^{-1} through fruit for αZAL to 5.12-5.26 ng kg^{-1} bw day^{-1} through beer consumption for βZAL considering LB and UB scenarios, respectively, contributing with 0.003% to 2.06% of the TDI (Table 5 and Table 6).

Other studies have estimated ZEA intakes in TDS and the EDI values were between 0.07 to 4.01 ng kg^{-1} bw day^{-1} through chocolate and bread consumption (Leblanc et al., 2005; Cano-Sancho et al., 2012; Sirot et al., 2013; Yau et al., 2016) and the risk assessment values (%TDI) reported in several TDS ranged from 0.01% to 29.94% (Tam et al., 2011; Huong et al., 2016).

3.5. Patulin and sterigmatocystin

PAT was mainly found in dried fruit, wine and fruit juices with incidence ranging from 5% to 40% (Table 4). The highest concentration was found in beer samples (43.19 μg L^{-1}) and the lowest concentration was quantified in dried fruit samples at 17.90 μg kg^{-1}. PAT was previously reported in fruit based products

with incidence between 5% and 50%. (Leblanc et al., 2005; Cano-Sancho et al., 2012; Sirot et al., 2013; FSAI, 2016).

The average daily exposure to PAT was estimated to range between 0.10 ng kg^{-1} bw day^{-1} across dried fruit intake to 3.70 ng kg^{-1} bw day^{-1} through wine consumption at LB and UB scenarios respectively contributing with 0.02% to 0.93% of the TDI (Table 5 and Table 6).

STG was only detected in dried fruit with an incidence of 7% and mean levels of 3.83 μg kg^{-1} (Table 4). No available data of STG in dried fruit were found. However, a study performed in the Netherland reported presence of STG in chocolate samples at 0.5 μg kg^{-1} (López et al., 2016).

3.6. Emerging mycotoxins

Enniatins (ENNs) were found in 2% of vegetable samples to 45% of coffee samples (Table 4). The highest mean concentration was found in dried fruit (16.68 μg kg^{-1}) for $ENNA_1$ and low concentration was detected in meat samples (1.38 μg kg^{-1}) for ENNB. BEA was found in 5% of meat to 35% of coffee samples. The highest mean contents were found in dried fruits samples (33.15 μg kg^{-1}), while lowest mean concentration was detected in cereal samples with 4.03 μg kg^{-1}.

ENNs were also previously reported in TDS performed in the Netherland, where ENB and $ENNB_1$ were found in apple sauce and cereal-based composites at mean concentrations of 6.6 and 58 μg kg^{-1} respectively and BEA was detected in dried fruits and tomato products reaching 2 μg kg^{-1} (López et al., 2016).

The average daily exposure to ENNs was estimated to range between 0.012 ng kg^{-1} bw day^{-1} through dried fruits for ENNB to 2.36 ng kg^{-1} bw day^{-1} through cereal ENNB, while the estimated daily intake for BEA reached 7.10 ng kg^{-1} bw day^{-1} through meat consumption (Table 5).

3.7. Alternaria toxins

Alternaria toxins were detected mainly in beverages samples (Table 4). AME was detected in 60% of wine samples and 21% of fruit juices at mean concentration of 7.55 and 8.54 μg L^{-1} respectively. AOH was detected in 25% of tea beverages and 90% of beer samples at mean contents up to 22.01 μg L^{-1}. López et al., (2016) detected also *Alternaria toxins* in TDS performed in the Netherland where AME, AOH were detected in a large variety of analysed wine, tomato products and dried fruits up to 8.1 μg kg^{-1}.

The average daily exposure to AOH and AME was estimated to range between 0.15 ng kg^{-1} bw day^{-1} day by tea to 12.75 ng kg^{-1} bw day^{-1} through beer consumption (Table 5). Sprong et al., (2016) reported in The Netherlands TDS, daily intake in percentile 50 between 2.30 to 8.3 ng kg^{-1} bw day^{-1} kg bw day for AOH by wine and tomato products consumption and 2.6 to 13.9 ng kg^{-1} bw day^{-1} for AME through pasta and dried fruits consumption.

Table 5: Estimated dietary exposure to mycotoxins by the Valencia´s population in the different scenarios proposed

	Cereals		Vegetables		Fruits		Dried fruits		Legumes		Meat		Fish		Beer		Wine		Fruit juice		Coffee		Tea		Milk	
Mycotoxin	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB
AFB_1	-	-	-	-	-	-	0.0023	0.003	-	-	-	-	-	-	-	-	-	-	0.14	0.14	-	-	-	-	-	-
AFB_2	-	-	-	-	-	-	0.008	0.015	-	-	-	-	-	-	0.43	0.45	-	-	0.19	0.19	-	-	1.73	1.77	-	-
AFG_1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.18	0.25	-	-	-	-	-	-	0.05	0.06	-	-
AFG_2	0.03	0.05	-	-	-	-	-	-	0.06	0.09	0.24	0.26	-	-	-	-	-	-	0.06	0.06	-	-	0.17	0.20	-	-
AFM_1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.002	0.003
AME	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.85	0.89	0.47	0.48	-	-	-	-	-	-
AOH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12.72	12.75	0.61	0.62	0.20	0.24	-	-	0.15	0.18	-	-
BEA	0.21	0.23	0.06	0.08	-	-	0.098	0.10	-	-	7.09	7.10	-	-	-	-	-	-	-	-	-	-	-	-	-	-
DAS	-	-	-	-	-	-	-	-	-	-	-	-	0.18	0.21	-	-	-	-	-	-	-	-	-	-	-	-
DON	2.9	2.92	3.06	3.08	0.034	0.04	-	-	0.2	0.4	1.87	1.90	1.53	1.55	4.64	4.71	1.03	1.07	-	-	-	-	-	-	-	-
3ADON	0.06	0.08	-	-	-	-	-	-	-	-	-	-	0.19	0.21	-	-	-	-	-	-	0.02	0.04	0.05	0.12	-	-
15ADON	0.18	0.2	0.54	0.57	-	-	-	-	0.13	0.17	-	-	0.45	0.47	-	-	0.23	0.26	-	-	0.02	0.03	0.12	0.16	-	-
ENNA	-	-	-	-	-	-	-	-	-	-	-	-	0.29	0.31	-	-	-	-	-	-	-	-	-	-	-	-
$ENNA_1$	0.13	0.15	-	-	-	-	0.017	0.018	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
ENNB	2.33	2.36	0.06	0.08	-	-	0.012	0.06	-	-	0.11	0.12	-	-	-	-	-	-	-	-	0.07	0.08	-	-	-	-
$ENNB_1$	-	-	0.06	0.09	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HT-2	3.45	3.47	2.54	2.56	2.86	2.89	0.30	0.31	0.89	0.90	1.45	1.47	-	-	0.92	1.23	0.10	0.11	0.62	-0.66	-	-	-	-	-	-
NEO	0.76	0.79	1.04	1.05	0.76	0.85	0.17	0.18	0.16	0.18	-	-	0.27	0.29	0.45	0.49	1.45	1.47	-	-	1.17	1.19	-	-	-	-
NIV	0.48	0.51	-	-	-	-	5.67	5.68	-	-	1.74	1.76	-	-	0.81	0.90	0.30	0.37	-	-	-	-	-	-	-	-
OTA	-	-	-	-	-	-	0.006	0.007	-	-	3.20	3.23	0.04	0.05	0.28	0.31	0.08	0.12	0.26	0.27	0.11	0.14	-	-	-	-
PAT	-	-	-	-	0.17	0.26	0.10	0.12	-	-	-	-	-	-	0.45	0.46	3.68	3.70	3.43	3.49	-	-	-	-	-	-
STG	-	-	-	-	-	-	0.001	0.005	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T-2	1.68	1.7	-	-	-	-	-	-	-	-	-	-	-	-	0.07	0.14	-	-	-	-	-	-	-	-	-	-
ZEA	0.34	0.36	-	-	0.13	0.22	-	-	-	-	-	-	-	-	0.17	0.24	-	-	-	-	-	-	-	-	-	-
α-ZAL	-	-	-	-	0.009	0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-ZAL	-	-	1.74	1.76	0.3	0.36	0.13	0.14	1.0	1.03	-	-	-	-	5.12	5.26	-	-	0.07	0.09	-	-	1.36	1.49	-	-
β-ZOL	-	-	-	-	0.009	0.094	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 6: Risk characterization-derived mycotoxin intake in the population of the region of Valencia

