VNIVERSITAT DE VALÈNCIA

Facultat de Farmàcia

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

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Tesi Doctoral Internacional

ANALYSIS AND RISK ASSESSMENT OF MYCOTOXINS IN COFFEE

ANÁLISIS Y EVALUACIÓN DEL RIESGO DE MICOTOXINAS EN CAFÉ

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Presentada per:

Ana García Moraleja

Dirigida per:

Dr. Jordi Mañes Vinuesa

Dra. Emilia Ferrer García

VNIVERSITAT DE VALÈNCIA Facultat de Farmàcia

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El Dr. Jordi Mañes Vinuesa, Catedràtic de Universitat de l'àrea de Nutrició i Bromatologia i la Dra. Emilia Ferrer García, Professora Titular de l'àrea de Toxicologia,

CERTIFIQUEN QUE:

Dª Ana García Moraleja, llicenciada en Farmàcia, ha realitzat baix la seua direcció el treball "Analysis and risk assessment of mycotoxins in coffee", i autoritzen la seua presentació per a optar al títol de Doctora per la Universitat de València. I, perquè així conste, expedeixen i signen el present certificat.

Burjassot (València), febrer 2016

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Aquest treball ha sigut plasmat en 3 articles publicats en les següents revistes:

- Simultaneous determination of mycotoxin in commercial coffee. *Food Control* 57 (2015) 282-292. Impact factor: 2.806.

- Development of a new method for the simultaneous determination of 21 mycotoxins in coffee beverages by liquid chromatography tandem mass spectrometry. *Food Research International* 72 (2015) 247–255. Impact factor: 2.818.

- Analysis of mycotoxins in coffee and risk assessment in Spanish adolescents and adults. *Food and Chemical Toxicology* 86 (2015) 225-233. Impact factor: 2.895.

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

- Avaluació del risc de micotoxines emergents de *Fusarium* (AGL2010-17024/ALI).

- Estudi de les micotoxines i metabòlits en aliments i mostres biològiques, de la toxicitat i dels processos de descontaminació (AGL2013-43194-P).

- Red nacional sobre les micotoxines i fongs toxicogènics y dels seus processos de descontaminació.

La present Tesi Doctoral Internacional forma part del Programa de Doctorat Internacional "Chemistry, Toxicology and Healthiness of foods" dirigit per el Prof. Pietro Damiani i la Prof.ssa Lina Cossignani de la Università degli Studi di Perugia.

Never regard a study as a duty, but as the enviable opportunity to learn to know the liberating influence of beauty in the realm of the spirit for your own personal joy.

Albert Einstein

A mi familia

A Enrico

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List of abbreviations

15-aDON	15-acetyl deoxynivalenol
3-aDON	3-acetyl deoxynivalenol
AcN	*Acetonitrile*
AECOSAN	Agencia Española de Consumo Seguridad Alimentaria y Nutrición
AFB_1	*Aflatoxin B1* (Aflatoxina B_1)
AFB_2	*Aflatoxin B2* (Aflatoxina B_2)
AFG_1	*Aflatoxin G1* (Aflatoxina G_1)
AFG_2	*Aflatoxin G2* (Aflatoxina G_2)
AFs	*Aflatoxins* (Aflatoxinas)
AOAC	*Association of Official Analytical Chemicals*
BEA	*Beauvericin* (Beauvericina)
CE	Comisión Europea
CEN	Comité Europeo de Normalización
CIA	Columna de Inmuno Afinidad
CL	Cromatografía Líquida
CL-EM/EM	Cromatografía Líquida acoplada a Espectrometría de masas en tándem
CONTAM	Panel Científico de Contaminantes en Alimentos
DAS	Diacetoxyscirpenol
DC	*Decaffeinated Coffee*
DSR	Desviación Standard Relativa
DOM-1	De-epoxy-deoxynivalenol

DON	Deoxynivalenol
EC	*European Commission*
EDI	*Estimated Daily Intake*
EFSA	*European Food Security Authority* (Autoridad Europea de Seguridad Europea)
EM	Espectrometría de Masas
ENA	*Enniatin A* (Eniatina A)
ENA_1	*Enniatin A_1* (Eniatina A_1)
ENB	*Enniatin B* (Eniatina B)
ENB_1	*Enniatin B_1* (Eniatina B_1)
ENs	*Enniatins* (Eniatinas)
FAO	*Food and Agriculture Organization* (Organización de las Naciones Unidas para la Alimentación y la Agricultura)
FB_1	*Fumonisin B_1* (Fumonisina B_1)
FB_2	*Fumonisin B_2* (Fumonisina B_2)
FB_3	*Fumonisin B_3* (Fumonisina B_3)
FBs	Fumonisins (Fumonisinas B)
HPLC	*High Performance Liquid Chromatography* (Cromatografía Líquida de Alta Resolución)
HT-2	*HT-2 Toxin* (Toxina HT-2)
IARC	*International Agency for Research on Cancer* (Agencia Internacional de investigación del Cáncer)
IC	*Instant Coffee*
ICS	*Instant Coffee with Sugar*
ICSM	*Instant Coffee with Sugar and Milk*
IDE	Ingesta Diaria Estimada

IDT	Ingesta Diaria Tolerable
ISO	*International Standard Organization* (Organización Nacional de Estandarización)
IST	Ingesta Semanal Tolerable
IT	*Ion Trap*
JECFA	*Joint FAO/WHO Expert Committee on Food Additives*
LC-MS/MS	*Liquid Chromatography Tandem Mass Spectrometry*
LMP	Limite Máximo Permitido
LOD	*Limit of Detection* (Límite de Detección)
LOQ	*Limit of Quantification* (Límite de Cuantificación)
ME	Micotoxinas Emergentes
MeOH	*Methanol*
ML	*Maximum Limit*
MRM	*Multiple Reaction Monitoring*
NEB	Nefropatía Epidémica de los Balcanes
NEO	Neosolaniol
NIV	Nivalenol
NRC	*Natural Roasted Coffee*
OMS	Organización Mundial de la Salud
OTA	*Ochratoxin A* (Ocratoxina A)
PTWI	*Probably Tolerable Weekly Intake*
QqQ	*Triple Quadrupole*
R^2	Coeficiente de correlación
RM	Reacción de Malliard

RSD *Relative Standard Deviation*

SCF *Scientific Committee for Food* (Comité científico de los alimentos)

SS *Suppression of the signal*

SSE *Suppression of the signal / Enhancement*

STG *Sterigmatocystin* (Sterigmatocystina)

T-2 *T-2 Toxin* (Toxina T-2)

TDI *Tolerable Daily Intake*

TRC *Torrefacto Roasted Coffee*

TRCs Tricotecenos

TRs *Trichothecenes*

UE Unión Europea

UTE *Ultra-Turrax Extraction*

ZEA *Zearalenone*

RESUMEN

La población en general espera tener una alimentación cada vez más sana y segura, con alimentos nutritivos y asequibles. Desafortunadamente, antes durante y después de la cosecha, las materias primas y los productos procesados están sometidos a la contaminación por microorganismos y hongos y, en consecuencia, por sus metabolitos secundarios, las micotoxinas. El café es uno de estos productos. La presente tesis doctoral aborda nuevas técnicas analíticas para desvelar los contenidos de estas micotoxinas en café, y evalúa el "riesgo" que representa un producto tan fuertemente instaurado en la alimentación de numerosas sociedades a lo largo de todo el mundo.

Entre las diferentes metodologías analíticas estudiadas se han seleccionado dos de ellas. La primera consiste en una extracción de multimicotoxinas con acetonitrilo/agua como extractante, que permite el análisis simultaneo de 18 micotoxinas en café tostado. La segunda consiste en la extracción con acetato de etilo/ácido fórmico, que permite la extracción simultanea de 21 micotoxinas desde la bebida de café preparada para su consumo. La técnica instrumental consiste en una cromatografía líquida acoplada a espectrometría de masas de triple cuadrupolo (CL-EM/EM). Las propuestas se han validado obteniendo resultados satisfactorios de acuerdo con la normativa aplicable europea en cuanto a los requisitos exigidos para los métodos analíticos (CE/657/2002).

La aplicación de ambos métodos analíticos a muestras comercializadas revela un alto número de muestras contaminadas por

micotoxinas: con rangos de contaminación en café tostado desde 0,10 µg/kg hasta 25,86 mg/kg, y en bebida de café desde 0,69 µg/kg hasta 282,89 µg/kg. Entre las micotoxinas encontradas cabe destacar la presencia de ocratoxina A (OTA), la única micotoxina legislada en café, detectada incluso a niveles superiores a los límites máximos establecidos (5,0 µg/kg) en un pequeño número de muestras. No obstante, una parte de las muestras analizadas no presenta contaminación por ninguna de las micotoxinas analizadas.

Los diferentes tipos de café estudiados no muestran diferencias significativas en la mayoría de los casos. Si bien, algunas micotoxinas como OTA o deoxinivalenol entre otras, si muestran diferencias en función del tipo de procesado o del tipo de café.

Con los datos de contaminación de café combinados con la base de datos de consumo de la EFSA, se ha calculado la ingesta diaria estimada de acuerdo con las recomendaciones de la OMS. Además, se ha realizado una evaluación del riesgo mediante comparación de la ingesta diaria estimada con parámetros toxicológicos de ingesta diaria tolerable e ingesta semanal tolerable. Los resultados muestran que el café no supone un riesgo potencial para los consumidores en lo que respecta a la ingesta de micotoxinas. Sin embargo, sus contenidos deben ser tenidos en cuenta para futuros estudios de contaminantes en dieta total.

SUMMARY

Nowadays population desires a healthy and safety diet, with nutritive and affordable foods. Unfortunately, before, during and/or after harvesting, food products are subject to contamination by microorganism and fungi, and consequently by their secondary metabolites, the mycotoxins. Coffee is an agricultural food product and is subject to contamination. This Doctoral Thesis develops new analytical technics for quantification of mycotoxins contents in coffee. In addition, implement the risk assessment of this widely consume and deeply ingrained around the world product.

Among the different analytical methodologies, two of them are selected. One for the analysis of roasted coffee, consisting in an extraction with acetonitrile/water for the simultaneous analysis of 18 mycotoxins; The other one for the analysis of coffee brews consisting in an extraction with ethyl acetate/formic acid 5% for the simultaneous analysis of 21 mycotoxins. Instrumentation consist in a liquid chromatography tandem mass spectrometry LC-MS/MS. Both analytical methods achieve satisfactory results according quality standards of the European Union regulation (CE/657/2002).

The analyses of real samples indicate the presence of the studied mycotoxins in coffee beverages at concentrations ranging from 0.10 µg/kg to 25. 86mg/kg in roasted coffee samples, and from

0.69 µg/kg to 282.89 µg/kg in coffee brew. Ochratoxin A, the only mycotoxin regulated in coffee, is detected in samples, and in some cases concentration levels exceed the maximum limit established (5.0 µg/kg). However, some samples are mycotoxin free.

The most coffee types do not show significant differences in the degradation of mycotoxins. Nevertheless, ochratoxin A and deoxynivalenol between others, show different behaviour by the different coffee types and coffee brewing methods.

The estimated daily intake is calculated based on the recommendations of WHO. Data on mycotoxin concentrations in coffee samples, and food consumption data available from EFSA data base, have been used for the estimation. In addition, risk assessment has been performed via comparison with the tolerable daily intake or the tolerable weekly intake and with available studies of total diet. The results show that coffee intake does not represent a potential risk for consumers regarding individual mycotoxin contamination. However, contamination of coffee by mycotoxins likely affects the exposition of the total diet, and should be considered in future total diet studies.

1. INTRODUCTION

1. INTRODUCCIÓN

1.1. El café

El consumo de café apareció inicialmente en Etiopía y posteriormente en el siglo XV en Yemen, su consumo se fue extendiendo por África, posteriormente Europa y finalmente América (Jeszka-Skowron et al. 2015). En la actualidad, el café es la bebida más conocida a nivel mundial después del agua, y una de las materias primas más importantes en el mercado internacional (Barbin et al. 2014), también el té es ampliamente consumido en Asia, no obstante los valores de producción a nivel mundial no alcanzan a los valores del café, y mucho menos en lo que respecta al mercado internacional, puesto que el té es principalmente consumido en los países productores, siendo el café un producto tropical consumido en los principales países desarrollados de Europa y América del Norte (FAO, 2015; ICO, 2016). De hecho, el café es el segundo producto del mercado internacional después del petróleo (Lee et al. 2015). En los últimos años, productores de las zonas tropicales, se están interesando en la certificación de calidad para la comercialización de este alimento; en estos momentos, el 38% de la producción mundial cuenta con algún certificado de calidad (Tscharntke et al. 2015).

La planta de café o cafeto, crece bien en áreas tropicales sin grandes variaciones ambientales (Piyapromdee et al. 2014), principalmente en el área comprendida entre las latitudes 25º norte y 25º sur, en el conocido cinturón del café (Narita e Inouye, 2014). Es cultivado en 80 países de 4 continentes. Brasil es el mayor productor, con el 30% de la producción mundial en 2015, seguido de Vietnam, Colombia, Indonesia y algunos países africanos y de Centro América como Etiopía y Honduras (ICO, 2016). Además, la mayoría de las plantaciones pertenecen a pequeños agricultores, proveyendo una importante fuente de empleo y desarrollo local en las regiones productoras (Bicho et al. 2013). Estos cultivos representan gran cantidad del área agroforestal. La fluctuación en los precios y el aumento de la demanda de café por el mercado mundial están generando cierta presión a los agricultores, que aumentan e intensifican el área de los cultivos (De Beenhouwer et al. 2013).

El comercio del café comienza con los pequeños agricultores y hasta el consumidor final pasa por diferentes etapas, como se puede ver en la **figura 1** (Piyapromdee et al. 2014).

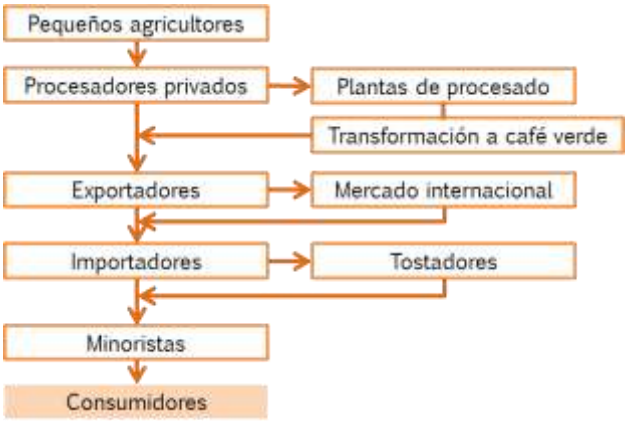


Figura 1: diagrama de flujo comercio de café.

Un total de 85 especies diferentes de cafeto son conocidas, aunque solo tres de ellas son útiles para uso comercial: *arabica*, *canephora* (Robusta), y *liberica* (Bohn et al. 2012). Entre las principales especies del café las más importantes a nivel comercial y económico son arábica y robusta, siendo arábica la mejor considerada en cuanto a la calidad (Barbin et al. 2014). La producción mundial de café contempla principalmente un 70% de café arábica, y 30% de café robusta (Bandyopadhyay et al. 2012). La especie arábica es más apreciada a nivel comercial debido a un sabor más fino y delicado que la robusta (Barbin et al. 2014). El café arábica crece en el 85% de los países productores, predominantemente en el continente americano, y representa aproximadamente del 69 al 74% de la producción mundial del café. El café robusta crece principalmente en países asiáticos y africanos (Bicho et al. 2013).

El procesado del café conlleva la selección de las semillas, fermentación, lavado y secado, posteriormente el tostado del grano verde (**figura 2**) (Bohn et al. 2012).

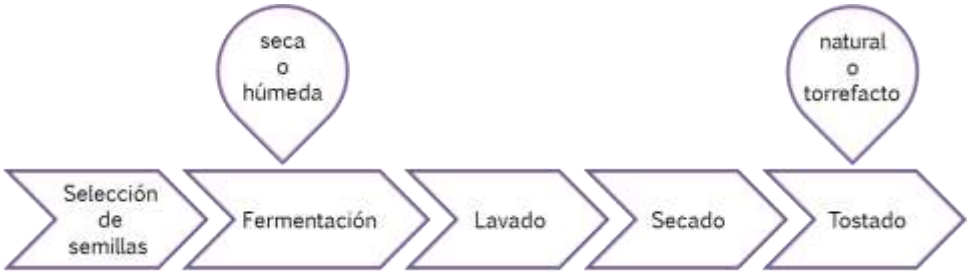


Figura 2: Procesado del café desde la planta.

El proceso de fermentación en el aroma suele ser negativo, pero es un paso imprescindible para eliminar la capa de mucílago. Existen dos tipos, el húmedo y el seco. El húmedo tiene como resultado un producto más aromático con atributos frutales y ácidos, menos amargo, quemado y con notas de madera. No obstante, la fermentación en seco o natural es la más usada en los principales países productores. Durante ambos tipos de fermentación se han identificado especies de hongos pertenecientes a géneros: *Candida, Pichia*, *Hanseniaspora, Saccharomyces, Penicillium, Fusarium* y *Aspergillus,* así como bacterias: *Klebsiella, Erwinia, Hafnia, Lactobacillus, Aeromonas, Enterobacter* y *Pseudomonas* (Lee et al. 2015; Silva et al. 2000).

Las semillas de café son tostadas con calor seco a temperaturas entre 200 y 300ºC, con agitación constante (Narita et al. 2014). Principalmente se usan dos tipos de tostado, el natural y el torrefacto, siendo la diferencia entre ambos la adicción de azucares, de hasta un 15% en peso, durante el tostado torrefacto (Real Decreto 1676/2012). Dependiendo del tipo de tostado los granos de café varían en volumen entre el 40-69% (arábica), y 48-57% (robusta). El contenido de cafeína en el grano de café verde en café arábica y robusta respectivamente es de 1,5 y 2,4%. No obstante, durante el tostado el contenido de cafeína se reduce hasta 1,3 y 2,2% respectivamente. En general, el café verde de la variedad robusta presenta valores más elevados de pH, sólidos solubles, cafeína, ácidos cafeínicos totales, ácidos dicafeinolíticos totales y ácidos feruloilquínicos (Bicho et al. 2013).

La producción de café soluble requiere una mayor tecnología que la producción de café molido. El proceso industrial del café soluble requiere una concentración, que se puede realizar por evaporación térmica, liofilización o por pulverización y posterior envasado (Weschenfelder et al, 2015).

El grado de tostado se usa para cuantificar la calidad de café, que se clasifica en ligero, medio o alto. La composición de algunos compuestos carbonatados como ésteres vinílicos, lactonas, ésteres,

aldehídos, cetonas y ácidos en la bebida dependen del grado de tostado que se haya aplicado, no siendo de gran importancia el tiempo o la temperatura de tostado, pero si el incremento de la temperatura desde la introducción del grano de café verde hasta alcanzar la temperatura de tostado (Barbin et al., 2014).

Durante el tostado se producen diferentes cambios químicos, siendo de gran importancia la formación del aroma del café, mediante la Reacción de Maillard (RM) y otras reacciones térmicas (Lee et al., 2015) como la caramelización de los azúcares o la degradación de la cafeína. Por ello, el café de tostado ligero contiene más cantidad de cafeína que el café con tostado fuerte. Además, el aceite aromático cafeol que caracteriza el aroma del café aparece tras el tostado a altas temperaturas, pero no antes (Bohn et al. 2012). Se producen también numerosas reacciones: de degradación de polifenoles por hidrólisis generando ácidos fenólicos libres, y formación de ácido clorogénico en lactonas por la RM (Bandyopadhyay et al. 2012). La trigonelina se degrada a compuestos aromáticos como piridinas y pirroles (Lee et al. 2015).

No obstante, la calidad de la bebida de café y las características físicas, químicas y sensoriales se ven afectadas, además de por las condiciones y grado de tostado, por diversos factores, como la calidad

industrial, la variedad o mezclas de variedades de café, y por las condiciones de almacenamiento. Igualmente, durante la preparación tienen importancia la temperatura, presión y volumen de agua, el grado de molido, la cantidad de café molido usado para la preparación de la taza y el grado de compactación (Severini et al. 2015). Los principales tipos de procesado de café son: cafetera tradicional italiana, máquina eléctrica para cápsulas monodosis, café turco y café soluble. Por otro lado, las sensaciones percibidas durante el consumo de café varían dependiendo de la motivación de cada consumidor a la hora de tomar su taza de café. Los consumidores de café con motivación hedonista disfrutan más del sabor y aroma además del efecto. Los consumidores funcionales no valoran la experiencia sensorial, valorando únicamente el efecto estimulante de la cafeína (Labbe et al., 2015).

La calidad de las semillas del café depende de la presencia de defectos, tamaño y composición química (Bicho et al., 2013). Además, el contenido en agua del café verde es de aproximadamente 12,5%, por ello el grano de café es susceptible de ser contaminado por microrganismos y hongos, que pueden alterar las cualidades organolépticas del producto final. En consecuencia, el contenido en agua del café verde se toma como estándar de referencia para estimar su calidad. En cuanto a la composición, se usan marcadores químicos

en el grano para predecir la calidad de la bebida, estos son la cafeína, la trigonelina, acido 5-cafeinólico, celulosa, lípidos del café y sacarosa (Barbin et al. 2014).

El flavor del café para los consumidores es el indicador más importante de calidad, seguido del cuerpo y del aroma. La complejidad y matices del flavor están muy influenciados por el cultivo, procesado y preparación del café. En cuanto a la composición en compuestos volátiles y no volátiles, el tostado juega un factor importante (Sunarharum et al. 2014). El control de calidad es imprescindible, ya que se conocen adulteraciones de café en polvo o molido con achicoria, malta, higos, cereales, maltodextrinas, glucosa o mezclando café de diferentes regiones (Barbin et al. 2014). En la actualidad se está estudiando un control de calidad mediante nariz electrónica para la clasificación de perfiles aromáticos que tipifiquen los tipos de café, los diferentes grados del tostado, el tipo de envasado y el efecto de la preparación de la bebida de café (Severini et al. 2015) en sustitución de los paneles de catadores.

La composición química del extracto de café es una mezcla compleja de fitoquímicos, lípidos, carbohidratos etc.… además de cafeína, teobromina, y paraxantina (Bohn et al. 2012). Se encuentran también presentes minerales: Ca, K, Mg, Na, Cl, S, P; oligoelementos:

Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Sr, Zn; y elementos traza: Al, As, B, Ba, Br, Cd, Hg, Pb, Sn. Las concentraciones de todos ellos vienen influenciadas por el terreno de cultivo (Pohl et al. 2013). Los compuestos bioactivos más importantes son derivados fenólicos (ácido clorogénico), metilxantinas (cafeína), diterpenos (cafestrol), ácido nicotínico (vitamina B_3) y su precursor la trigolenina (Jeszka-Skowron et al. 2015).

El café está considerado entre los alimentos más ricos en polifenoles (Bandyopadhyay et al. 2012). Además, debido a su alto consumo, es el mayor contribuidor a la dieta de compuestos antioxidantes, con un aporte del 60% entre la población consumidora (Bohn et al. 2014). El compuesto fenólico más abundante del café es el ácido cafeico y su derivado el ácido clorogénico (Bandyopadhyay et al. 2012). Los principales ácidos clorogénicos son: hidroxicinamatos, ácido felúrico, ácido p-cumarínico (Bohn et al. 2012). Estos polifenoles son los responsables del sabor amargo y astringente de esta bebida, y también de las propiedades antioxidantes con efectos beneficiosos frente a trastornos relacionados con el estrés oxidativo, pudiendo tener relación con el cáncer, enfermedades cardiovasculares y el envejecimiento (Bandyopadhyay et al. 2012).

La cafeína, 1.3.7-trimetilxantina, es el compuesto bioactivo más característico del café. Un adulto que consume entre 3 - 4 tazas de café al día ingiere de 300 a 400 mg de cafeína (Eilat-adar et al. 2013). Si bien este dato es variable ya que el contenido en cafeína depende del tipo de semilla, del tostado y del tipo de preparación (Frost-Meyer et al. 2012).

La fracción de lípidos saponificables del café está constituida principalmente por glicéridos, fosfolípidos y diterpenos esterificados. La insaponificable contiene esteroles, diterpenos libres y tocoferoles. Los principales diterpenos son cafestrol y kahweol (Tsukui et al. 2014). La cantidad de cafestrol y kahweol en la taza de café depende del tipo de preparación, teniendo en café filtrado menor cantidad de estos compuestos que el café hervido. Sus efectos negativos radican en el aumento del colesterol total y las lipoproteínas de baja densidad (LDL); sus efectos positivos son la capacidad antinflamatoria que podría conllevar efectos antiangiogénicos, kahweol también reduce la óxido nítrico sintasa, y ejerce protección frente al estrés oxidativo (Frost-Meyer et al. 2012).

Otros compuestos encontrados en la bebida de café con efectos biológicos son: melanoidinas y trigonelina (Frost-Meyer et al. 2012); que in vitro han demostrado una actividad biológica, como

antioxidantes, quimioprotectores, antihipertensivos, hipoglucemiantes, prevención del envejecimiento celular, y anticarcinógenas (Ludwig et al. 2014). Además, aproximadamente otros 1000 compuestos volátiles y 70 olores se han identificado en bebida de café (Severini et al. 2015).

Mientras que los polifenoles son los responsables del sabor amargo y astringente de esta bebida (Bandyopadhyay et al. 2012), el ácido clorogénico y las lactonas contribuyen al amargor de la bebida (Narita et al. 2014). Estando estrechamente relacionado el contenido en lípidos y proteínas con el cuerpo de la bebida final de café; el ácido clorogénico con la acidez, el favor y la claridad, y en general la calidad con la cafeína, trigonelina, ácido clorogénico y polisacáridos como sacarosa y proteínas en café tostado (Barbin et al. 2014). El tipo de preparación de la taza de café afecta altamente a las características organolépticas de la bebida, sobretodo en la concentración de ácidos orgánicos, sólidos, y contenido de cafeína (Severini et al., 2015). Muchos consumidores añaden leche a la bebida preparada, en este caso los polifenoles interaccionan con las proteínas de la leche y se reduce la percepción de la astringencia (Bandyopadhyay et al. 2012).

Debido a que el café es de consumo habitual su composición tanto en compuestos deseables como tóxicos se tiene que mantener

controlada para garantizar su calidad y su seguridad (Pohl et al. 2013). En los últimos años se ha ido estudiando las propiedades de los compuestos del café y se han reportado actividades antioxidantes, inhibición de alfa-amilasas, inhibición de lipasas y antihiperglucemiante entre otras (Narita et al. 2014).

Existe controversia entre el efecto del café en las enfermedades cardiovasculares. El consumo moderado a largo plazo no aumenta el riesgo de enfermedades cardiovasculares, y algunos estudios demuestran que incluso podría tener efectos preventivos (Bohn et al. 2012). El consumo de café se ha relacionado con efectos beneficiosos frente a la diabetes mellitus tipo 2, y la prevención de cáncer hepático, de endometrio, colorectal, y de mama (Ludwig et al. 2014). Se ha encontrado una asociación inversa entre el consumo de café y el riesgo de cáncer hepático. Y estudios recientes parecen indicar que no hay relación entre el consumo de café y cáncer de pecho y próstata (Bohn et al. 2014). No obstante, hay estudios que encuentran indicios de que podría presentarse como un factor de riesgo de cáncer gástrico (Liu et al. 2015).