Mycotoxin	Cereals		Vegetables		Fruits		Dried fruits		Legumes		Meat		Fish		Beer		Wine		Fruit juice		Coffee		Tea	
	TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)		TDI (%)	
	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB
DON	0.29	0.29	0.30	0.31	0.003	0.004			0.02	0.04	0.18	0.19	1.53	1.55	0.46	0.47	0.10	0.11	-	-	-	-	-	-
3ADON	0.006	0.008	-	-	-	-	-	-	-	-	-	-	0.19	0.21	-	-	-	-	-	-	0.002	0.004	0.005	0.01
15ADON	0.01	0.02	0.054	0.057	-	-	-	-	0.01	0.02	-	-	0.45	0.47	-	-	0.023	0.026	-	-	0.002	0.003	0.01	0.03
HT-2	3.45	3.47	2.54	2.56	2.86	2.89	0.3	0.31	0.89	0.90	1.45	1.47	-	-	0.92	1.2	0.09	0.11	0.62	0.66	-	-	-	-
NIV	0.04	0.05	-	-	-	-	0.47	0.49	-	-	0.14	0.15	-	-	0.81	0.90	0.02	0.03	-	-	-	-	-	-
OTA	-	-	-	-	-	-	0.03	0.04	-	-	19.0	19.0	0.34	0.36	1.63	1.84	0.48	0.70	1.53	1.58	0.65	0.82	-	-
PAT	-	-	-	-	0.04	0.06	0.02	0.03	-	-	-	-	-	-	0.11	0.12	0.92	0.93	0.84	0.85	-	-	-	-
T-2	1.68	1.70	-	-	-	-	-	-	-	-	-	-	-	-	0.07	0.14	-	-	-	-	-	-	-	-
ZEA	0.13	0.14	-	-	0.070	0.089	-	-	-	-	-	-	-	-	0.06	0.08	-	-	-	-	-	-	-	-
α-ZAL	-	-	-	-	0.003	0.0032	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
β-ZAL	-	-	0.69	0.70	0.120	0.125	0.05	0.06	0.40	0.41	-	-	-	-	2.05	2.06	-	-	0.02	0.04	-	-	0.54	0.60
β-ZOL	-	-	-	-	0.030	0.032	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.8. Occurrence mycotoxins in food from Mediterranean diet

To have an indication of how mycotoxins were spread out among analysed samples and to compare distribution many food groups selected, a boxplot multivariate statistical technique representing series of numerical data through its quartiles was used. Results interpretation in terms of variables as mean mycotoxins contents by identifying possible significant differences, asymmetries, atypical values and homogeneity with logarithmic transformation on the values of mycotoxins contents showed that the diet components resulted on different behaviours according to the studied mycotoxins and revealed also noticeable pairing of some mycotoxins in some food groups. The variables with the highest probability of pairing were HT-2 and βZAL with legumes, OTA with meat, DON, NEO with wine, AOH, βZAL and DON with beer, PAT with fruit juice, BEA with coffee and finally NIV and dried fruits (Figure 1).

Regarding data obtained in the estimation of the coefficients on the tandem mycotoxins and food groups, considerable value was found for NIV. This means that the mycotoxin contribution of dried fruit on diet is not constant but depends on the presence or absence of NIV. Contamination of dried fruits by NIV will have a positive effect on the average compared to the reference level, keeping the rests of the variables constant.

Even some studies have already been conducted on Mediterranean diet related to flavonoids and nutrients intake (Vasilopoulou et al., 2005; Tresserra-Rimbou et al., 2014; Davis et al., 2015), to the best of our knowledge, no data were available before this study on mycotoxins intake and Mediterranean diet.

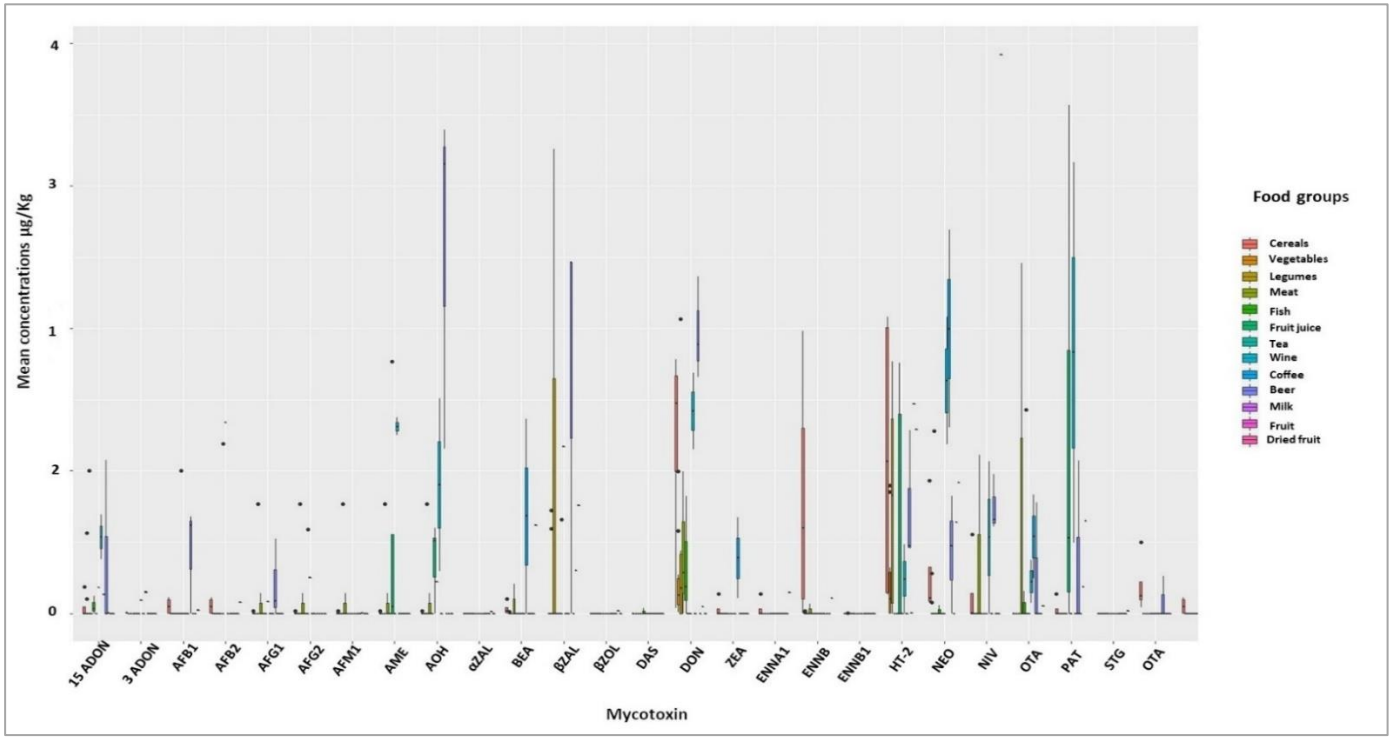

Figure 1: Boxplot of the average levels µg kg^{-1} to mycotoxins in different food groups.

4. Conclusions

In this study, 26 out of 31 mycotoxins tested were found mainly in beers, cereals and dried fruits at low concentration levels. The estimated dietary exposure of adult population to the mycotoxins analysed was found well below the current recommendations. The findings indicate that the mycotoxins levels measured in this study were unlikely to pose notable health risk to population living in Valencia. By identifying the principal food groups that contribute to mycotoxins exposure, it appears that cereals, dried fruits, beers and meat should be included in monitoring programs and particular attention should be paid to NIV, DON and HT-2 screening.

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Conflict of interest

The authors declare no conflict of interest and informed consent was obtained from all individual participants included in the study.

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4. DISCUSIÓN GENERAL

4. DISCUSIÓN GENERAL

De acuerdo a los objetivos planteados, en el trabajo de investigación llevado a cabo a lo largo de esta Tesis Doctoral se ha realizado la validación de los métodos analíticos, basados en LC y GC acoplados a espectrometría de masas en tándem para la determinación de micotoxinas en distintas matrices alimentarias. Para realizar el EDT se han tomado muestras de alimentos representativos de la dieta Mediterránea consumidos por la población valenciana, se ha evaluado la exposición de los consumidores, y se ha realizado la evaluación del riesgo.

4.1. Validación de la metodología analítica

La metodología analítica para la determinación de micotoxinas en alimentos ha sido optimizada y validada de acuerdo con la recomendación EC 657/2002, donde se establece que una sustancia puede ser identificada por LC-MS/MS y CG-MS/MS con al menos dos transiciones, una de cuantificación y otra de confirmación. La fragmentación de los iones precursores ha sido optimizada mediante la aplicación de diferentes energías de colisión, seleccionando las dos transiciones con mayor proporción entre la señal cromatográfica y el ruido de fondo (S/N) y con la mínima interferencia de los componentes de la matriz. Los criterios de identificación empleados han sido el tiempo de retención, la masa exacta de la micotoxina (o su correspondiente aducto) y su patrón de fragmentación típico. La cuantificación de cada micotoxina se ha realizado con la transición de cuantificación, ratificada por la transición de confirmación.

La determinación de AFs (B_1, B_2, G_1, G_2), ENs (A, A_1, B, B_1), BEA, FBs (B_1, B_2) AOH, AME, STG y OTA se realiza por LC-MS/MS con trampa de iones (IT) en modo positivo. La separación se lleva a cabo con columna Gemini C_{18} (150 x 4.6 mm, 5 µm), con metanol y agua acidificados en ácido fórmico al 0.1% como fase móvil. El método optimizado permite la separación de las 15 micotoxinas en un tiempo total de 20 min.

La determinación de AFM_1 se realiza mediante LC-MS/MS en tiempo de vuelo (qTOF), en modo positivo, que permite identificar con la masa exacta de la micotoxina (o su correspondiente aducto) con hasta cinco cifras decimales, empleando columna Gemini C_{18} (150 x 4.6 mm, 5 µm), utilizando acetonitrilo y agua acidificados en ácido fórmico al 0.1% como fase móvil.