Efectos negativos del café que no se pueden ignorar son: aumento de la actividad del sistema nervioso central, ansiedad, insomnio, y bajo peso en recién nacidos en madres consumidoras;

todos ellos ampliamente relacionados con la cafeína (Frost-Meyer et al. 2012). De hecho, se desaconseja el uso de café en mujeres embarazadas (Eilat-adar et al. 2013).

1.2. Micotoxinas

Los hongos y otros organismos similares causan serias y a veces intratables enfermedades en humanos y animales; además dan pérdidas económicas por infección de plantas y desvalorización comercial (Thornton et al. 2015).

Podemos enmarcar las micotoxinas como metabolitos secundarios producidos mayoritariamente por hongos filamentosos que parasitan alimentos, principalmente cereales y otros productos agrícolas (Kokkonen y Jestoi, 2009). Hasta el momento se conocen aproximadamente unas 400 micotoxinas (Sulyok et al. 2010). Entre las más estudiadas están las aflatoxinas (AFs), zearalenona (ZEA), tricotecenos (TRCs), fumonisinas, ocratoxinas y patulina. La contaminación por hongos se puede dar durante el cultivo de la planta, principalmente con especies del género *Fusarium, Alternaria* y *Cladosporium* o durante el almacenamiento, con predominio de *Aspergillus, Penicillium* y *Trichoderma* (Varga et al., 2013). La producción de micotoxinas no es continua y está influenciada por las

condiciones climatológicas (Kandhai et al. 2011). En la actualidad existe un aumento de factores influenciados por el cambio climático como insectos, parásitos, condiciones del suelo, que están potenciando la colonización fúngica y por tanto la producción y presencia de micotoxinas en productos agrícolas (Tirado et al. 2010). Es importante indicar que la presencia de un hongo en los productos alimentarios no implica la existencia de micotoxinas, ya que la producción de micotoxinas se realiza bajo condiciones específicas de temperatura, humedad y susceptibilidad del producto contaminado, y la ausencia de hongo tampoco implica la ausencia de micotoxinas (Bryden 2012).

Sus funciones en el hongo productor no son del todo conocidas, probablemente desempeñen funciones ecológicas y reguladoras, pero con resultados tóxicos para otros organismos como bacterias, plantas a las que parasitan, y consumidores (Reverberi et al. 2010). Son contaminantes inevitables de los alimentos; aunque seamos capaces de evitar el crecimiento fúngico en las etapas más propensas a la producción de micotoxinas, puede haber una contaminación en el trasporte, almacenamiento, y/o procesado de los productos alimentarios (Kandhai et al., 2011). *La Food and Agricultural Organization* (FAO) estima que aproximadamente el 25% de los productos de la agricultura están contaminados en cierta cantidad por micotoxinas (FAO, 2016), si bien esta cifra podemos considerarla

infravalorada por no tener en cuenta la presencia de otros metabolitos potencialmente tóxicos, como por ejemplo las micotoxinas emergentes (ME) de las que aún existen pocos datos sobre su toxicidad. De hecho, el estudio de micotoxinas es de importancia global creciente, son muchos los organismos que cada vez más dedican sus esfuerzos a estudiarlas. Por ejemplo, planteado como un enfoque holístico del problema de las ME, se ha creado el proyecto MYCONET que tiene el objetivo de desarrollar un sistema de detección de ME, principalmente de *Fusarium*, en productos presentes en los mercados europeos (Kandhai et al. 2011).

Los procesos de calidad son necesarios para minimizar la exposición a micotoxinas (Bueno et al. 2015). Se ha creado una lista con los principales indicadores para prever la existencia micotoxinas. Estos indicadores son, a nivel ambiental: la temperatura, la humedad relativa, y ventilación entre otros; a nivel de las organizaciones competentes y/o empresas productoras: conciencia de la seguridad alimentaria, uso de fungicidas, legislación aplicable a las buenas prácticas de manufactura y exigencias de valores máximos en alimentos (Kandhai et al. 2011).

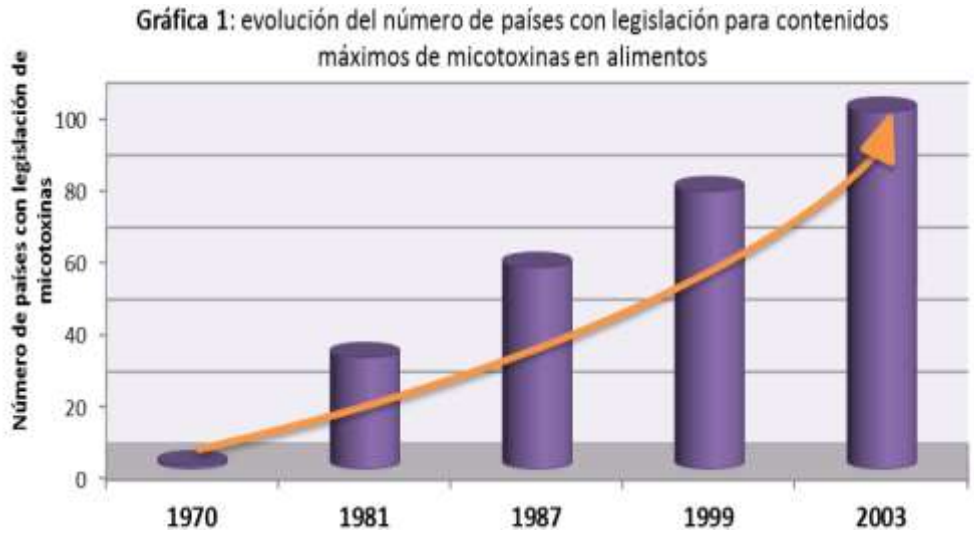
Su alta variabilidad química convierte a las micotoxinas en un amplísimo conjunto de moléculas con efectos muy diversos en la

salud. Además, es uno de los problemas más frecuentes que nos encontramos a la hora de estudiar su posible resistencia a tratamientos de detoxificación (Köppen et al., 2010).

Las enfermedades que causan se denominan micotoxicosis. Son enfermedades no transmisibles, y los tratamientos tan solo son de tipo sintomático. Estas toxinas tienen un carácter estacional, y están relacionadas cada una con alimentos determinados, entrando en contacto con humanos por vía alimentaria (Marín et al. 2013). Para las micotoxinas más toxicas se han establecido valores de referencia toxicológicos como la Ingesta Diaria Tolerable (IDT). Para las micotoxinas que han demostrado ser genotóxicas y cancerígenas para el ser humano, como las AFs, se ha adoptado el enfoque del margen de exposición. Para otras micotoxinas menos conocidas, no es posible caracterizar el peligro por falta de datos, según explica la Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN, 2015).

Además de por sus efectos en la salud humana, las micotoxinas representan un gran problema a nivel mundial, ya que causan pérdidas económicas al afectar la productividad agrícola y animal. A su vez, tienen una repercusión directa en el comercio internacional de alimentos. En la actualidad cerca de 100 países tienen una legislación

específica para micotoxinas, si bien con valores diversamente restrictivos ya que no se ha llegado a un consenso entre ellos. La disparidad de valores de límites máximos permitidos (LMP) genera trabas al comercio, siendo la Unión Europea (UE) quien tiene los niveles más restrictivos y Estados Unidos uno de los más permisivos (Yao et al, 2015). La FAO dentro de sus funciones de normalización internacional en el ámbito en las Naciones Unidas, realizó un estudio a nivel mundial en cuanto a la legislación se las micotoxinas en los alimentos en 2003 (FAO, 2004), recopilando información acerca de las respectivas normas de los LMP en los 5 continentes. Desde 1970 en que se fijó el primer valor límite para micotoxinas ha ido creciendo el número de países que han ido estableciendo reglamentaciones al respecto (**gráfica 1**).



La FAO anima a los organismos competentes a realizar una normalización de los LMP de micotoxinas de forma armonizada para facilitar el comercio internacional. Ya existen asociaciones como la Asociación de Naciones del Sudeste Asiático (ANSEA), MERCOSUR (Argentina, Brasil, Paraguay y Uruguay), y la UE que han armonizado recientemente sus reglamentos en el contexto de LMP de micotoxinas en alimentos. Esta reglamentación no es completa, debido a la existencia de varios factores, tanto de naturaleza científica como socioeconómica, como son: disponibilidad de datos toxicológicos, disponibilidad de datos relativos a la presencia de las micotoxinas en diversos productos básicos, disponibilidad de métodos analíticos, y necesidad de un abastecimiento suficiente de alimentos, entre otros.

Por otro lado, tanto la *International Standard Organization* (ISO) como el Comité Europeo de Normalización (CEN) o la *Association of Official Analytical Chemicals* (AOAC International) cuentan con varios métodos normalizados de análisis y buenas prácticas, por ejemplo: determinación de OTA en cereales y derivados (EN ISO 15141-1:2000); determinación de OTA en cebada y café tostado (EN 14132: 2003/AC:2007); determinación de patulina en zumos (EN 14177:2004); técnicas de examen de la muestra para el análisis de micotoxinas (CEN/TR 15298:2006) entre otros. Específicamente para el café, el *Codex alimentarius* adoptó en 2009 un Código de buenas prácticas

para prevenir y reducir la contaminación de Ocratoxina A (CAC/RCP 69-2009).

En España, el primer Real Decreto por el que se establecieron los LMP de AFs en alimentos para consumo humano fue publicado en 1988. Desde la entrada del libre comercio en la UE se ha ido armonizando la reglamentación concerniente a contaminantes en alimentos en todos los países miembros. En 2006, la Comisión Europea (CE) publicó el reglamento 1881/2006 con las cantidades máximas de contaminantes en alimentos, entre ellos las micotoxinas. Este reglamento contempla las AFs, ocratoxina A (OTA), patulina, deoxinavalenol (DON), ZEA, fumonisinas B (FBs), toxinas T-2 y HT-2 (CE, 1881/2006). En el mismo, también se especifica en el séptimo párrafo que las restricciones de micotoxinas no son solo en alimentos de consumo humano, sino también en alimentos de uso veterinario.

En la **Tabla 1** se muestra el LMP para los alimentos con niveles más y menos restrictivos respectivamente en el reglamento 1881/2006 y sus posteriores modificaciones.

Además, en la UE existe legislación aplicable a la determinación de micotoxinas en alimentos referente a controles oficiales y a la metodología de análisis (**Tabla 2**).

Tabla 1: Legislación Europea con respecto a límites máximos de micotoxinas en alimentos.

Micotoxina	LMP[a]	µg/kg[b]	Producto	Reglamento
AFB1[f]	Min[c]	0,10	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad	1881 / 2006 CE
	Max[d]	12,0	Almendras, pistachos y huesos de albaricoque que vayan a someterse a un proceso de selección, u otro tratamiento físico, antes del consumo humano directo o de su utilización como ingredientes de productos alimenticios	165 / 2010 CE
	Cf[e]	-	-	-

[a]LMP: Límite Máximo Permitido de micotoxinas en alimentos.
[b]Concentración expresada en µg/kg
[c]Min: LMP más bajo para esa micotoxina.
[d]Max: LMP más alto para esa micotoxina.
[e]Cf: LMP establecido para café o derivados.
[f]AFB_1: aflatoxina B_1
[g]AFT: aflatoxinas totales
[h]OTA: ocratoxina A
[i]DON: deoxinivalenol
[j]ZEA: zealarenona
[k]FBs: suma de fumonisinas B_1 y B_2
[l]HT-2 y T-2: suma de micotoxinas HT-2 y T-2
[m]ACC: Alcaloides del cornezuelo del centeno.