La determinación de TCs: DON, 3ADON, 15ADON, NIV, NEO, DAS, T-2, HT-2 FUS-X, las micotoxinas ZON y sus metabolitos: αZAL, βZAL, αZOL, αZOL y PAT se realiza por GC-MS/MS con triple cuadrupolo (QqQ). Previo al análisis es necesario realizar un proceso de derivatización mediante una mezcla de agentes sililantes (BSA + TMCS + TMSI). La derivatización tiene por objetivo la eliminación de los hidrógenos activos de los grupos reactivos permitiendo un aumento de la volatilidad de los analitos. Como productos de la reacción se generan derivados trimetilsililo (TMS), los cuales presentan una mayor estabilidad térmica respecto al compuesto original. La separación se lleva a cabo con una columna capilar HP-5MS (30m x 0,25 mm x 0,25 µm) en modo de vaporización programable por temperatura (PTV) a 250 ° C empleando helio como gas transportador a una presión fija de 20.3 psi. El método optimizado permite la determinación de 15 micotoxinas en un tiempo total de 23.15 min.

Para la extracción se han utilizado diferentes procedimientos, optimizando en todos los casos los parámetros analíticos de límite de detección (LOD), límite de cuantificación (LOQ), linealidad, recuperación y efecto matriz.

4.1.1. Extracción en matrices sólidas

La extracción por QuEChERS permite disminuir el uso de disolventes respecto a los métodos tradicionales de extracción y purificación. También permite disminuir el tiempo de análisis, a la vez que ofrece resultados seguros, por lo que es una alternativa eficaz y robusta para su utilización en matrices alimentarias complejas.

El método QuEChERS se ha empleado para la extracción de 31 micotoxinas en cereales, vegetales, frutas, frutos secos, legumbres, carne y pescado. Debido al elevado contenido de grasas y/o aceites en las muestras, se adiciona 2 % ácido fórmico al agua para facilitar la separación de los compuestos. Se usan 2g de muestra, previamente triturados, 10 mL de agua y de acetonitrilo. En la fase de purificación se utiliza C_{18} para eliminar lípidos, proteínas y carbohidratos. Se han obtenido recuperaciones para las micotoxinas estudiadas en todas las matrices ensayadas entre 60 y 119 % y valores de LOD entre 0,15 y 3 µg/kg y LOQ entre 0.5-10 µg/kg. El efecto matriz, evaluado para cada tipo de matriz alimentaria estudiada, muestra un aumento de la señal en pescado, especialmente para las micotoxinas ZON, 3ADON y NEO con valores hasta 158% y una supresión de la señal para AFG_1, AFG_2 y FB_2 en legumbres.

4.1.2. Extracción en matrices líquidas

Para la determinación de micotoxinas en matrices líquidas se han utilizado diferentes métodos de extracción: DLLME, extracción líquido-líquido y QuEChERS.

La determinación de 30 micotoxinas en muestra de cerveza vino, cava, sidra, zumo y té, se realiza mediante la extracción DLLME. 5 mL de muestra previamente desgasificada (para las matrices cerveza, cava y sidra), se adicionan los disolventes dispersantes (metano/acetonitrilo) y disolventes extractantes (acetato de etilo/cloroformo) ofreciendo ventajas de ser eficaz, sencilla y rápida. La linealidad es adecuada para todas las micotoxinas r2 >0.990. LOD y LOQ oscilan entre 0.03-2.34 μg/L y 0.1-7.81 μg/L, respectivamente. El EM oscila entre 71% a 140%, mostrando el mayor aumento de la señal para AME en muestras de té.

Para análisis de micotoxinas en muestras de café se ha utilizado un método extracción líquido-líquido. Debido a la composición de alto contenido de pigmentos que se encuentran en el café se ha realizado un proceso de clarificación previo a la extracción utilizando Solución Carrez, que permite la precipitación de proteínas y eliminación de turbidez. A 20 mL de muestra clarificada se añaden los disolventes metanol, acetonitrilo y mezcla de acetato de etilo con ácido fórmico. Las curvas de calibración muestran una linealidad adecuada (r2>0.995). Los porcentajes de recuperación oscilan entre 79%-127%, correspondiendo el valor más alto para AOH. Los valores de LOD y LOQ oscilan entre 0.03-3 μg/L y 0.1-10 μg/L, respectivamente. El efecto matriz oscila de 44%-109%, la mayor supresión se observa para STG.

Con respecto al análisis de AFM_1 en leche se ha utilizado un método QuEChERS. Para ello se ha utilizado 10 mL de leche con adición de disolvente acetonitrilo. En la fase de purificación se utiliza PSA y C_{18} para eliminar interferencias como proteínas presentes en la matriz. Las recuperaciones obtenidas en rango de 71% -84%. La linealidad es adecuada, con valores de (r2>0.995). Los valores de LOD de 0.009% y LOQ de 0.03%. La evaluación del efecto matriz muestra una supresión de la señal del 56%.

Por lo tanto, para la cuantificación de micotoxinas en todas las muestras analizadas se utilizan extractos de las matrices adicionadas con soluciones preparadas evaluadas para compensar las variaciones de la señal producidas por el efecto matriz y permitir una cuantificación adecuada.

4.2. Presencia de micotoxinas en alimentos.

Una vez validado el método de extracción de micotoxinas, se procedió a su análisis en alimentos. Se seleccionaron los grupos de alimentos más consumidos durante la semana, incluidas las bebidas y se distinguieron de acuerdo con los componentes principales de la dieta mediterránea (Tabla 1) seguida principalmente por la población valenciana, que se caracteriza por un modelo nutricional que incorpora principalmente alimentos de origen vegetal, como frutas y verduras, cereales, legumbres y frutos secos, y establece un consumo de pescado y aves al menos dos veces por semana y limitado de carne roja. La selección de los alimentos, se realiza en base a alimentos consumidos al menos dos veces por semana.

Tabla 1: Clasificación de las muestras de alimentos analizados

Grupos de alimentos	Alimentos	Muestras analizadas (N)
Cereales	Arroz	38
	Pasta	22
	Pan	15
	Platos preparados	13
Vegetales	Calabacín	8
	Berenjena	9
	Zanahoria	5
	Cebolla	11
	Pimientos	20
	Maíz dulce	6
	Setas	6
	Tomate	16
	Patatas	54
	Sopa de vegetales	6
Frutas	Manzana	10
	Naranja	10
	Uva	10
	Fresa	10
Frutos secos	Almendras	5
	Nueces	5
	Dátiles	5
	Cacahuetes	5
	Higos	5
	Pasas	5
Legumbres	Alubias	2
	Lentejas	4
	Garbanzo	2
	Guisantes	7
	Judías	9
Carne	Ternera	4
	Cerdo	9
	Pollo	9
Pescado y mariscos	Pescado	29
	Calamar	14
	Gambas	10
Cerveza	Cerveza	20
	Cerveza sin alcohol	10
	Cerveza con limón	10
Vino	Vino	20
	Vino sin alcohol	10
	Vino con limón	10
Otras bebidas alcohólicas	Cava	10
	Sidra	10

	Sidra sin alcohol	10
Zumo	Manzana	10
	Piña	7
	Melocotón	7
	Mezcla de frutas	7
	Naranja	7
Café	Cafe	20
Té	Té	20
Leche	Leche	20
TOTAL		596

4.2.1. Presencia de micotoxinas en matrices sólidas

- *Cereales*

Se han analizado un total de 88 muestras de cereales. Las micotoxinas detectadas son AFG_2, ZEA, los TCs DON, 3ADON, 15ADON, HT-2, T-2, NEO y NIV, y las micotoxinas emergentes ENB, ENA_1 y BEA. La prevalencia oscila del 3% (3ADON) al 60% (DON). Respecto a la legislación, ninguna muestra supera los límites máximos: 4 μg/kg para AFs, 750 μg/kg para DON y 75 μg/kg para ZEA (EC, 1881/2006).

De los TCs, T-2 presenta la concentración mayor (19.70 μg/kg), con incidencia del 10% y HT-2 presenta incidencia del 40% con contenidos de 9.20 μg/kg. Los estudios disponibles en la bibliografía muestran incidencias y contenidos similares para T-2 y HT-2, de 20% a 70% y de 27 μg/kg a 24.80 μg/kg, respectivamente (Cano-Sancho et al., 2012).

Los contenidos medios de DON son bajos, con media de 4.22 μg/kg. Diferentes estudios muestran incidencias y contenidos muy variables, desde 3% a 100%, y concentraciones de 2.4 a 524.17 μg/kg (Leblanc et al., 2005; Cano-Sancho et al., 2012; Beltrán et al., 2013; Raad et al., 2014; Sirot et al., 2013; Yau

et al., 2016; López et al., 2016), llegando a ser los contenidos mucho mayores que los obtenidos en el presente estudio.

NIV y NEO presentan incidencia de 16% y 44% y concentraciones medias de 4.57 μg/kg y 2.70 μg/kg, respectivamente. Leblanc et al., (2005) y Sirot et al., (2013) encuentran en cereales incidencias inferiores al 5%, pero concentraciones superiores (20 μg/kg) a las obtenidas en nuestro estudio.

Las micotoxinas emergentes $ENNA_1$, BEA y ENNB presentan incidencias de 6, 7 y 39% y concentraciones medias de 2.85, 4.03 y 4.63 μg/kg, respectivamente. López et al., (2016), solo han detectado $ENNB_1$ del total de ENNs, pero a concentraciones mucho mayores (58 μg/kg).

AFG_2 se ha detectado en el 5% de las muestras, con concentración media de 1.64 μg/kg. Yau et al., (2016), encuentran incidencias y contenidos similares, (del 3% y de 0.05 a 0.24 μg/kg, respectivamente). Sin embargo, Huong et al., (2016) describen incidencias de AFG_2 muy elevadas (67%).