Tabla 1: Continuación

Micotoxina	LMP[a]	µg/kg[b]	Producto	Reglamento
AFT[g]	Min	4,0	Frutos secos, distintos de los higos secos, y productos derivados de su transformación, destinados al consumo humano directo o a ser usados como ingredientes en los productos alimenticios	1058 / 2012 CE
	Max	15,0	Cacahuetes destinados a ser sometidos a un proceso de selección, u otro tratamiento físico, antes del consumo humano directo o de su uso como ingredientes de productos alimenticios	1881 / 2006 CE
	Cf	-	-	-
OTA[h]	Min	0,5	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad	1881 / 2006 CE
	Max	80,0	Extracto de regaliz, para uso alimentario, especialmente en bebidas y confitería	105 / 2010 CE
	Cf	5,0	Café tostado en grano y café tostado molido	1881 / 2006 CE
		10,0	Café soluble	

Tabla 1: Continuación

Micotoxina	LMP[a]	µg/kg[b]	Producto	Reglamento
Patulina	Min	10,0	Zumo de manzana y productos sólidos elaborados a base de manzanas, incluidos la compota y el puré de manzana destinados a los lactantes y niños de corta edad.	1881 / 2006 CE
	Max	50,0	Zumos de frutas, zumos de frutas concentrados reconstituidos y néctares de frutas y bebidas espirituosas	1881 / 2006 CE
	Cf	-	-	-
DON[i]	Min	200,0	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad	1881 / 2006 CE
	Max	1750,0	Trigo duro, avena y maíz no elaborados	1126 / 2007 CE
	Cf	-	-	-
ZEA[j]	Min	20,0	Alimentos elaborados a base de cereales y alimentos infantiles para lactantes y niños de corta edad	1881 / 2006 CE
	Max	200,0	Maíz destinado al consumo humano directo, harina de maíz, maíz molido, maíz triturado y aceite de maíz refinado	1881 / 2006 CE
	Cf	-	-	-

Tabla 1: Continuación

Micotoxina	LMP[a]	µg/kg[b]	Producto	Reglamento
FBs[k]	Min	200,0	Alimentos elaborados a base de maíz y alimentos infantiles para lactantes y niños de corta edad	1881 / 2006 CE
	Max	4000,0	Maíz no elaborado	1126 / 2007 CE
	Cf	-	-	-
HT-2[l] Y T-2	Min	15,0	Alimentos elaborados a base de cereales para lactantes y niños de corta edad	Rec. 165 / 2013 UE
	Max	1000,0	Avena no transformada (con cáscara)	Rec. 165 / 2013 UE
	Cf	-	-	-
ACC[m]	Antes del 1 de julio de 2017 se estudiarán para productos de la molienda de cereales entre otros, unos contenidos máximos adecuados y factibles, que ofrezcan un elevado nivel de protección de la salud humana			1940 / 2015 CE
	Cf	-	-	-
Citrinina	2000		Complementos alimenticios a base de arroz fermentado con levadura roja *Monascus purpureus*	212 / 2014 CE
	Cf	-	-	

Tabla 2: Cuadro resumen con la legislación aplicable

669/2009 CE	Establece las normas relativas a la intensificación de los controles oficiales que deben realizarse, en los puntos de entrada de los países de riesgo (ejemplo: cacahuetes de Brasil por riesgo de contaminación por micotoxinas).
401/2006 CE	Métodos de muestreo para el control oficial del contenido de micotoxinas en los productos alimenticios. Criterios aplicables a la preparación de las muestras y los métodos de análisis para el control oficial del contenido de micotoxinas en los productos alimenticios

1.2.1. Aflatoxinas

El descubrimiento de AFs fue con la enfermedad X del pavo (Yao et al. 2015). Desde entonces, se han identificado cerca de 20 AFs, pero solo las B_1, B_2, G_1, y G_2 (CAS-RN 1162-65-8; 7220-81-7; 1165-39-5; 7241-98-7) se han encontrado frecuentemente en alimentos. Las AFs son derivados difuranocumarínicos. La nomenclatura B o G corresponde a la fluorescencia azul o verde observada tras la aplicación de radiación UV. AFB_1 es la aflatoxina más prevalente (Yao et al. 2015), considerándose el carcinógeno natural más potente (Chiewchan et al. 2015). Son metabolizadas en hígado por enzimas del citocromo P_{450} (Marin et al. 2013), transformándose en AFs M_1 y M_3, y

pueden ser excretados, tanto en animales como en humanos, por la leche, orina y heces (Chiewchan et al. 2015).

Son producidas por varias especies del género *Aspergillus spp.* (*flavus, parasiticus, nonius, ochraceus*…) (Raiola et al, 2015). Las cosechas más susceptibles de ser contaminadas por micotoxinas son cereales (maíz, arroz y trigo) y frutos secos (Yao et al, 2015), produciendo micotoxinas en un rango de temperaturas entre 12 - 40 ºC, aunque el óptimo de temperaturas oscila entre 24 – 28 ºC (Chiewchan et al. 2015).

Las mejores condiciones ambientales para el crecimiento de AFs son alta temperatura y humedad, típico de las zonas tropicales y subtropicales cálidas, áridas y semiáridas. Son también importantes para la contaminación la presencia de insectos que pueden dañar los frutos y hacerlos más susceptibles de la conminación por hongos (Yao et al. 2015). *Aspergillus spp*. coloniza principalmente las partes aéreas de las plantas (Marin et al. 2013), no obstante, la contaminación por AFs se puede dar durante el crecimiento de la planta, o en cualquier momento desde la recolección hasta el consumo, siendo el hábitat natural de *Aspergillus spp*. el suelo, vegetación en descomposición, heno y granos con contaminación microbiológica. Estos hongos proliferan poco en zonas frías, si bien bajo condiciones de estrés como

lluvias fuertes o inundaciones aumentan las probabilidades de producción de AFs. Con frecuencia se ha comunicado la detección de AFs en muestras que superan los LMP por la legislación. En la mayor parte de los casos provienen de países de regiones tropicales (Chiewchan et al. 2015).

Las AFs son carcinógenos extremos en todas las especies animales investigadas (ratones, peces, monos…) (Marin et al. 2013). De hecho, están reconocidas como las micotoxinas más peligrosas para la salud pública, causando efectos tóxicos tanto tras el consumo agudo como crónico en humanos (Chiewchan et al. 2015). El hígado es el órgano más afectado por su toxicidad. Cada año aparecen entre 550.000 y 600.000 nuevos casos de cáncer hepático, de los cuales entre 25.000 a 155.000 podrían estar atribuidos a la ingesta de AFs (Chiewchan et al. 2015). En efecto las AFs están clasificadas como grupo 1 (carcinógeno para humanos) por la *International Agency for Research on Cancer* (IARC). El comité científico de los alimentos (*Scientific Committee for Food*, SCF) de la CE concluyo, teniendo en cuenta estudios previos de análisis del riesgo, que una ingesta diaria de 1ng/kg pc/día de AFs o inferior representa un riesgo para la salud humana (CE, 1996).

Los primeros síntomas de aflatoxicosis aguda son fiebre, malestar y anorexia puede evolucionar a dolor abdominal, vómitos, hepatitis y en algunos casos la muerte (Raiola et al, 2015), estos casos de toxicidad aguda son raros, para ello son necesarias altas dosis de AFs, esto ocurre esporádicamente en países en vías de desarrollo. Por ejemplo, en Kenia en 2004 se diagnosticaron 317 casos de intoxicación con AFs, de los cuales 125 no sobrevivieron (Marin et al. 2013).

La toxicidad crónica es más común, los efectos dependen de factores como la edad, especie, género, estado nutricional, dosis y tiempo de exposición; en animales se produce una baja productividad (desciende el número de huevos, bajo peso…), en humanos: cáncer hepático, efectos en el sistema reproductor, encefalopatía con degeneración grasa de las vísceras, fibrosis del intersticio pulmonar, carcinogenicidad, aumento la respuesta inflamatoria, pero también causa la reducción de la eficiencia de la inmunización en niños con una consecuente susceptibilidad a infecciones. Además, muestra efectos sinérgicos con el virus de la hepatitis B generando carcinoma hepático (Marin et al, 2013; Raiola et al 2015).

Las AFs son moléculas muy termoestables, con altas temperaturas de degradación (entre 237-306ºC) y temperaturas superiores a 150ºC para degradaciones parciales. Ya que no son

fácilmente eliminables por el cocinado doméstico, es importante reducir al máximo la presencia de moho y evitar que se produzcan las micotoxinas (Chiewchan et al. 2015; Milani et al. 2014). Efectivamente, en la actualidad, la forma más eficaz de reducir la presencia de AFs en alimentos es emplear prácticas de control de crecimiento del hongo, durante la cosecha, recolecta, almacenamiento y procesado. Para ello se ha usado radiación gamma que inactiva al hongo, la ultravioleta a 254 nm puede inactivar al *A. flavus*. También se han usado tratamientos físicos y químicos. Hasta el momento, el secado se considera la mejor herramienta por su relación coste / efectividad para reducir la aparición de AFs (Chiewchan et al. 2015).

1.2.2. Ocratoxina A

Las ocratoxinas forman uno de los grupos de micotoxinas mas importantes para el hombre (Malir et al. 2013) de los cuales el más abundante es la OTA (CAS-RN 303-47-9) un pentaketido (skrinjar et al. 2013) consistente en una dihidrocumarina clorada unida al grupo 7-carboxil de la L-fenilalanina (Rhouati et al. 2013; Marin et al. 2013).

Las ocratoxinas son micotoxinas que se producen durante el almacenamiento en climas tropicales por hongos del genero *Aspergillus* y en áreas templadas o frías por hongos del genero *Penicillium.* Estos hongos los podemos encontrar tanto en plantas

como en cereales, mayoritariamente en arroz, soja, café, cacao (Marin et al. 2015; Rhouati et al. 2013), dependiendo de factores ambientales como: condiciones climáticas, latitud, vectores (insectos), heridas en la planta que permitan la penetración del hongo, prácticas de cultivo incluido es uso de fungicidas y productos fitosanitarios, tipo de almacenamiento, etc. (Skrinjar et al. 2013).

La producción de OTA aparece durante el crecimiento de la planta, siembra, almacenamiento o procesado (Rhouati et al. 2013). Su producción está relacionada con la temperatura, y la actividad de agua, aunque existen fluctuaciones impredecibles. OTA se ha encontrado en productos de origen vegetal (cereales, café, cacao…), animales (cerdo), incluso en pescados ahumados. En general, las principales fuentes son: cereales 58%, vino 15%, carne de cerdo 3%, y el café, que juega un rol variable según el grado de consumo, entre el 1-10%. El hongo puede contaminar también ambientes con humedad, de hecho, se ha detectado OTA en las salidas de ventilación de edificios (Malir et al. 2013).

Es una molécula estable que no se destruye bajo condiciones normales de cocción, tostado y fermentación (Rhouati et al. 2013). Temperaturas superiores a 250ºC durante varios minutos son necesarias para reducir su concentración, pudiéndose reducir

aproximadamente un 20% con el horneado, mientras que el hervido no la degrada (Malir et al. 2013).

Tiene efectos en animales y humanos, se ha relacionado con carcinogénesis, nefropatías crónicas, inmunotoxicidad, mielotoxicidad, teratotoxicidad, y genotoxicidad (Skrinjar et al. 2013; Rhouati et al. 2013), Además puede presentar sinergismo con otras micotoxinas. Es el compuesto más tóxico de las ocratoxinas, ya que estructuralmente es similar a la fenilalanina, de hecho, los mecanismos de toxicidad 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 muchos tipos celulares (Marin et al. 2013). La contaminación por OTA se ha relacionado con nefropatías en cerdos, con la nefropatía epidémica de los Balcanes (NEB) y alta incidencia de tumores en el tracto urinario (Marin et al. 2015). OTA está clasificada como grupo 2B (posible carcinógeno en humanos) por la IARC. El panel científico de contaminantes en alimentos (CONTAM) de la Autoridad Europea de Seguridad Alimentaria (EFSA) proveen una ingesta semanal tolerable (IST) es de 120 ng/kg pc/semana (EFSA, 2006).

1.2.3. Fumonisinas

Las Fumonisinas son diésteres del ácido propanotricarboxílico y polihidroxiaminas de cadena larga. Unos 18 análogos de fumonisinas han sido identificados y clasificados en las series A, B, C y P dependiendo de su estructura química. Las más estudiadas en alimentos son la B_1, B_2 y B_3 (CAS-RN 116355-83-0, 116355-84-1 y 136379-59-4 respectivamente) (Bryla et al. 2013).

Las FBs son micotoxinas producidas principalmente por hongos del género *Fusarium*, principalmente por *F. verticilloides* y *F. proliferatum*. (Bryla et al., 2016; Gallo et al. 2015). La contaminación de FBs se produce en todos los continentes. Para su síntesis el hongo necesita azúcares en el medio, especialmente amilopectina, pH neutro y presencia de nitrógeno; además de factores ambientales adecuados, temperatura (20-30ºC) y actividad de agua alta (0.95–0.99) (Bryla 2013). Se encuentran FBs frecuentemente contaminando maíz y sus derivados, cerveza, arroz, polvo de ajo, polvo de cebolla, te negro, higos de Túnez, cacahuetes y semillas de soja (Scott, 2012). Se calcula que más de la mitad del maíz y derivados alrededor del mundo están contaminados por FBs (Bryla et al. 2013). Aun siendo más frecuente la contaminación en granos que en harinas y derivados, que no son ingeridos directamente por el hombre, platean un problema para los

animales de granja (Milani et al. 2014), ya que causan grandes pérdidas económicas por disminución de la producción cárnica (Ahangarkani et al. 2014).

Las FBs son relativamente estables a tratamientos térmicos (Scott, 2012), una depleción significativamente de sus concentraciones se daría en procesos a 150ºC. El tostado en el caso del maíz produce reducciones de entre el 6-35% (Milani et al. 2014).

La fumonisina B_1 (FB_1) es una molécula carcinogénica, neurotóxica y embriotóxica (Scott, 2012). Estudios in vitro demuestran que la FB_1 es la más tóxica de todas las FBs, interfiere el proceso de crecimiento celular, permeabilidad de membrana y biosíntesis de esfingolípidos. Su toxicidad en humanos no está del todo clara, aunque se ha visto una fuerte correlación entre consumo de FBs y aumento de casos de cáncer esofágico en granjeros de África del Sur, África central, China y norte de Italia cuya dieta es rica en maíz, reduce el peso corporal en niños y pueden ser causantes de dolor intestinal y diarreas (Bryla et al. 2013). Las FBs están clasificadas como grupo 2B (posible carcinógeno para humanos) por la IARC. La IDT de FBs es de 2000 ng/kg pc/día (EFSA, 2014a).

1.2.4. Tricotecenos

Los TRCs comprenden una familia de unos 200 metabolitos secundarios (Raiola et al. 2015) relacionadas estructuralmente por su anillo tetracíclico sesquiterpeno 12,13-epoxytrichothec-9-ene. Se dividen en grupos de la A la D dependiendo en variaciones en su grupo funcional hidroxil y acetil. El tipo A están representados por HT-2 y T-2 (CAS-RN: 26934-87-2, 21259-20-1), el grupo B están representados por NIV, DON, 3aDON, 15aDON, DAS y NEO (CAS-RN: 23282-20-4, 51481-10-8, 50722-38-8, 88337-96-6, 2270-40-8, 36519-25-2,). Los TRCs C y D incluyen algunos TRCs de menor importancia (Marin et al. 2013).