ZEA está presente en el 7% de las muestras, con contenidos de 5 μg/kg. Los estudios disponibles en bibliografía muestran incidencias variables, del 2% al 44%, y contenidos similares a los obtenidos en este estudio, de 0.19 a 7.56 μg/kg (Sirot et al., 2013; Cano-Sancho et al., 2016; Yau et al., 2016).

Por lo tanto, las incidencias de micotoxinas en cereales obtenidas en este estudio son, en general, similares a las obtenidas por otros autores en EDT y los contenidos son similares o menores.

- *Vegetales*

Se han analizado un total de 141 muestras de vegetales, detectándose BEA, DON, 15ADON, ENNB, ENNB1, HT-2, NEO y βZAL. Las incidencias son mucho menores que en cereales, del 2% para $ENNB_1$ y 15ADON al 13% para DON. Los contenidos medios de las muestras positivas oscilan de 4.76 a 38.41 μg/kg para BEA y βZAL, respectivamente.

Los contenidos de DON (7.23 μg/kg) son similares a los obtenidos por Sirot et al., (2013) en vegetales (1.5 μg/kg) y, mucho menores a los obtenidos por Cano-Sancho et al., (2012) en maíz dulce (114 μg/kg). Además, estos autores encuentran también altos contenidos de HT-2 (11% incidencia a concentraciones de 31.60 μg/kg), respecto a los nuestros (6% incidencia a concentraciones de 10.34 μg/kg).

βZAL ha sido detectada con incidencia del 7% y contenidos de 38.41 μg/kg. Los resultados disponibles para ZEA y sus metabolitos en vegetales son variables, en general, menores a los encontrados en este estudio: Yau et al., (2016) y Sirot et al., (2013) no detectan ZEA y ninguno de sus metabolitos y Cano Sancho et al., (2012) y Leblanc et al., (2005) encuentran contenidos de 4.9 a 17 μg/kg.

La incidencia y contenidos de BEA en vegetales (7% y 4.76 μg/kg, respectivamente) son similares a los obtenidos por López et al., (2016), en muestras de tomate (2.4 μg/kg).

Las incidencias de micotoxinas en vegetales son bajas y, en general, similares a las obtenidas por otros autores en EDT. Los contenidos son similares o menores, excepto para βZAL.

- *Frutas*

Se han analizado un total de 40 muestras de frutas detectándose DON, HT-2, NEO, PAT, ZEA, α-ZAL, β-ZAL, β-ZOL. Mayor prevalencia presenta HT-2 (40%), y menor α-ZAL y β-ZOL (3%). Los contenidos más altos son de β-ZAL (62.67 μg/kg) y los menores de DON (8.43 μg/kg).

PAT se detecta en el 10% de las muestras, a concentraciones medias de 20.76 μg/kg, resultados similares a los obtenidos en muestras de frutas de Irlanda (21.30 μg/kg) (Food Safety Authority of Ireland, 2016). Por otra parte, Sirot et al., (2013) encuentran valores menores, de 0.04 a 6.09 μg/kg.

- *Frutos secos*

En las 30 muestras analizadas se detectan AFB_1, AFB_2, BEA, $ENNA_1$, ENNB, HT-2, NEO, NIV, OTA, PAT, STG y β-ZAL. La micotoxina detectada con mayor incidencia y concentración es NIV (50% y 212 μg/kg), mientras que presenta menor incidencia STG (7%) y menor concentración AFB_1 (2.30 μg/kg).

Los contenidos de NIV en frutos secos son mucho mayores que los obtenidos por Leblanc et al., (2005) que obtiene concentraciones medias de 16 μg/kg.

Las AFB_1 y AFB_2 presentan incidencias y concentraciones de 10-13% y 2.30-4.75 μg/kg, respectivamente, no superando los límites máximos permitidos

(15 μg/kg) (Comisión Europea, 1881/2006), resultados similares a los obtenidos por Cano-Sancho et al., (2012) con 11% y 20% de incidencia y concentraciones de 2.66 μg/kg y 8.85 μg/kg en muestras de cacahuetes y pistachos. No obstante, en un EDT (FSANZ, 2001) realizado en alimentos de Nueva Zelanda se han obtenido concentraciones medias de AFB_2 y AFB_1 de 6 μg/kg y 38 μg/kg respectivamente en cacahuetes, excediendo los niveles máximos permitidos.

Respecto a la presencia de OTA en frutos secos, diferentes estudios realizados en España, Líbano y Países Bajos (Cano-Sancho et al., 2012; Raad et al., 2014; López et al., 2016), han mostrado contenidos ligeramente inferiores (1.8 μg/kg) a los encontrados en este estudio (3.11 μg/kg).

- *Legumbres*

Se han analizado un total de 24 muestras detectándose las micotoxinas DON, 15ADON, HT-2, NEO, AFG_2 y βZAL. La incidencia menor es de 8% (15ADON y AFG_2) y la mayor de 54% (HT-2) y los contenidos en las muestras positivas de 6.92 a 26.51 μg/kg para DON y β-ZAL, respectivamente.

DON se ha detectado en el 21% de las muestras y presenta contenidos medios inferiores a los obtenidos por otros autores (31.25 μg/kg) (Raad et al., 2014). Por el contrario, los contenidos de AFG_2 (10.39 μg/kg) son mayores a los obtenidos por Yau et al., (2016) y Huong et al., (2016) (1.8-2.90 μg/kg).

- *Carne*

En las 22 muestras de carne de ternera, cerdo y pollo se han detectado DON, HT-2, NIV, ENNB, BEA, AFG_2 y OTA. Las incidencias oscilan entre

5% (BEA) a 18% (DON, NIV y OTA). Los valores medios encontrados desde 1.38 μg/kg para ENNB a 54.09 μg/kg para OTA.

La incidencia de OTA es igual o menor que la mostrada por otros estudios (de 7% a 64%). No obstante, los contenidos son mayores que los obtenidos por otros autores (de 0.01 μg/kg a 2.68 μg/kg) (Leblanc et al., 2005; Sirot et al., 2013; Huong et al., 2016; Yau et al., 2016).

Los contenidos medios de AFG_2 (3.13 μg/kg) son similares a los obtenidos por Huong et al., (2016) (4 μg/kg).

- *Pescados*

En las 54 muestras de pescado, calamares y gambas se han detectado DON, 3ADON, 15ADON, NEO, ENNA, OTA y DAS. Los resultados obtenidos muestran una incidencia desde 4% (NEO, ENA, OTA y DAS) hasta 19% (DON), y concentraciones en rango de 4.30 μg/kg a 12.43 μg/kg, para OTA y NEO, respectivamente.

Existen escasos estudios sobre la presencia de micotoxinas en pescado y marisco. Un EDT realizado en Viet Nam encuentra mayores incidencias (50% - 100%) y similares concentraciones de OTA (2.24 μg/kg y 4.85 μg/kg). No obstante, a diferencia de estos autores, en nuestro estudio no se detectan AFs en muestras de pescado y mariscos (Huong et al., 2016).

4.2.2. Presencia de micotoxinas en matrices líquidas

- *Cerveza*

Se han analizado 40 muestras clasificadas en cerveza con alcohol, sin alcohol y cerveza con limón, detectándose AFB1, AFG1, AOH, DON, 15ADON, T-2, HT-2, NIV, NEO, ZON, βZAL, OTA y PAT. AOH es la micotoxina más prevalente (90%). La concentración mayor es de PAT (43.18 μg/L), y las menores incidencias T-2 (3%) y concentraciones para AFG_1 (1.16 μg/L).

Estos resultados están de acuerdo con los publicados por Prellé et al. (2013) y Bauer et al., (2016), que detectan AOH con alta incidencia (del 30 al 100%), y con concentraciones variables (0.56-23.2 μg/L), similares a las obtenidas en nuestro estudio (22.01 μg/L).

Así mismo, incidencias similares de DON (56% hasta 87%) y concentraciones muy variables, entre 2.1 y 73.2 μg/L, han sido publicadas por otros autores (Papadopoulou-Bouraoui et al., 2004; Rodríguez-Carrasco et al. 2015; Juan et al., 2017; Bertuzzi et al. 2011; Bogdanova et al., (2018), llegando a ser los contenidos mucho mayores que los obtenidos en el presente estudio (8.98 μg/L).

La incidencia de AFB_2 y AFB_1 es de 35 a 60%, respectivamente, con contenidos de 1.17 y 1.47 μg/L. Incidencias y concentraciones similares (64 % y 0.07 a 10.60 ng/L) han sido obtenidas por otros autores (Burdaspal y Legarda 2013; Mabli et al., 2007; Benesova et al., 2012). Sin embargo, Molina-García et al., (2011) y Matumba et al., (2011) han obtenido contenidos muy elevados (35,5 μg/L).

- *Vino*

Se han analizado un total de 40 muestras clasificadas en vino con alcohol, sin alcohol y con limón. Las micotoxinas detectadas son AOH, AME, βZAL, DON, 15ADON, HT-2, NIV, NEO, OTA y PAT. El 88% de las muestras de vino presenta contaminación con al menos una micotoxina. AOH es la micotoxina más prevalente, con una incidencia del 52%, β-ZAL y HT-2 las menos prevalentes (5%). Los contenidos mayores son de β-ZAL y PAT con 25.86 y 24.64 μg/L, respectivamente y la concentración más baja, de 1.13 μg/L en OTA, no superandose el límite máximo establecido para OTA en vino de 2 μg/kg (EC, 1881/2006).