Los TRCs A y B son sintetizados por varias especies de *Fusarium,* pero también por especies de *Trichoderma* (Raiola et al. 2015); sin embargo, los más importantes HT-2 y T-2 están producidos por *F. sporotrichioides, F. langsethiae, F. acuminatum,* y *F. poae*. Los principales productores de DON son *F. graminearum, F. culmorum*, y *F. cereales*. Estos hongos son importantes patógenos que contaminan los campos de cultivo en zonas de climas templados (Marin et al. 2013).

A nivel celular los TRCs se unen a los ribosomas e inhiben la síntesis de proteínas, en las células inmunitarias resulta con la producción de citoquinas proinflamatorias, los efectos postprandiales

son anorexia, dolor abdominal, diarrea, vómitos, pérdida de peso y cambios neuroendocrinos e inmunológicos (Lebrun et al. 2015). La IARC los clasifica como grupo 3 (no clasificable por su toxicidad en humanos). Sus IDT son para NIV 1200 ng/kg pc / día, para DON y sus metabolitos 1000 ng/kg pc/día, DAS 2000 ng/kg pc/día, suma de HT-2 y T-2 100 ng/kg pc/día (EFSA, 2014a).

Los TRCs en general son moléculas muy estables ya que no se degradan durante el almacenamiento, molienda, y algunos procesados (Marin et al 2013), no obstante, se ha visto una clara reducción de DON y T-2 tras el procesado térmico de alimentos (He et al., 2010)

1.2.4.1. Tricotecenos tipo B:

Entre los TRCs B los que tienen mayor impacto en la seguridad alimentaria son: DON, NIV, 3aDON, 15aDON, 4-ANIV, y fusaralenona-X (Pascuali y Migheli, 2014). DON es el menos tóxico de los TRCs, y es uno de los contaminantes más comunes en cereales en todo el mundo (Marin et al. 2013). No obstante, los productos contaminados con DON normalmente también están contaminados con sus metabolitos 15-acetyl deoxinivalenol y 3-acetyl deoxinivalenol (15-aDON y 3-aDON respectivamente), y en el caso de animales el DON puede ser

transformado en de-epoxy-deoxinivalenol (DOM-1), un metabolito con menor toxicidad (Wang et al. 2014). Se encuentra principalmente en cereales como trigo, maíz y cebada, y con menor frecuencia en arroz (Raiola et al. 2015).

En animales hay estudios que demuestran que bajas ingestas del DON estimulan el sistema inmune, pero a valores mayores lo deprimen. Además de manera aguda el DON afecta a la barrera intestinal en pollos, y causa dolor abdominal y diarrea (Raiola et al, 2015). Experimentos *in vivo* han demostrado un aumento del tamaño hepático, niveles altos de proteínas hepáticas en suero y albumina, y reducción del tamaño del tiroides (Marin et al. 2013). En humanos, sus efectos agudos son dolor abdominal, diarrea, mareo, dolor de cabeza, irritación de garganta, náuseas y vómitos. Los efectos crónicos son pérdida de peso, anorexia, malnutrición e inmunosupresión (Wang et al. 2014).

La contaminación de cereales por DON se puede prevenir separando los granos enmohecidos antes del almacenado; pudiendo alcanzar una reducción del 74%. La molienda aumenta la contaminación, ya que favorece la penetración del hongo. Durante el horneado se realiza una reducción de entre el 24-71% (Milani et al. 2014)

1.2.4.2. Tricotecenos tipo A:

Diacetoxyscirpenol (DAS), T-2 y HT-2 y sus metabolitos deacetilados son toxinas de *Fusarium*, en la actualidad representan un elevado riesgo para la salud humana (Yang et al. 2013).

La toxicidad de HT-2 ha sido menos estudiada, sin embargo, T-2 se metaboliza rápidamente a HT-2. T-2 es un potente inhibidor de la síntesis de proteínas en la mitocondria, estudios *in vivo* e *in vitro* demuestran efectos inmunosupresores y citotóxicos. Tienen fuerte efectos tóxicos en mucosas y membranas de la piel (Marin et al. 2013). Producen anorexia y vómitos, además de letargia, ataxia, hemorragias, sepsis y fallo cardiopulmonar (Lebrun et al. 2015). El DAS también presenta una alta toxicidad en animales, la combinación de T-2 con DAS puede producir lesiones orales (Yang et al. 2013).

Exposiciones prolongadas causan enfermedades conocidas como *Alimentary Toxic Aleukia* (ATA) caracterizada por la progresiva aparición de síntomas, que van desde ardor de boca y faringe, diarrea, náuseas, dolor abdominal y vómitos, hasta una destrucción asintomática de la médula ósea, causando progresiva leucopenia, linfocitosis y granulopenia; todo esto lleva a inmunosupresión, con sepsis y fiebre. Al final aparece neumonía que puede acabar en muerte del paciente (Marin et al. 2013).

1.2.5. Micotoxinas Emergentes

Las ME de *Fusarium* fueron descubiertas posteriormente a TRCs o fumonisinas (Tolosa et al. 2013), incluyen un conjunto de metabolitos secundarios como fusaproliferina, eniatinas (ENs): eniatina A, eniatina A_1, eniatina B y eniatina B_1 (ENA, ENA_1, EN_B y ENB_1, respectivamente), y beauvericina (BEA), entre otros (Serrano 2013). Son hexadepsipéptidos cíclicos, lo que le da a la molécula un carácter hidrófilo y lipófilo. Los aminoácidos valina e isoleucina se unen por enlaces peptídicos, y tienen enlaces éster intramoleculares tipo lactona (Jestoi, 2008).

Las especies que producen mayoritariamente estas toxinas son del genero *Fusarium*, en concreto, *F. verticilloides, F. proliferatum, F. subglutinans, F. oxysporum, F. poae,* y *F. avenaceum* (Sifou et al., 2011). La producción tiene lugar en diferentes zonas geográficas en todo el mundo, Italia, Túnez, Portugal, Marruecos, África del sur, Polonia, Noruega, España, y Croacia (Ficheux et al. 2012). Generando contaminación por micotoxinas en cereales, productos derivados de cereales (Vaclavikova et al. 2013), y en frutos secos; en concentraciones desde µg/kg hasta decenas de mg/kg (Tolosa et al. 2013).

Estas micotoxinas actúan como ionóforas y perturban el balance iónico fisiológico y pH de la membrana celular (Oueslati et al., 2011).

En la molécula hay una concentración de carga negativa, que puede formar complejos con iones metálicos (Jestoi, 2008). Por su carácter lipófilo es capaz de incorporarse a la membrana celular y formar poros de gran afinidad por iones como K^+, Na^+, Mg^{++}, y Ca^{++} alterando la homeostasis de la célula (Meca et al. 2011). La BEA es tóxica para varias líneas celulares humanas, puede inducir apoptosis y fragmentación del ADN. Además, parece ser un inhibidor selectivo de la colesterol acetiltransferasa (Oueslati et al., 2011). No obstante, el panel CONTAM de la EFSA ha publicado una opinión científica (EFSA, 2014b), declarando que existen escasos datos toxicológicos para establecer la IDT.

En algunos casos, el procesado de alimentos altera los niveles de contaminación de ME, tras la molienda no se degradan, pero parte de ellas se eliminan con el salvado y solo un 40% permanece en la harina (Vaclavikova et al. 2013); Y durante la cocción de productos derivados de cereales, como la pasta también se produce una reducción de gran parte de ME (Serrano et al. 2015).

1.3. Micotoxinas en café

Los estudios disponibles que evalúan la presencia de micotoxinas en café utilizan técnicas espectrométricas, con diferentes tipos de detección: ultravioleta visible, fluorescencia o espectrometría de masas (EM), el método de detección más utilizado es la EM. Respecto a la extracción, la mayoría de los autores debido a la complejidad de la matriz utilizan una purificación previa la determinación. Sibanda et al. (2002) analiza café verde mediante purificación de la muestra con columnas de inmunoafinidad (CIA) comerciales (Ocratest) y detección con Cromatografía Líquida de alta resolución (HPLC, *High Performance Liquid Chromatography*). Ventura et al., (2003) estudian OTA en café verde y tostado, analizado por HPLC con detector de fluorescencia, y confirmado con EM. Noba et al., (2009) estudian muestras de café en lata listo para tomar, analizando mediante cromatografía líquida (CL) acoplada a EM, y Noonim et al., (2009) estudian la presencia de FB en granos de café mediante un test comercial tipo ELISA y posterior confirmación con CL-EM/EM. Estos autores han observado que la técnica ELISA plantea falsos positivos y sobreestimación de las concentraciones. Desmarchelier et al. (2014) optimizan un método para el análisis simultáneo de OTA y AFs B_1, B_2, G_1, G_2, mediante extracción con QuEChErs y purificación con CIA para

la detección con CL-EM. En la bibliografía consultada no existen estudios de multimicotoxinas en muestras de café.

Respecto a las micotoxinas analizadas, la mayoría de autores estudian la presencia de OTA, que como ya hemos visto en el apartado de micotoxinas (**Tabla 1**) es la única micotoxina legislada en café según el reglamento 1881/ 2006 de la UE, si bien es cierto, algunos autores también analizan otras micotoxinas. Los análisis de micotoxinas en café hasta la fecha estudian tanto muestras de café verde, café tostado como de café preparado para su consumo, con resultados diversos. En el estudio del café verde llevado a cabo por Batista et al., (2003) detectan contaminación en 5 de las 40 muestras analizadas con concentraciones entre 0,64 y 4,14 µg/kg y una concentración media 2,45 µg/kg. En un estudio con 16 muestras de café tostado, Bandeira, (2012) detecta la presencia de OTA en el 31% de las muestras con concentraciones entre 0,09 a 9,00 µg/kg. Casal et al., (2014) analizan 10 muestras de café soluble detectando OTA en concentraciones entre 0,15 y 11,80 µg/kg. En el análisis de 30 muestras de café listo para su consumo Noba et al., (2009) detectan contaminación en todas las muestras con concentraciones de hasta 0.037ng/ml. Otros autores (Sibanda et al., 2002, Ventura et al., 2003) tras el estudio de café verde y tostado no detectan contaminación por OTA.

Algunos autores han señalado la presencia de OTA en muestras con concentraciones superiores a los LMP (5,0 µg/kg en café tostado y 10,0 µg/kg en café soluble): Nielsen et al. (2015) estudian café tostado detectando OTA en concentraciones que exceden la legislación vigente en el 10% de las muestras analizadas, con un máximo de 21,0 µg/kg; Bandeira et al. (2012) detectan 2 muestras de café tostado con concentraciones superiores a 5,0 µg/kg; Tozlovanu y Pfohl-Leszkowicz (2010) detectan contaminación en el 70% de las muestras de café tostado con concentraciones entre 0,5 y 3 µg/kg, y una muestra presenta una contaminación de 11,9 µg/kg. Por lo tanto, los estudios disponibles muestras que la OTA está presente en el café y que algunas muestras exceden los niveles máximos permitidos.

Los estudios de FB llevados a cabo en muestras de café verde detectan FB_2, FB_4, y FB_6 a concentraciones superiores a 164 µg/kg (Nielsen et al., 2015), y en concentraciones entre 1,90 a 9,70 µg/kg en el 33% de las muestras (Noonim et al., 2009). Tras el proceso de tostado, las FB no han sido detectadas (Nielsen et al., 2015).

Las AFs también son estudiadas con incidencias del 76,5% y 54,6% en muestras de café verde y café tostado y concentraciones medias de 4,28 y 2,85 µg/kg, respectivamente (Soliman, 2002).

Como se puede observar, los estudios de micotoxinas en café son llevados a cabo principalmente en el café como materia prima. No obstante, como ya se ha comentado en el apartado anterior, algunos tratamientos afectan al contenido de micotoxinas en alimentos. De hecho, es posible encontrar estudios acerca de los efectos durante el procesado del café, encontrado resultados dispares: La Pera et al. (2008) describen una reducción de la cantidad de OTA desde el 65 al 100%, con diferencias entre los diferentes tipos de procesado, por ejemplo, el procesado con cafetera tradicional tipo moka produce reducciones más intensas que el café tradicional turco. Santini et al. (2011) detectan que durante la preparación de la bebida parte de la concentración de OTA pasa desde el café tostado a la bebida, pero no más del 80%. En cambio, Tozlovanu y Pfohl-Leszkowicz (2010) tras el procesado detectan concentraciones desde el 13 al 141% de la concentración inicial, es decir, observan desde la reducción hasta el aumento de las concentraciones de OTA, posiblemente por extracción y concentración de la misma. Otras micotoxinas también se ven afectadas por el tostado, como las FB que sufren una destrucción total (Nielsen et al., 2015), o las AFs en café verde, que dependiendo de la temperatura y el tipo de tostado pueden sufrir degradaciones del 42,2 al 55,9% (Soliman, 2002).

1.4. Evaluación del riesgo

La estimación de la ingesta es un elemento esencial para la cuantificación del riesgo. La evaluación de la ingesta diaria estimada (IDE) combina datos de consumo con los datos de contaminantes en los alimentos.

La evaluación de la exposición a un determinado contaminante se lleva a cabo teniendo en cuenta el consumo de alimentos de la dieta total, para ello es necesaria la evaluación del aporte de cada alimento que puede contribuir a dicha exposición. Y mediante la suma de los aportes de los diferentes alimentos, que consume una determinada población, conocer la exposición.