Estos resultados están en concordancia con datos publicados en varios estudios sobre la alta incidencia de AOH y AME en muestras de vino: AME hasta 93% y valores medios de hasta 1.0 μg/L (Zwickel et al., 2016; Asam et al., (2010) y AOH 60% y concentraciones entre 0.03 y 7.7 μg/L (Zwickel et al., 2016; Scott et al., 2006; Scussel et al., 2012; Asam et al., 2010).

La presencia de OTA en vino se ha descrito en diferentes estudios con incidencias similares (50% -100%) a las nuestras (45%), y concentraciones mayores, de hasta 8,6 μg/L (Lasram et al., 2013; Ostry et al., 2015; Rodríguez-Cabo et al., 2016; De Jesus et al., 2017; Mariño-Repizo et al., 2017). Además, en muestras de vino dulce, los contenidos de OTA publicados por otros autores llegan a valores de 15,25 μg/L (Mateo et al., 2007), superando los límites máximos.

Los TCs en muestras de vino presentan incidencias de 10-60%, a concentraciones entre 8.85 y 21.26 μg/L, identificándose 15ADON, DON, HT-2, NIV y NEO. Limitados estudios han incluido la determinación de

micotoxinas *Fusarium* en vino. Al-Taher et al., (2013) identificaron T-2 en el 11% de las muestras de vino con niveles medios de 0,3 μg/L.

- *Cava y sidra*

Se han analizado un total de 10 muestras de cava y 20 muestras de sidra con alcohol y sin alcohol. A pesar de la elevada prevalencia de OTA en muestras de cava (80%), la concentración encontrada es la menor (1.36 μg/L). AOH presenta la incidencia más baja (10%) y la concentración más alta (21.56 μg/L).

En muestras de sidra, βZAL presenta la mayor incidencia y concentración media con 60% y 61.48 μg/L, respectivamente. La incidencia más baja se encontró para PAT (20%) y los contenidos más bajos para ZEA con 11.53 μg/L. En la sidra sin alcohol, solo se detectó PAT con una incidencia del 30% y una concentración media de 35.86 μg/L.

Existen pocos estudios que analicen PAT en cava y sidra. Harris et al., (2009) detectan PAT en el 19% de muestras de sidra a concentraciones medias de 36.9 μg/L y Leblanc et al., (2005) cuantificaron el PAT en el 50% de las muestras de bebidas alcohólicas, incluida la sidra, a niveles medios de 19.50 μg/L, similares a los obtenidos en el presente estudio, que en sidra con alcohol se detecta PAT con una incidencia del 20% y una concentración media de 25.79 μg/L.

- *Zumos de fruta*

En las 38 muestras de zumo las micotoxinas detectadas son AFB_1, AFB_2, AOH, AME, HT-2, OTA, PAT y βZAL. PAT es la micotoxina que presenta mayor incidencia y concentración media con 37% y 28.63 μg/L, mientras que,

βZAL solo se encuentra en el 5% de las muestras en una concentración media de 23.22 μg/L.

Los estudios presentes en bibliografía en diferentes tipos de zumos de fruta muestran incidencias menores de PAT (de 5% a 14%) pero a concentraciones muy superiores a las encontrados en este estudio, desde 33.7 a 1234 μg/L (Cho et al., 2010; Lee et al., 2014; Li et al. 2018). Respecto a la legislación, los valores de PAT detectados en muestras de zumos no superan el límite máximo destinado al consumo de zumo de frutas a base de manzana de 50 μg/kg (EC, 1881/2006).

OTA presenta una incidencia del 13%, similar a la descrita por otros autores (hasta 25%), que también describen concentraciones bajas que oscilan de 0.057 a 1.9 μg/L (Akdeniz et al., 2013; Asadi et al., 2018). Con relación a la legislación, el zumo de uva es el único tipo de zumo donde la Comisión Europea ha establecido un límite para OTA (2 μg/kg), por lo que a pesar de que en el presente estudio las muestras contaminadas sobrepasan este límite (6.25 μg/L), ninguna muestra positiva es de zumo de uva.

Las micotoxinas del grupo TCs están generalmente presentes en granos y productos a base de granos y, muy raramente en zumos. Sin embargo, López et al., (2016) encuentran la toxinas T-2 en el de zumo de manzana a 14 μg/L y de las toxinas HT-2 por debajo de LOQ (<20 μg/L). Esos autores atribuyen los contenidos a la contaminación cruzada durante la fabricación del zumo de manzana. En el presente estudio se detecta HT-2 en el 16% de las muestras a concentraciones de 19.53 μg/L.

- *Café, té y leche*

Se han analizado 20 muestras de café positivas para BEA, 3ADON, ENNB, NEO, y OTA. NEO presenta mayor incidencia y concentración media 50% y 28.61 μg/L, respectivamente. 3ADON presenta la menor incidencia (10%), mientras que la concentración mas baja se ha detectado para ENNB con 2.14 μg/L. Con respecto a la legislación, OTA es la única micotoxina que tiene establecido un límite máximo en café, de 10 μg/kg. Ninguna de las muestras analizadas supera el límite (EC, 1881/2006).

Las incidencias y contenidos de OTA en café presentados por otros autores son similares (hasta 49% a concentraciones que oscilan de 0.04 a 2.10 μg/L) (Leblanc et al., 2005; Raad et al., 2014; Cano-Sancho et al., 2012; López et al., 2016).

Las micotoxinas detectadas en 20 muestras de té son AFB_2, AFG_1, AFG_2, AOH, 3ADON y βZAL. AFB_2 es la micotoxina más prevalente con 35% y la concentración más alta se ha detectado para βZAL con 35.61 μg/L. Sin embargo, 3ADON solo se ha detectado en 10% y la concentración más baja se encontrado para AFG_1 con 1.33 μg/L.

En leche solo se detecta la presencia de AFM_1, con incidencia del 20% y concentración media de 0.03 μg/L. La incidencia es menor y los contenidos son similares a los obtenidos por otros autores, con incidencias hasta 94% a concentraciones que oscilan de 0.03 a 0.22 μg/L (Urieta et al., 1996; Cano-Sancho et al., 2012; Raad et al., 2014).

4.3. EDT: Evaluación del riesgo por exposición a micotoxinas

La exposición de la población a micotoxinas por los alimentos depende de la composición de la dieta en la zona geográfica considerada y de la contaminación por micotoxinas de los alimentos consumidos por dicha población. Se ha realizado una evaluación de la exposición basada en el enfoque determinista. Para ello, se calcula la ingesta diaria estimada (IDE) a partir de los datos de consumo de alimentos y de los contenidos de micotoxinas en los mismos.

Los datos sobre consumo real de alimentos se han obtenido a partir de la base de datos de consumo en hogares españoles del Ministerio de Agricultura, Alimentación y Medio Ambiente (MAPAMA, 2017), expresado en Kg de peso corporal (Kg pc), considerando el peso medio de la población adulta de 70 Kg.

Para evaluar la exposición a micotoxinas de la población valenciana se toman los datos de todas las muestras analizadas. Para ello, se han considerado dos escenarios posibles: nivel bajo de exposición (LB), asignando valor de cero a aquellas muestras cuyos contenidos de micotoxinas son menores que el LOD o LOQ y nivel alto de exposición (UB), asignando el valor del LOD a aquellas muestras cuyos contenidos de micotoxinas son menores que el LOD o LOQ (EFSA, 2010).

Se ha realizado la evaluación del riesgo comparando los valores de IDE obtenidos con los valores de ingesta diaria tolerable (IDT) establecidos (Comité Mixto FAO/OMS de Expertos en Aditivos Alimentarios, JECFA; Comité Científico sobre la Alimentación Humana, SCF; Autoridad Europea de Seguridad Alimentaria, EFSA). La caracterización del riesgo se expresa como % TDI. Respecto a las micotoxinas que aún no tienen establecida la IDT, se ha

calculado únicamente la IDE. Para las AFs, micotoxinas carcinógenas genotóxicas, se aplica el principio ALARA (As Low As Reasonably Achievable), es decir, tan bajo como sea razonablemente aceptable.

Las IDE obtenidas para las AFs varían, considerando los escenarios LB y UB, desde 0,002 – 0.003 ng kg^{-1} bw $día^{-1}$ para los frutos secos y leche a 1,73-1.7 ng kg^{-1} bw $día^{-1}$ por consumo de té, respectivamente. Estos resultados son similares a los obtenidos por Huong et al., (2016) y Yau et al., (2016) (entre 0.1 y 2.8 ng kg^{-1} bw $día^{-1}$) en población de Viet Nam y China, respectivamente por consumo de leguminosas y a los obtenidos por Cano-Sancho et al., (2012) y Raad et al., (2014) (entre 0.045 y 0.182 ng kg^{-1} bw $día^{-1}$) por consumo de frutos secos en España y el Líbano, respectivamente. No obstante, las IDE de cereales (0.03-0.05 ng kg^{-1} bw $día^{-1}$) son menores que las descritas en la bibliografía para cereales y productos a base de cereales (de 0,52 a 22 ng kg^{-1} bw $día^{-1}$) (Huong et al., 2016; FSAI, 2016; Raad et al., 2014).