Además, el estudio de un alimento de manera individual presenta una mayor flexibilidad para poder calcular la exposición en diferentes segmentos de la población. Por ejemplo, el café es consumido en bajas cantidades con respecto a la dieta total y determinados segmentos de la población no lo consumen; sin embargo, una parte de la población consume café de manera diaria y en algunos casos en grandes cantidades (OMS, 2009).

La EFSA ha desarrollado de forma armonizada una compilación de datos de consumo de alimentos en 22 estados miembros. Esta

recopilación de datos de consumo clasifica los alimentos y bebidas de una manera estandarizada. Además, el consumo de alimentos de manera individual en diferentes segmentos de la población como niños, adolescentes, adultos, etc., y representando también los diferentes percentiles de consumo. Esto supone una herramienta para la estimación del riesgo probabilística y determinística. Para la presente tesis se ha usado la base de datos de la EFSA, en concreto los datos correspondientes a España, para respetar la zona geográfica en que fueron tomadas las muestras usadas para realizar el estudio de la evaluación del riesgo. Los datos de consumo de café correspondientes a España fueron recopilados por parte de la AECOSAN en 2009, abarcando a 1067 sujetos de los cuales 981 adultos y 86 adolescentes. Otros segmentos de la población no fueron considerados como es el caso de niños. En la **tabla 3** se muestran los datos de consumo de café obtenidos (EFSA, 2015).

Se han descrito diferentes formas de llevar a cabo la evaluación del riesgo. La Organización Mundial de la Salud (OMS) en 2009 publicó las recomendaciones del programa internacional de seguridad química (OMS, 2009). Según estas recomendaciones la evaluación de la exposición por la dieta se puede realizar: como evaluación de la población general con los datos de consumo de la media de la

Tabla 3: datos de consumo de café en individuos españoles, según la base de datos EFSA, conforme a la encuesta realizada por AECOSAN (EFSA, 2015)

	Consumo[a]			
	Café tostado		Café soluble	
	Adolescentes	Adultos	Adolescentes	Adultos
Individuos consumidores	13%	38%	16%	42%
Segmento de la población				
Media	0,0031	0,0135	0,0096	0,0246
95th	0,0274	0,0625	0,0444	0,0769
97.5th	0,0345	0,0778	0,1067	0,1167
99th	0,0462	0,0904	0,2564	0,4088

[a]Consumo expresado como g de café por kg de peso corporal (habitante) y día

población (se contempla un único valor para definir la exposición de la población) es decir, estudio determinístico, o teniendo en cuenta los diferentes percentiles de consumo en la población: 90th, 95th, 97.5th, estudio probabilístico.

Con el estudio probabilístico se puede caracterizar la exposición de toda la población de una forma más precisa, diferenciando los grupos de consumidores. Con la estimación determinística nos reporta un resultado más manejable para ser usado posteriormente, para compararlo con la IDT y hacer una aproximación del riesgo de la población.

Siendo el cálculo de la IDE determinística:

$$IDE = \frac{\text{Consumo de café diario por un individuo medio en una población}}{\text{Contenido en micotoxinas del alimento caso de estudio}}$$

Estando el consumo expresado como gramos por kg de peso corporal y por día, y el contenido de micotoxinas expresado en nanogramos por gramo de alimento. La evaluación de la ingesta probabilística se basa en un complejo método matemático en el que se enfrentan dos rangos de datos con una distribución dependiente de los hábitos de consumo de la población y de la distribución de la contaminación del producto. Este cálculo se realiza frecuentemente con el método matemático Monte-Carlo, en el que se multiplican ambas variables dando como resultado una función que describe la IDE de la población. No obstante, los métodos probabilísticos están desaconsejados en el caso de toxicidad crónica, ya que entran en juego otras variantes como variaciones en el consumo a lo largo del tiempo de los individuos, cambio de hábitos, etc. Por ello, también se puede realizar un estudio determinístico refinado, en el que se realiza la evaluación de la IDE a los diferentes percentiles de la población según

su ingesta. De esta forma no se obtiene un valor único medio que describe a la población total, sino que se obtiene un rango de exposición, dependiente de la ingesta de cada individuo.

Además, se pueden marcar subescenarios en cuanto a la concentración de micotoxinas, pudiendo situarnos en la cota inferior (lower bound) considerando la concentración en las muestras por debajo del límite de cuantificación (LOQ) igual a cero, o en la cota superior (upper bound) considerando las muestras por debajo del límite de detección (LOD) con una concentración igual al LOD, y las muestras con resultados entre el LOD y el LOQ con valor igual al LOQ.

Con estos resultados se puede proceder a la evaluación del riesgo. Para ello se comparan los datos de la IDE con los datos de IDT, o con los de ingesta IST propuestos por la CE (CE, 1996; EFSA, 2006, EFSA 2014a, EFSA, 2015).

2. OBJECTIVES

2. OBJETIVOS:

En este contexto, el **objetivo** de la presente tesis es la evaluación del riesgo del consumo de café en cuanto a su contenido en micotoxinas.

Para alcanzar el objetivo propuesto se plantean los siguientes **objetivos parciales**:

1. Puesta a punto y validación de la metodología analítica para el análisis multimicotoxina en muestras de café tostado y café procesado.
2. Evaluación de la contaminación y presencia simultánea de micotoxinas en diferentes tipos de café y tras diferentes tipos de procesados.
3. Evaluación del riesgo de exposición a micotoxinas por consumo de café en los diferentes grupos de población.

OBJECTIVES:

In this context, the **objective** of the present study is to perform a mycotoxins risk assessment for coffee consumption.

To achieve this objective, the following **partial objectives** have been proposed:

1. Development and validation of new analytical methodologies to identify and quantify mycotoxins in roasted coffee and coffee beverage samples.
2. Evaluate the contamination levels and co-occurrence of mycotoxins in different coffee types and after different coffee preparations.
3. Perform a mycotoxins risk assessment for coffee consumption, attending different population groups.

3. RESULTS

3.1. Simultaneous determination of mycotoxin in commercial coffee

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Simultaneous determination of mycotoxin in commercial coffee

*Ana García-Moraleja, Guillermina Font, Jordi Mañes, Emilia Ferrer**

Laboratory of Toxicology and Food Chemistry, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

* Corresponding author:
Emilia Ferrer
Tel.: 0034 963544950
fax: 0034 963544954.
E-mail address: emilia.ferrer@uv.es

ABSTRACT

Mycotoxins are secondary metabolites produced by filamentous fungi that usually contaminate food products. Coffee is a natural product susceptible to mycotoxin contamination. The present study evaluates the presence of nivalenol, deoxynivalenol, T-2 and HT-2 Toxin, diacetoxyscirpenol, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, fumonisin B_1, fumonisin B_2, ochratoxin A, zearalenone, enniatin A, enniatin A_1, enniatin B, enniatin B_1, and beauvericin in coffee samples, using liquid chromatography tandem mass spectrometry (LC-MS/MS). The results show that zearalenone was not present in any sample. In the positive samples the contents of fumonisins ranged from 58.62 to 537.45 µg/kg, emerging mycotoxins ranged from 0.10 to 3569.92 µg/kg, aflatoxins ranged from 0.25 to 13.12 µg/kg, and trichothecenes, excepting nivalenol, ranged from 5.70 to 325.68 µg/kg. Nivalenol presented the highest concentrations, from 0.40 to 25.86 mg/kg. Ochratoxin A ranged from 1.56 to 32.40 µg/kg, and five samples exceeded the maximum limit established by the European Commission.

Keywords: Mycotoxins, roasted coffee, torrefacto, instant coffee, mass spectrometry.

1. Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi, such as *Aspergillus spp., Penicillium spp.,* and *Fusarium spp.*, in the matrix where they grow (Rocha, Freire, Erlan Feitosa Maia, Izabel Florindo Guedes, & Rondina, 2014). Hypotheses about their functions on the producer fungus are still being studied, but they probably perform ecological functions against other organisms and protect fungi from oxidative stress (Reverberi, Ricelli, Zjalic, Fabbri, & Fanelli, 2010). Mycotoxins are classified into different groups depending on their molecular structure, producer fungus, and toxicity: Trichothecenes, which comprise nivalenol (NIV), deoxynivalenol (DON), diacetoxyscirpenol (DAS) and T-2 and HT-2 Toxin, are molecules that produce the inhibition of protein synthesis (Rocha, Freire, Erlan Feitosa Maia, Izabel Florindo Guedes, & Rondina, 2014). Aflatoxins are produced by *Aspergillus spp*. and are classified as aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), and aflatoxin M_1 (AFM_1). The International Agency for Research on Cancer (IARC, 2012) classified AFB_1, AFB_2, AFG_1, and AFG_2 as carcinogenic to humans, mostly in liver cells. Fumonisins B are classified as fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2) and are hepatotoxic and immunotoxic molecules (Köppen et al., 2010). Ochratoxin A (OTA) produces nephropathies and urothelial tumors in

humans (Amezqueta et al., 2012). Zearalenone (ZEA) has estrogenic effects (Afsah-Hejri, Jinap, Hajeb, Radu, & Shakibazadeh, 2013). Emerging mycotoxins, such as enniatin A, enniatin A_1, enniatin B, enniatin B_1, and beauvericin (ENA, ENA_1, ENB, ENB_1, and BEA, respectively) have only recently been studied (Serrano, Font, Mañes, & Ferrer, 2013), and some studies show that they can represent a potential risk for the human health because of their toxic effects in cell lines (Prosperini, Meca, Font, & Ruiz, 2012).

The stimulant properties of coffee, probably because of its caffeine content and the lifestyle trends of the population, have led to increased coffee beverage consumption each year (International Coffee Organization ICO, 2013). In Spain, a total of 80,000.00 tons of coffee (ground and instant coffee) are consumed annually (Ministry of Agriculture Food and Environment, 2014). Coffee is one of the most consumed food products, with an important economic and cultural role. It is cultivated in a total of 75 countries on four continents, with Brazil the largest manufacturer of coffee, with approximately one third of the world production (Cabrera & Gimenez, 2010). Fungi from *Fusarium* genera cause diseases in coffee plants (*Coffea arabica* and *Coffea canephora*). They penetrate through the aerial portions of the plants, but the first symptoms start on the roots (Nina, Smeltekop, Almanza, & Loza-Murguia, 2011). The contamination of coffee by

filamentous fungi can occur at various stages, harvesting, preparation, transportation, or storage, and in fermentation and drying, especially where the water activity is lower (Silva, Batista, & Schwan, 2008).

Some studies have reported mycotoxin contamination in coffee. The coffee legislation of mycotoxins (European commission (EC) 1881/2006) sets maximum limits (ML) of OTA: 5.0 µg/kg (in roasted coffee beans and ground roasted coffee, excluding soluble coffee), and 10.0 µg/kg (in soluble coffee-instant coffee). In fact the most studied mycotoxin in coffee is OTA (Paterson, Lima, & Taniwaki, 2014). The presence of non-regulated mycotoxins in coffee as aflatoxins, fumonisins, patulin and sterigmatocystin has been studied (Rahmani, Jinap, & Soleimany, 2009). A study of green coffee reported concentrations of FB_2 ranging from 9.70 to 13.00 µg/kg (Noonim, Mahakarnchanakul, Nielsen, Frisvad, & Samson, 2009). Nielsen, Ngemela, Jensen, Medeiros, and Rasmussen (2015) studied green, roasted and instant coffee (n = 57) and reported concentrations of OTA and FB_2 (mean: 2.83 µg/kg and maximum: 8.30 µg/kg; mean: 25 µg/kg and maximum: 136 µg/kg respectively). A qualitatively study has been conducted in 30 green coffee samples, results showed incidences of 10, 17, 23, 7, 17, and 7% of OTA, (AFB_1 + AFB_2), AFG_1, PAT and STG respectively (Bokhari & Aly, 2009). Other studies reporting non contamination of coffee samples with OTA and AFs or not

reporting data from real samples has been published (Desmarchelier et al., 2014; Khayoon, Saad, Salleh, Manaf, & Latiff, 2014; Sibanda, De Saeger, Barna-Vetro, & Van Peteghem, 2002; Ventura et al., 2003). Publications about method validations or multi matrix studies reporting concentrations of OTA in green and roasted coffee show contamination levels ranging from 0.09 to 9.00 µg/kg (Bandeira, 2012); ranging from 0.64 to 4.14 µg/kg (Batista, Chalfoun, Prado, Schwan, & Wheals, 2003); of 23.70 µg/kg (Imperato, Campone, Piccinelli, Veneziano, & Rastrelli, 2011); ranging from 0.80 to 5.40 µg/kg (Lobeau, De Saeger, Sibanda, Barna-Vetro, & Van Peteghem, 2005).

A recent study on mycotoxins and climate change (Paterson et al., 2014) advised the necessity of urgent consideration of mycotoxins in coffee. As far as we know, no data have been published to evaluate the simultaneous presence of different groups of mycotoxins produced by *Fusarium* and/or *Aspergillus* (aflatoxins, OTA, ZEA, fumonisins, trichothecenes, and emerging mycotoxins) in coffee.

In this context, the aim of the present study was to evaluate the contamination levels and co-occurrence of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, DON, NIV, DAS, T-2, HT-2, ZEA, ENA, ENA_1, ENB, ENB_1, and BEA in different coffee samples using Ultra Turrax liquid extraction

and Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) with triple quadrupole (QqQ) mass analyzer determination.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (AcN) and methanol (MeOH) were provided by Merck (Darmstadt, Germany). Ammonium formiate (99%) was supplied by Panreac Quimica (Barcelona, Spain) (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were passed through a 0.45 mm cellulose filter from Scharlau (Barcelona, Spain). The standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Individual stock solutions of ENA_1, ENB, ENB_1, and BEA with a concentration of 1000 mg/L, and ZEA, NIV, DON, FB_1, FB_2 and ENA with a concentration of 500 mg/L, were prepared in MeOH. Aflatoxins and OTA were prepared at 500 mg/L, and DAS, T-2 and HT-2 at 100 mg/L were prepared in AcN. The solutions were stored in glass-stoppered bottles in the dark in secure conditions at -20ºC. These stock solutions were diluted with AcN/MeOH [50:50] to obtain the appropriate working concentrations before LC-MS/MS analysis.