La caracterización del riesgo para OTA varía del 0,03% al 19.0% para frutos secos y carne, respectivamente. Esta evaluación del riesgo es igual o menor a la obtenida por otros autores, que muestran resultados para cereales y carne del 0.01% a 29.41%, respectivamente (Leblanc et al., 2005; Sirot et al., 2013; Huong et al., 2016; Raad et al., 2014; Cano-Sancho et al., 2012; Tam et al., 2011; FSAI, 2016 y Sprong et al., 2016).

Para los TCs el menor valor de riesgo se obtiene para 15ADON (0.01%) y el mayor para HT2 (3.47%), ambos en cereales, resultados acordes a los obtenidos por Sirot et al., (2013) en arroz (0.23%) y Leblanc et al., (2005) en cereales, nueces y oleaginosas (2.30%).

Respecto a ZEA y sus metabolitos, el riesgo oscila de 0.003% para αZAL en frutas a 2.06% para βZAL en cerveza. Diferentes estudios han estimado un riesgo similar, de 0.07 % a 4.01% por cereales o productos a base de cereales y frutos secos (Tam et al., 2011; Huong et al., 2016; Leblanc et al., 2005; Cano-Sancho et al., 2012; Sirot et al., 2013; Yau et al., 2016).

PAT oscila de 0,02% en frutos secos a 0,93% en vino.

Las micotoxinas emergentes no tienen establecida IDT, por lo que la EDI obtenida para las ENNs oscila de 0,01 ng kg^{-1} bw $día^{-1}$ en frutos secos a 2.36 para la ENNB por consumo de cereales. La EDI de BEA mayor es de 7.10 ng kg^{-1} bw $día^{-1}$ por consumo de frutos secos.

La EDI calculada para las toxinas de *Alternaria* (AOH y AME) varían entre 0,15 ng kg^{-1} bw $día^{-1}$ día por ingesta de té a 12.75 ng kg^{-1} bw $día^{-1}$ para AOH por consumo de cerveza. Resultados similares obtienen Sprong et al., (2016) en EDT en Países Bajos, con IDE de 2.6 a 13.9 ng kg^{-1} bw day^{-1} para AME por consumo de pasta y frutos secos y de 2.30 a 8.3 ng kg^{-1} bw $día^{-1}$ kg bw día para AOH por consumo de vino y productos de tomate.

Por lo tanto, para todas las micotoxinas estudiadas en todos los alimentos se obtienen valores de riesgo iguales o menores que los descritos en los EDT disponibles en la bibliografía.

El análisis estadístico multivariante PCA es una herramienta que permite analizar conjuntamente un número grande de variables a través de un conjunto de indicadores, con el propósito de construir índices que permitan monitorizar y analizar el comportamiento de las micotoxinas y sus contenidos en los diferentes grupos de alimentos estudiados.

El análisis de componentes principales permite realizar combinaciones lineales de las variables estudiadas, número, contenido de micotoxinas y diferentes alimentos y correlacionarlas entre sí, e investigar la contribución de los alimentos estudiados a la ingesta de las micotoxinas identificadas. Este análisis ha mostrado que existe una notable relación de algunas micotoxinas en algunos grupos de alimentos. Así, los resultados mostraron una mayor contribución de legumbres a la ingesta de HT-2 y βZAL, de carne a la ingesta de OTA, de cerveza a la ingesta de AOH, βZAL y DON, de zumos de frutas a la ingesta de PAT, así como de frutos secos a la ingesta de NIV.

Se han realizado algunos estudios en dieta mediterránea relacionada con la ingesta de flavonoides y nutrientes (Vasilopoulou et al., 2005; Tresserra-Rimbou et al., 2014; Davis et al., 2015), pero no hay datos disponibles sobre la ingesta de micotoxinas y la dieta mediterránea.

4.4. Recomendaciones de alimentación saludable – dieta mediterránea

Los datos de consumo real de alimentos en la población no coinciden exactamente con la recomendación de raciones para una alimentación saludable basada en la dieta mediterránea. Es por ello que en el presente trabajo una vez analizados los contenidos de micotoxinas en alimentos que componen el EDT se ha comparado cada grupo de alimento con su recomendación de consumo (SENC, 2018) para observar si existe relación entre los alimentos y las micotoxinas estudiadas.

Posteriormente se ha realizado la evaluación de riesgos basándose en las raciones recomendadas para la alimentación saludable de la dieta mediterránea y

se ha comparado con la evaluación del riesgo realizada con el consumo real de alimentos y con los EDT disponibles en la bibliografía. Los resultados obtenidos se muestran en la Figura 1.

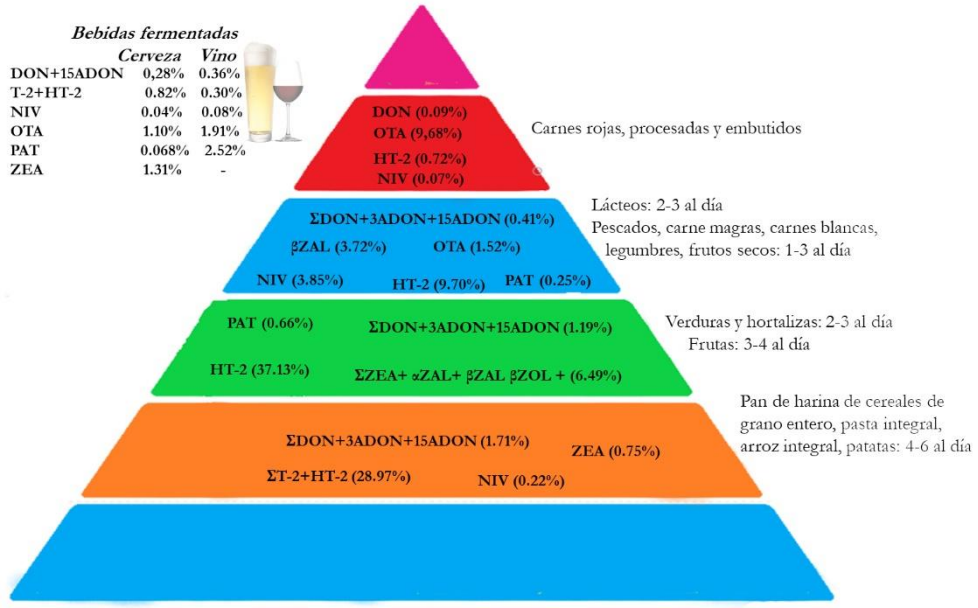

Figura 1: Caracterización del riesgo en los diferentes grupos de alimentos, según la recomendación de alimentación saludable de la dieta mediterránea.

Se ha evaluado si existen diferencias en la evaluación del riesgo considerando la dieta según el consumo actual de la población (EDT, apartado 4.3), respecto al consumo según las recomendaciones de la alimentación saludable de la dieta mediterránea (SENC, 2018). Los resultados obtenidos se muestran en la tabla 2.

Tabla 2: Caracterización del riesgo para micotoxinas considerando el consumo actual (EDT) y el consumo recomendado para la alimentación saludable en la dieta mediterránea.

Micotoxinas	**Consumo actual Rango TDI (%)**	**Recomendaciones consumo DM Rango TDI (%)**
OTA	23.70 -24.34	12.97 – 14.21
ZEA, αZAL, βZAL	4.10 – 4.29	7.31-12.97
PAT	1.93 – 1.99	3.22-3.49
TCs		
DON, 3ADON, 15ADON	3.65-3.82	3.07-4.10
NIV	1.48-1.62	2.87-4.26
T2+HT2	14.87-15.41	57.31-78.05

En general, los datos de la evaluación del riesgo para las micotoxinas estudiadas (ZEA, αZAL, βZAL, PAT, DON, 3ADON, 15ADON y NIV) son similares según el consumo actual de alimentos que según las recomendaciones.

No obstante, el riesgo de OTA que implica la ingestión de carne se ha reducido a la mitad según las recomendaciones de la dieta Mediterránea, ya que el consumo recomendado es inferior. Así mismo, los valores de riesgo obtenidos para OTA son similares a los descritos en otros estudios EDT (Huong et al., 2016; Cano-Sancho et al., 2012; Raad et al. 2014; Yau et al. 2016; Tam et al., 2011; Beltrán et al. 2013; Leblanc et al., 2005; Sirot et al., 2013; FSAI, 2016; Sprong et al., 2016).

Por el contrario, el riesgo de T2 +HT-2 que implica el consumo de cereales, frutas, verduras y legumbres ha aumentado de manera proporcional al aumento del consumo de dichos grupos de alimentos según las recomendaciones de la Dieta Mediterránea. Los datos disponibles en la bibliografía (Cano-Sancho et al.

2012; Yau et al. 2016; Beltrán et al., 2013; Raad et al., 2014; Leblanc et al., 2005; Sirot et al., 2013; Sprong et al., 2016) muestran que en los EDT los valores del riesgo para T2 + HT2 son similares a los obtenidos en este estudio, tanto según el consumo actual que con las recomendaciones dieta mediterránea.

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5. CONCLUSIONES

5. CONCLUSIONES

1. La revisión bibliográfica realizada sobre estudios de dieta total revela que OTA, DON, NIV, T2 y HT2 fueron las micotoxinas más determinadas en los diferentes alimentos analizados. A pesar de la frecuencia relativamente alta de micotoxinas detectadas en los diferentes EDT, los valores de IDE calculados estaban por debajo de los valores de TDI establecidos, excepto para algunas muestras puntuales de cereales y productos de carne donde TCs y OTA sobrepasaron sus respectivos TDI.

2. La metodología analítica basado en GC-MS/MS y LC-MS/MS resultó ser una herramienta muy útil para la determinación de las 31 micotoxinas estudiadas en los diferentes grupos de alimentos obteniendo unos límites de detección y cuantificación del orden de μg/kg.

3. Se han obtenido resultados satisfactorios en cuanto a linealidad, y exactitud para las técnicas de extracción optimizadas; QuEChERS para la extracción de micotoxinas desde cereales, vegetales, frutas, frutos secos, legumbres, carne, pescado y leche, extracción liquida-liquida para el café y microextracción liquida-liquida dispersiva para cerveza, vino, zumos y té.

4. El 49% de los 328 platos de menús listos para su consumo y el 95% de las 110 bebidas contenían al menos una micotoxina siendo el deoxinivalenol la micotoxina con mayor incidencia seguido de NEO, HT2 y AOH en los alimentos listos para su consumo y de AOH, AME, OTA, y PAT en bebidas.

5. Los contenidos medios de las micotoxinas en las muestras positivas fueron inferiores a 50 μg/kg excepto para OTA en carne, B-ZAL en fruta y NIV en frutos secos donde se alcanzaron valores de hasta 212 μg/kg.

6. El análisis estadístico ha mostrado que las micotoxinas halladas varían según los grupos de alimentos resultando en una mayor contribución de legumbres a la ingesta de HT-2 y βZAL, de carne a la ingesta de OTA, de cerveza a la ingesta de AOH, βZAL y DON, de zumos de frutas a la ingesta de PAT, así como de frutos secos a la ingesta de NIV.

7. 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.

8. Los datos de riesgo basándose en las raciones recomendadas para la dieta mediterránea mostraron valores de TDI similares a los obtenidos a través del consumo real de alimentos con justificadas variaciones relacionadas con la reducción de la ración de carne y el aumento de cereales, frutas, verduras y legumbres.

9. La presencia frecuente de micotoxinas aunque a niveles bajos, pone de manifiesto la necesidad de incluir la monitorización de micotoxinas en estudios de dieta total.

ANEXO I

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Review

Dietary exposure assessment to mycotoxins through total diet studies. A review

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ABSTRACT

Mycotoxins are secondary metabolites of fungi that contaminate food in several stages and their increasing presence in food chain demand further control. Assessment of mycotoxins human exposure through processed diet is an important component of food safety strategies. The present review explores and summarises total diet studies (TDS) carried out in different countries focusing on mycotoxins determination. TDS were classified by samples preparation, mycotoxins analysis and dietary exposure evaluation. Most of reviewed TDS performed multi-mycotoxins analysis in composite samples mainly, prepared taking into account local culinary habits. High performance liquid chromatography coupled with fluorescence detector was the predominant and the most sensitive technique used for determination. Ochratoxin A was the most analyzed mycotoxin, followed by trichothecenes, aflatoxins, zearalenone, fumonisins, patulin, enniatins, and beauvericin respectively. *Alternaria* toxins and ergot alkaloids were also included. Food commonly analyzed were cereals, meat, vegetables, fruits, nuts and beverages. The findings in food were in below the current European legislation, except for some sporadic samples of wine and milk meaning less than 1% of total analyzed samples. Dietary exposure was evaluated, through the estimated daily intake mycotoxin evaluation and risk assessment concluded that relatively scarce toxicological concern was associated to mycotoxins exposure. However, a special attention should be paid to meat and cereal products high percentile consumers.

1. Introduction

In the last years, food safety policies based on scientific evidence are being implemented in food industries to protect consumer's interest (Lee et al., 2015). The European Food Safety Authority (EFSA) through the Council and European Parliament established several scientific advice and technical support in all areas impacting on food safety sharing information, data and best practices, for emerging risks identification and coherent communication development (European Commission, 178/2002; EFSA, 2009). The monitoring or surveillance data often focus on individual chemical substances in raw food commodities and don't often provide a direct assessment of dietary population exposure. There is scarce data in literature behaving toward ready-to-eat foods analysis, leading sometimes to an over-estimation of the amount of contaminants presents in food chain (EFSA, FAO & WHO, 2011). In this sense, De Nijs et al. (2016) suggested food consumption surveys, food monitoring studies, duplicate diet studies and total diet studies (TDS) as useful strategies to be used for mycotoxin exposure estimation.

Many countries regularly conduct TDS to evaluate population exposure to different food contaminants. (Moy and Vannoort, 2013; Betsy et al., 2012; Lee et al., 2015). In this sense, international committees EFSA, FAO & WHO (2011) published harmonized guidance document to improve dietary exposure assessment's efficiency and accuracy. Selection of representative food of the overall diet and culinary preparation of ready-to-eat dishes are critical steps in TDS implementation which very often combined analytical results with food consumption data for relatively accurate estimation of dietary exposure to such contaminants. The wide varieties of chemical substances present in diet make the establishment of priorities in TDS approach necessary. In particular, for mycotoxins, analysis is recommended in selected food items (Fig. 1) (WHO, 2002; WHO, 2015).

Mycotoxins are compounds produced by fungi growing mostly on plant products during production and storage. The Rapid Alert System for Food and Feed of the European Union reports mycotoxins on the second position according to the total number of hazard notifications (RASFF, 2017). The most important mycotoxins in food and animal feed are: aflatoxins (AFs), produced by *Aspergillus* species; Ochratoxin

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toxins

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Article

Evaluation of Mycotoxin Residues on Ready-to-Eat Food by Chromatographic Methods Coupled to Mass Spectrometry in Tandem

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Abstract: Simultaneous determination of twenty-seven mycotoxins in ready-to-eat food samples using "Quick Easy Cheap Rough and Safe" (QuEChERS) extraction and chromatographic methods coupled to mass spectrometry in tandem is described in this study. Mycotoxins included in this survey were aflatoxins (B_1, B_2, G_1, G_2), enniatins (A, A_1, B, B_1), beauvericin (BEA), fumonisins (FB_1, FB_2), sterigmatocystin (STG), deoxynivalenol (DON), 3-acetyl-deoxynivalenol (3-ADON), 15-acetyl-deoxynivalenol (15-ADON), nivalenol (NIV), neosolaniol (NEO), diacetoxyscirpenol (DAS), fusarenon-X (FUS-X), zearalenone (ZEA), α-zearalanol (αZAL), β-zearalenone (βZAL), α-zearalenol (αZOL), β-zearalenol (βzol), T2, and HT-2 toxin. The method showed satisfactory extraction results with recoveries ranging from 63 to 119% for the different food matrix samples. Limits of detection (LOD_S) and quantification (LOQs) were between 0.15–1.5 μg/kg and 0.5–5 μg/kg, respectively. The method was successfully applied to the analysis of 25 ready-to-eat food samples. Results showed presence of deoxynivalenol at 36% of samples (2.61–21.59 μg/kg), enniatin B at 20% of samples (9.83–86.32 μg/kg), HT-2 toxin at 16% of samples (9.06–34.43 μg/kg), and aflatoxin G_2 at 4% of samples (2.84 μg/kg). Mycotoxins were detected mainly in ready-to-eat food samples prepared with cereals, vegetables, and legumes, even at levels below those often obtained from raw food.

Keywords: mycotoxins; ready-to-eat food; GC-MS/MS; LC-MS/MS; Valencia

Key Contribution: An analytical method based on QuEChERS extraction was developed to evaluate the presence of twenty-seven mycotoxins in ready-to-eat food by chromatographic methods coupled to mass spectrometry in tandem.

1. Introduction

Mycotoxins are a group of toxic compounds produced as secondary metabolites by certain fungi of the genus *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps* that grow under different climate conditions and have been reported in several food matrices like cereals, peanuts, meat, eggs, milk, and fruits [1,2]. Chronic exposure to some mycotoxins can produce carcinogenic, mutagenic, or teratogenic effects. Aflatoxins (AFs) are indicated as carcinogenic and hepatotoxic and fumonisins (FBs) and ochratoxin A (OTA) are possibly teratogenic, hepatotoxic, and nephrotoxic while patulin (PAT), zearalenone (ZEA), deoxynivalenol (DON), nivalenol (NIV), T-2, and HT-2 toxins are related to toxicological effects mainly on gastrointestinal tract, immune, and endocrine systems [3]. Emerging mycotoxins, such as enniatins ENs and beauvericin, are cytotoxic [4] and their potent cytotoxic activity

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Presence of mycotoxins in ready-to-eat food and subsequent risk assessment

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ABSTRACT

A study on a set of ready-to-eat meals (n = 328) based on cereals, legumes, vegetables, fish and meat was carried out to determine the natural presence of twenty-seven mycotoxins by both liquid chromatography and gas chromatography coupled mass spectrometry in tandem (MS/MS) after QuEChERS extraction. The occurrence of mycotoxins was headed by cereal samples with 35% of samples contaminated by at least one mycotoxin followed by vegetables (32%), legumes (15%) and lastly, 9% of fish and meat samples were contaminated. DON was the most detected mycotoxin in vegetables, meat, fish and cereals with an incidence of 13% 18% 19% and 60%, respectively, and the highest mean levels were found in fish (1.19 μg/kg) and vegetable (1.53 μg/kg), respectively. The highest levels means were for HT-2 toxin ranging from 4.03 to 7.79 μg/kg, in cereal and legume samples respectively. In this last, HT-2 toxin was also the most prevalent (54%). In meat samples, OTA resulted with highest value with 8.09 μg/kg. Likewise, PCA analysis revealed a high correlation between the mycotoxins and the food groups analyzed. The findings indicate that there is no toxicological concern associated with exposure to mycotoxins for consumers as all levels were in accordance with the legislation.