2.2. Sample collection

Commercial samples of coffee (103 samples) were purchased from different supermarkets located in Valencia (Spain). One sample of green coffee beans was kindly provided from a coffee roaster (Asturias, Spain). All samples were collected during 2013-2014. The method of sampling was accomplished according to the Commission Regulation (EC401/2006) for the official control of the ML established for OTA in roasted coffee beans, ground roasted coffee and soluble coffee. The samples were classified according roasting process: 28 samples of natural roasted coffee and 75 samples of torrefacto roasted coffee (special industrial roasted coffee with additional sugar to increase the flavor). Also, the samples were classified according the caffeine content: 52 samples of caffeinated coffee and 40 samples of decaffeinated coffee. These samples were traditional packing and pre-portioned capsules. In addition, 11 milk and coffee pre-portioned coffee capsules (composed by soluble coffee and milk powder) were monitored.

2.3. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA) consisting of an autosampler, a quaternary pump, a

pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Version 4.1, was used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm x 4.6 mm I.D., 5 mm particle size) analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a C_{18} guard column (4 mm x 2 mm I.D.), using a gradient that started at 80% A (5 mM ammonium formiate in H_2O) and 20% B (MeOH), increased linearly to 95% B in 10 min, followed by a linear decrease to 80% B in 5 min, then to 70% B in 10 min. Afterward, the initial conditions were kept constant for 5 min. The flow rate was 0.2 ml/min **Fig 1** shows the chromatograms from a spiked sample during validation of the method as a proof of the method is suitable and the separation accurate enough. Chromatograms include the mass-to-charge ratio (m/z) of precursors and product ions and specify the retention time of each mycotoxin. The analysis was performed in positive and negative ion mode. The electrospray ionization source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens, 0.5 V; source temperature, 100 ºC; desolvation temperature, 300 ºC; desolvation gas (nitrogen 99.99% purity) flow, 800 L/h; cone gas 50 L/h (nitrogen 99.99% purity). For the instrument parameters, full scans and daughter scans under positive and negative modes were used. In addition, each compound was also characterized by the retention time.

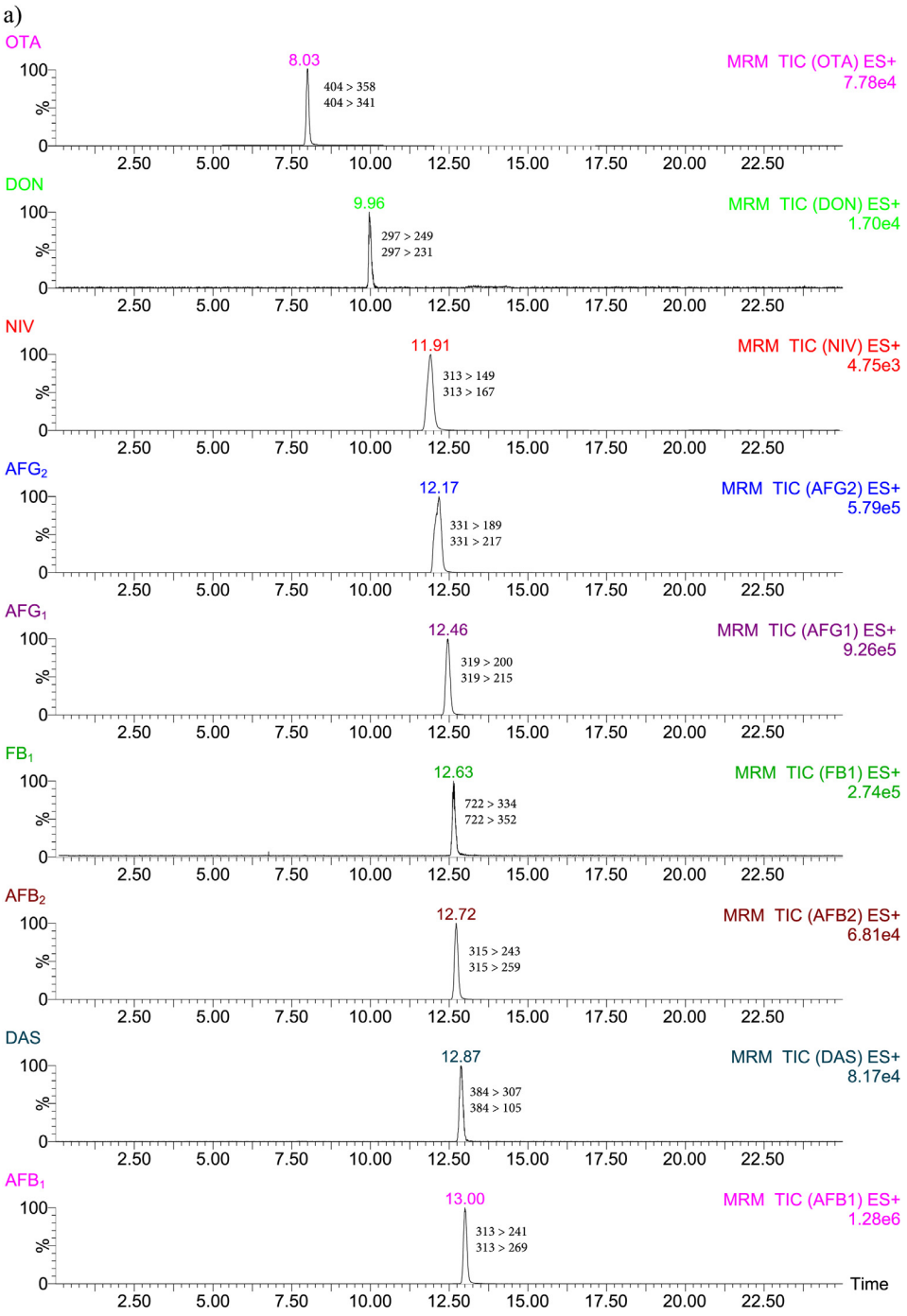

Fig. 1. a) Chromatogram of a 1 mg/kg spiked sample of each mycotoxin (OTA, DON, NIV, AFG_2, AFG_1, FB_1, AFG_2, DAS, AFB_1).

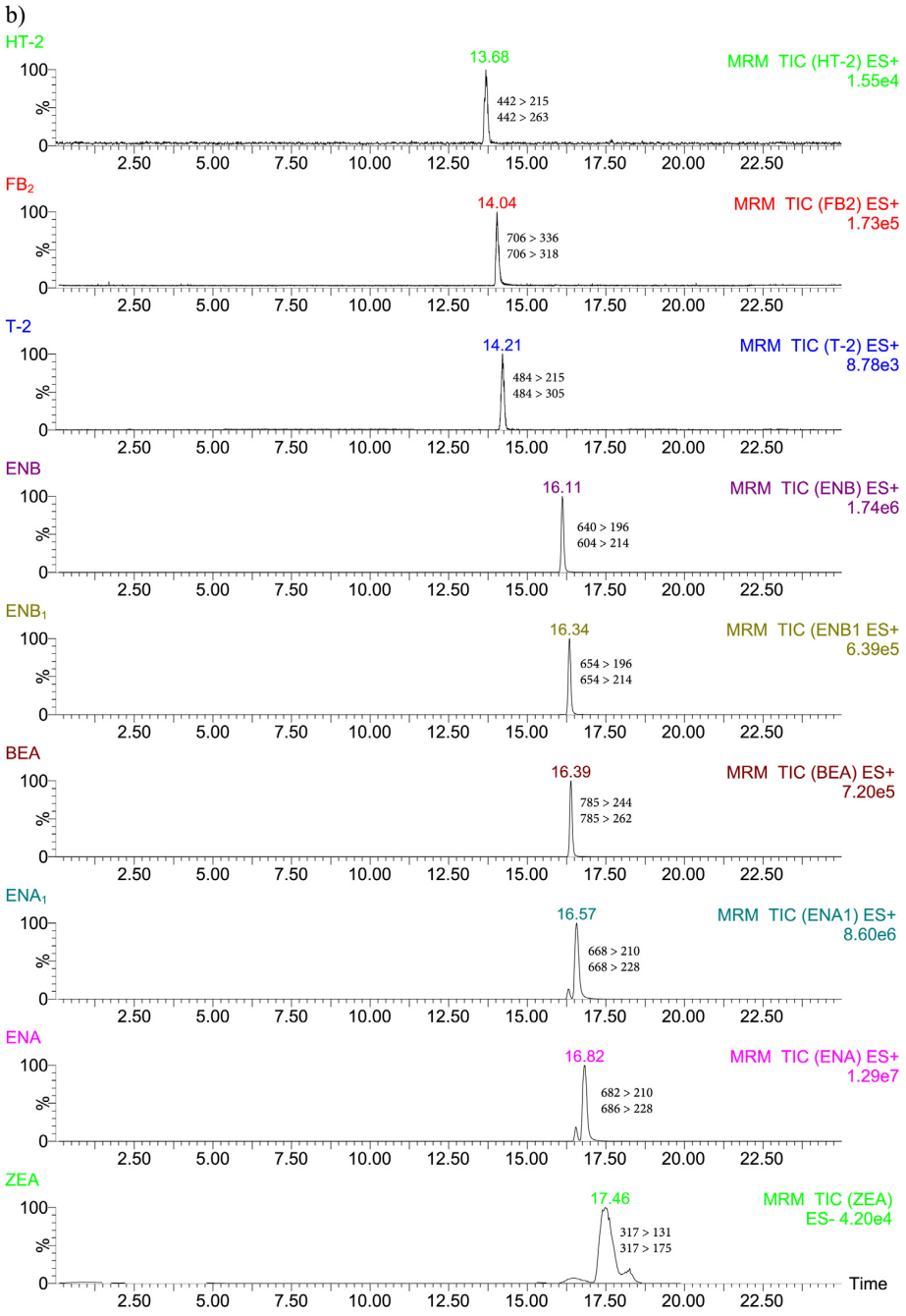

Fig. 1. b) Chromatogram of a 1 mg/kg spiked sample of each mycotoxin (HT-2, FB2, T-2, ENB, ENB1, BEA, ENA1, ENA, ZEA).

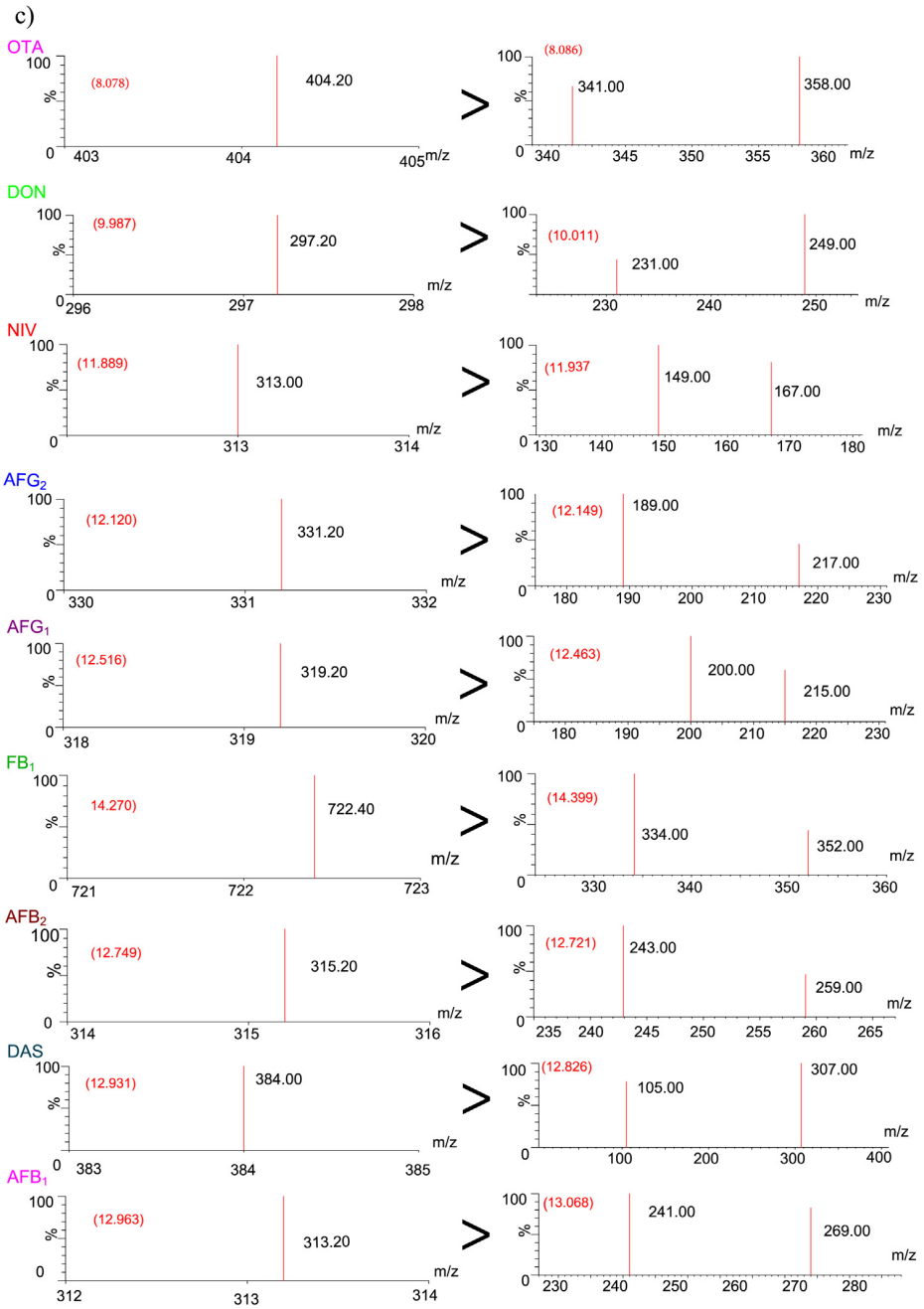

Fig. 1. c) Mass spectrum with precursor ion (left) and products ions (right) of each mycotoxin (OTA, DON, NIV, AFG_2, AFG_1, FB_1, AFG_2, DAS, AFB1).