1. Introduction

Ready-to-eat foods are becoming very popular worldwide as they give a consumer the convenience of use without any further preparation. Ready-to-eat foods may also contain organisms or dangerous substances due to the intentional addition of substances such as pesticides, veterinary drugs and other products used in primary production or toxic substances naturally present in foods that have been generated during the processed or storage, such as mycotoxins.

The Rapid Alert System for Food and Feed of the European Union places mycotoxins in the second position according to the total number of hazard notifications (RASFF, 2017).

Mycotoxins are secondary metabolites produced by fungi species belonging to various genera such as *Aspergillus, Penicillium and Fusarium*. There is proven relationship of mycotoxins consumption with teratogenesis, carcinogenesis, and mutagenesis. teratogenesis, carcinogenesis, and mutagenesis. Aflatoxin B_1 have been listed as carcinogen (Group I) by the International Agency for Research on Cancer (IARC, 2012), while Ochratoxin A (OTA) is classified by IARC in the Group 2B as possible human carcinogen (Marín et al., 2013). Reproductive issues were reported in farm animals exposed to zearalenone (ZEA) through the feed (Abrunhosa et al., 2016; Escrivá et al., 2015). Fumonisins are possibly teratogenic, hepatotoxic, and nephrotoxic (Alshannaq and Yu, 2017). Chronic exposure to deoxynivalenol (DON) is associated with delayed growth as well as immunotoxic and hematotoxic effects (Sirot et al., 2013). Nivalenol (NIV), T-2 and HT-2 toxins are related to toxicological effects mainly on gastrointestinal tract, immune and endocrine system (Juan-García et al., 2013). Emerging mycotoxins, such as enniatins (ENNs) and beauvericin (BEA) are cytotoxic and their potent cytotoxic activity was demonstrated in several mammalian cell lines even their toxicity in vivo is still to be proven (Prosperini et al., 2012).

Food consumption of food is the main route of human exposure to mycotoxins (Sirot et al., 2013). Mycotoxins have been investigated in several raw food stuffs (Stanciu et al., 2017; Juan et al., 2017; Danezis et al., 2016; Abrunhosa et al., 2016; Yang et al., 2014).

To determine the intake of mycotoxins on individual foods, which combine food consumption patterns and contamination level are often carried out (Peltonen et al., 2011). Some published studies focused on multi-mycotoxin presence applying total diet studies approach in order to obtain reliable information about the exposure of human population to these toxic compounds (Leblanc et al., 2005; López et al., 2016; Sirot et al., 2013; Raad et al., 2014; Yau et al., 2016; Huong et al., 2016; Tam et al., 2011). Nowadays, very few studies have been carried out on mycotoxins occurrence in cooked food (Sakuma et al., 2013; Carballo et al., 2018). In this sense, the risk assessment purpose of measuring the presence of contaminants is to characterise the distribution of one or

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Dietary exposure to mycotoxins through fruits juice consumption

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Abstract: A study on fruit juice products (apple, pineapple, apricot, orange and pear) was carried out to determine the natural occurrence of fifteen mycotoxins by gas chromatography coupled to tandem mass spectrometry (MS/MS). A developed multi-mycotoxin procedure was carried out by dispersive liquid-liquid microextraction (DLLME). 36% of the analyzed samples presented mycotoxin contamination. PAT was detected in orange, apple, mixed fruits and pineapple juices with prevalence of 86%, 60%, 29%, 14% at mean concentrations of 34.57 µg/L, 33.41 µg/L, 8.59 µg/L, 8.02 µg/L, respectively. One orange juice sample, exceeded the maximum level (ML) established by EU for PAT (50 µg/L). HT-2 toxin was found in mixed juice (43%) at mean level of 22.38 µg/L. Overall no toxicological concern was associated to mycotoxins exposure for children and adult population and the results obtained highlight the necessity for rigorous monitoring studies on HT-2 in fruit juice.

Keywords: mycotoxins, fruit juice, daily intake

Resumen: Exposición a micotoxinas a través del consumo de zumo de frutas.

Se presenta un estudio sobre zumos de frutas a base de manzana, piña, albaricoque, naranja y pera para determinar la presencia natural de quince micotoxinas mediante cromatografía de gases acoplada a espectrometría de masas en tándem (EM/EM). El procedimiento desarrollado de multi-micotoxinas se llevó a cabo mediante micro-extracción líquida-líquida dispersiva (DLLME). El 36% de las muestras analizadas presentaron contaminación con micotoxinas y una muestra de jugo de naranja, superó el nivel máximo (ML) establecido por la UE para PAT (50 µg/L). Se detectó PAT en naranja, manzana, frutas mezcladas y jugos de piña con una prevalencia de 86%, 60%, 29%, 14% a concentraciones promedio de 34.57 µg/L, 33.41 µg/L, 8.59 µg/L, 8.02 µg/L, respectivamente. La toxina HT-2 estaba presente en el jugo mixto (43%) a un nivel medio de 22.38 µg/L. En general, ninguna preocupación toxicológica se asoció con la exposición a micotoxinas en la población de niños y adultos, los resultados ponen de relieve la necesidad de estudios rigurosos de monitoreo de HT-2 en el zumo de fruta.

Palabras claves: micotoxinas, zumos de frutas, ingesta diaria

Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi as *Aspergillus, Penicillium, Fusarium and Claviceps*. More than 400 mycotoxins are known and only some of them represent a real threat to food security. The most relevant are aflatoxins (AFs), ochratoxin A (OTA), Patulin (PAT) fumonisins (FBs), zearalenone (ZEA) and trichothecenes (TCs) [1]. Chronic exposition to some mycotoxins can produce carcinogenic, mutagenic, teratogenic, cytotoxic, neurotoxic, nephrotoxic, immunosuppressive and estrogenic effect. Their seriousness effects depend largely on the ingested amounts and duration of exposure that may result from simultaneous ingestion of various mycotoxins [2]. Mycotoxins can be present along the entire process of food production; in field, before and after the harvest, during processing, storage and also in a finished product [3].

Various factors affect the levels of contamination of mycotoxins in fruit and fruit products such as type and variety of fruit, climate conditions, geographical location, year production treatments before and after the harvest, use and pesticides, damage to the surface of the fruit, and storage conditions [4]. European Food Safety Authority (EFSA) have established maximum permitted levels for certain mycotoxins as aflatoxins B_1, B_2, G_1, G_2 y M_1, OTA, PAT, ZEA, DON and fumonisins B_1 and B_2, T-2 and HT-2 toxins [5]. In fruits and fruit juices, only PAT and OTA are legislated. A maximum up to 50 µg/kg is set for Patulin in reconstituted concentrated fruit juices and fruit nectar (Table1)

Table 1. *Summary of maximum levels specified for patulin in fruit and fruit juice*

Food products	Maximum level µg/kg
Fruit juices, concentrated fruit juices as reconstituted and fruit, nectars, spirit drinks, cider and other fermented drinks derived from apples or containing apple juice	50
Apple juice and solid apple products	10

While for Ochratoxin A a maximum level of 2 µg/kg is set up in grape juices, reconstituted concentrated grape juice, grape nectar, grape must and reconstituted concentrated grape must, intended for direct human consumption [5].

The presence of mycotoxins has been highly investigated in different fruit juices such as apple juice [6], orange juice [7], pear juice [8] apricot and peach juice [9] and berry juice [10]. The most of the method used for extraction mycotoxins in fruit juice are QuEChERS extraction [11], liquid-liquid extraction [12], and dispersive liquid-liquid microextraction [13]. DLLME offers some advantages over traditional technique being simple, fast and low cost technique extraction [14].

Regarding analytical method for mycotoxins determination, liquid chromatography mass spectrometry in tandem (LC-MS/MS) and gas chromatography coupled to mass spectrometry detector (GC-MS/MS) have become the most extensively technique used for determination of mycotoxins in biological and food samples [15, 16]. The gas chromatographic techniques offers some advantages as lower detection limits and greater selectivity [17].

In this sense the aim of the present study was to evaluate the presence of fifteen mycotoxins DON, 3-AcDON, 15-AcDON, NEO, DAS, NIV, ZON, α-ZOL, β-ZOL,α-ZAL, β-ZAL, FUS X, T-2, HT-2 and PAT and to carry a risk exposure of the population to these mycotoxins through the fruit juice consumption.

Material and methods

Chemicals and reagents

Solvents (acetonitrile, hexane, chloroform and methanol) were supplied by Merck (Darmstadt, Germany). Deionized water (<18.2 MΩ cm resistivity) was obtained in the laboratory using a Milli-QSP® Reagent Water System (Millipore, Beadford, MA, USA).

Ammonium formate (99%) and formic acid (≥98%), sodium chloride were supplied by Sigma Aldrich (Madrid, Spain). Syringe nylon filter (13mm diameter 0.22 µm pore size) were obtained from Analysis Vinicos S.L. (Tomelloso Spain). The derivatization reagent composed of BSA (N,O-bis(trimethylsily) + TMCS (trimthylcholorosilane) + TMSI (N-trimethylsilyimidazole) (3:2:3) was obtained from Supelco (Bellefonte, PA). Sodium dihydrogen phosphate and disodium phosphate, used to prepare phosphate buffer, were acquired from Panreac Quimica S.L.U. (Barcelona, Spain).

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