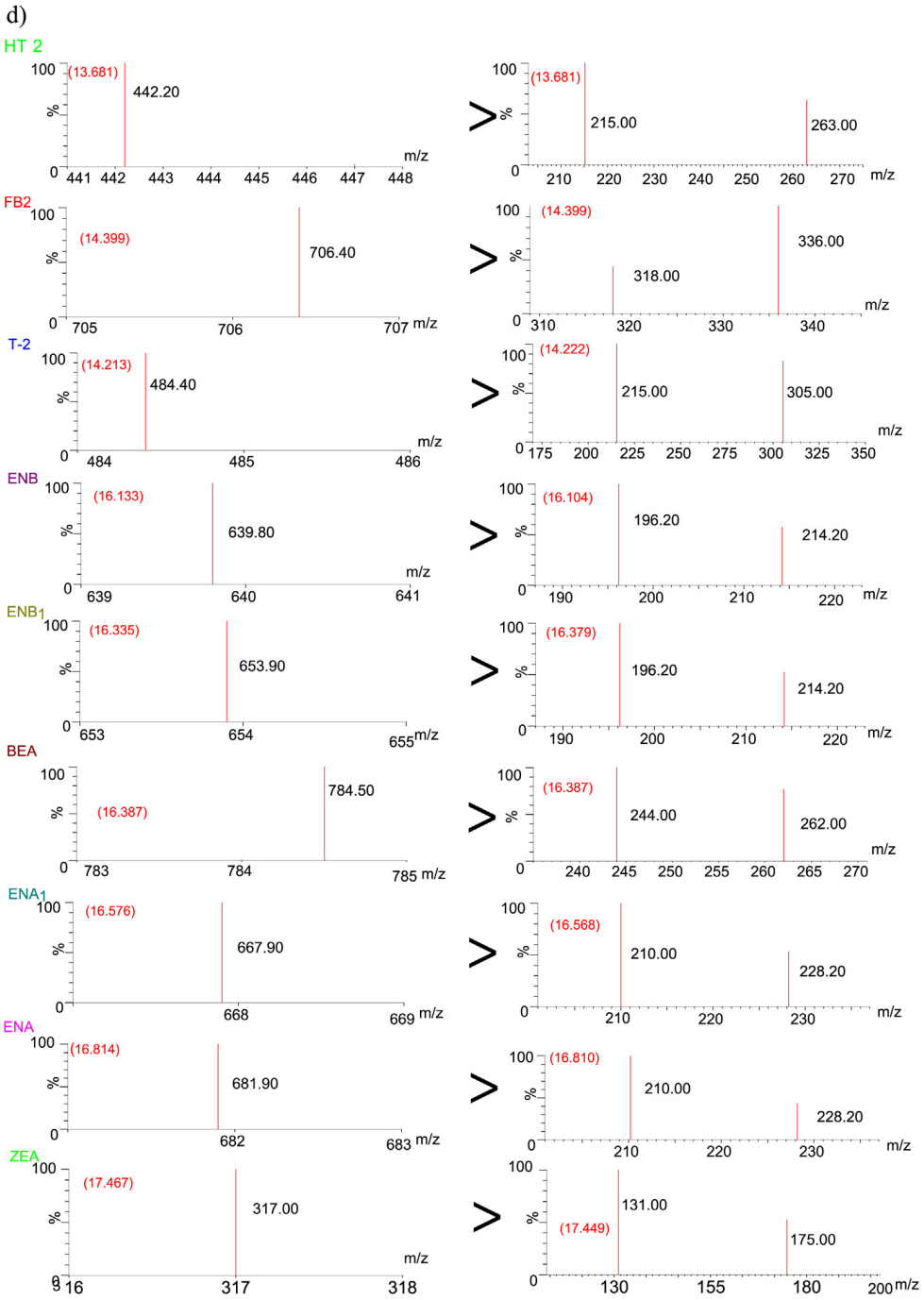

Fig. 1.) Mass spectrum with precursor ion (left) and products ions (right) of each mycotoxin (HT-2, FB2, T-2, ENB, ENB_1, BEA, ENA_1, ENA, ZEA).

The criteria adopted for accepting the analysis was a retention time deviation lower than 2.5% compared to the standard. Ideal fragmentation conditions were determined by testing different cone voltages and collision energies for each compound during infusion of pure standard. The selected parameters that gave the most abundant fragment ion (collision energies (eV) and cone voltages (V)) are shown in **Table 1**. The analyzer settings were as follows: resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, -3 and 1; multiplier, 650; collision gas (Argon 99.995% purity) pressure, 3.83 x 10^{-3} mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass spectrometer was operated in Multi Reaction Monitoring (MRM) mode using two transitions (Quantification and Confirmation) and the ion ratio between transitions, according with the European criteria (EC657/2002). The selected precursor and product ions are shown in **Table 1**. The precursor ion was the protonated form in most cases, excepting DAS, HT-2 and T-2, which were ammonium adducts

2.4. Sample preparation and extraction procedure

Conventional coffee samples were ground with a blender to obtain 0.1 cm particles. For pre-portioned coffee, 15 capsules of each

sample were opened and collected until the aliquots weighed 100 g. All aliquots were shaken vigorously.

For the extraction, 5 g of sample was extracted with 50 ml of AcN/H_2O [80:20] in Ultra Turrax (Ika T18 basic, Staufen, Germany) for 3 min. The extract was centrifuged for 15 min at 5 ºC and 4500 rpm. The supernatant was evaporated to dryness with a Büchi Rotavapor (R-200 Flawil, Switzerland). The reconstituted extract with 10 ml of MeOH was filtered and purified using C_{18} columns (Waters, Milford, Massachusetts) and powdered activated carbon by applying a slight vacuum. Then, the solvent was evaporated again with a Turbovap LV Evaporator (Zymark, Hoptikinton, USA). The extract was reconstituted with 1 ml of AcN/MeOH [50:50] and filtered with a 0.22 mm nylon filter (Membrane Solutions, Texas, USA) before injection on the LC-MS/MS (QqQ). All samples were processed in triplicate.

3. Results and discussion

3.1. Method validation

The analytical method was validated for coffee samples with a blank sample (mycotoxin-free coffee sample). The analytical parameters are shown in **Table 1**. The evaluation of the matrix effects

was performed using matrix-assisted calibration curves; the suppression of the signal (SS) was obtained for all mycotoxins (between 21 and 47%). The detection limit (LOD) for each mycotoxin was calculated using a signal-to-noise ratio of 3. The limit of quantification (LOQ) was calculated using a signal-to-noise ratio of 10. The accuracy was evaluated through recovery studies (n = 6) at two concentration levels: 10 LOQ and 100 LOQ (**Table 2**). For the evaluation of the linearity, calibration curves were constructed for all mycotoxins at six concentration levels from LOQ to 1 mg/kg for all mycotoxins. Special calibration curves for highly contaminated samples were made for ENB (from 1 to 10 mg/kg) and for NIV (from 5 to 50 mg/kg). The results showed good correlation coefficients $R^2 > 0.992$). The intra-day precision was assessed by six determinations at each addition level on the same day, whereas interday precision was assessed by one determination at each addition level for three days. The relative standard deviations ranged between 4 and 12% for the intra-day precision and between 5 and 15% for the inter-day precision. The recovery values ranged from 72 to 112%. Therefore, the results were in accordance with the limits established by the EC (EC657/2002).

Table 1
LC-MS/MS (QqQ) optimized parameters and analytical parameters of the validated method

	Cone (V)	Collision energy (eV)		Precursor ion (m/z)		Product ion		SS[c]	LOD (µg/kg)	LOQ (µg/kg)	Linearity (R^2)
		Q[a]	q[b]			Q[a]	q[b]				
ENA	25	25	25	682	$[M+H]^+$	210	228	40	0.15	0.50	0.999
ENA_1	35	35	35	668	$[M+H]^+$	210	228	43	0.08	0.25	0.997
ENB	35	35	35	640	$[M+H]^+$	196	214	33	0.15	0.50	0.999
ENB_1	13	13	12	654	$[M+H]^+$	196	214	40	0.02	0.50	0.998
BEA	30	30	20	785	$[M+H]^+$	244	262	46	0.03	0.10	0.998
NIV	22	35	35	313	$[M+H]^+$	149	167	45	75.00	85.24	0.997
DON	20	10	10	297	$[M+H]^+$	249	231	21	13.12	20.50	0.995
DAS	15	15	45	384	$[M+NH_4]^+$	307	105	22	3.00	5.00	0.999
HT-2	10	14	16	442	$[M+NH_4]^+$	215	263	34	10.00	35.50	0.995
T-2	10	15	45	484	$[M+NH_4]^+$	215	305	47	5.00	12.50	0.998
OTA	24	30	30	404	$[M+H]^+$	358	341	21	1.13	1.45	0.996
AFB_1	47	30	30	313	$[M+H]^+$	241	269	47	0.10	0.25	0.998
AFB_2	50	30	30	315	$[M+H]^+$	243	259	40	1.00	1.50	0.996
AFG_1	43	40	30	319	$[M+H]^+$	200	215	35	0.10	0.25	0.999
AFG_2	46	45	25	331	$[M+H]^+$	189	217	35	0.50	0.75	0.998
FB_1	30	30	30	722	$[M+H]^+$	334	352	31	16.00	53.33	0.993
FB_2	30	30	30	706	$[M+H]^+$	336	318	44	16.13	53.75	0.992
ZEA	25	25	25	317	$[M+H]^-$	131	175	22	6.00	12.50	0.993

[a]*Q:* Quantification ion
[b]*q:* Confirmation ion
[c]SS: Suppression of Signal (Matrix Effect) = (slope matrix-matched/slope standard in solvent)*100

Table 2
Experimental values of recovery ± intra-day and inter- day relative standard deviation

Mycotoxin	10 LOQ			100 LOQ		
	Concentration (µg/kg)	intra-day (n =6)	inter-day (n = 6)	Concentration (µg/kg)	intra-day (n =6)	inter-day (n = 6)
ENA	5.00	91 ± 4	90 ± 6	50.00	92 ± 5	91 ± 5
ENA_1	2.50	86 ± 9	88 ± 8	25.00	89 ± 4	89 ± 5
ENB	5.00	109 ± 9	112 ± 5	50.00	104 ± 7	106 ± 10
ENB_1	5.00	97 ± 11	95 ± 15	50.00	98 ± 5	98 ± 5
BEA	1.00	94 ± 4	94 ± 5	10.00	95 ± 3	95 ± 9
NIV	850.00	72 ± 4	76 ± 5	8500.00	73 ± 4	73 ± 9
DON	200.00	86 ± 8	85 ± 7	2000.00	89 ± 2	88 ± 4
DAS	50.00	82 ± 6	80 ± 9	500.00	84 ± 4	83 ± 6
HT-2	350.00	73 ± 4	73 ± 7	3500.00	71 ± 6	74 ± 6
T-2	125.00	82 ± 12	84 ± 11	1250.00	85 ± 10	84 ± 11
OTA	15.00	84 ± 8	78 ± 12	150.00	85 ± 4	83 ± 5
AFB_1	2.50	83 ± 7	81 ± 11	25.00	83 ± 5	84 ± 5
AFB_2	15.00	76 ± 9	75 ± 11	150.00	77 ± 3	76 ± 4
AFG_1	2.50	75 ± 5	74 ± 6	250.00	76 ± 4	76 ± 4
AFG_2	7.50	82 ± 5	78 ± 10	75.00	83 ± 3	79 ± 9
FB_1	500.00	84 ± 5	80 ± 12	5000.00	86 ± 4	85 ± 6
FB_2	500.00	88 ± 8	88 ± 7	5000.00	89 ± 4	88 ± 6
ZEA	125.00	77 ± 6	77 ± 7	1250.00	78 ± 4	76 ± 5

3.2. Monitoring study

3.2.1. Occurrence of mycotoxins

The samples analyzed in the present study were contaminated with at least six mycotoxins, excepting four samples (three caffeinated coffee samples and one decaffeinated coffee sample) that were mycotoxins-free. Most samples (61%) showed co-occurrence ranging from 10 to 12 mycotoxins. The occurrence and levels of the concentrations of mycotoxins in the samples are reported in **Fig. 2**. The MRM chromatogram and the product ions spectrum corresponding to a sample of proportioned caffeinated coffee naturally contaminated with OTA (11.43 µg/kg) is shown in **Fig. 3**

NIV was detected in most samples (96%). ZEA was not detected in any sample. ENB_1 and DAS were detected in a lower number of samples (12%). ENA_1, ENB, DON, HT-2, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, and FB_2 were detected in more than 50% of the analyzed samples.

3.2.2. Influence of coffee type

Incidence, mean, minimum and maximum concentration of each mycotoxin are shown according the different classification in **Table 3a** (caffeinated and decaffeinated), **Table 3b** (natural and torrefacto roasting), and **Table 3c** (milk added coffee).

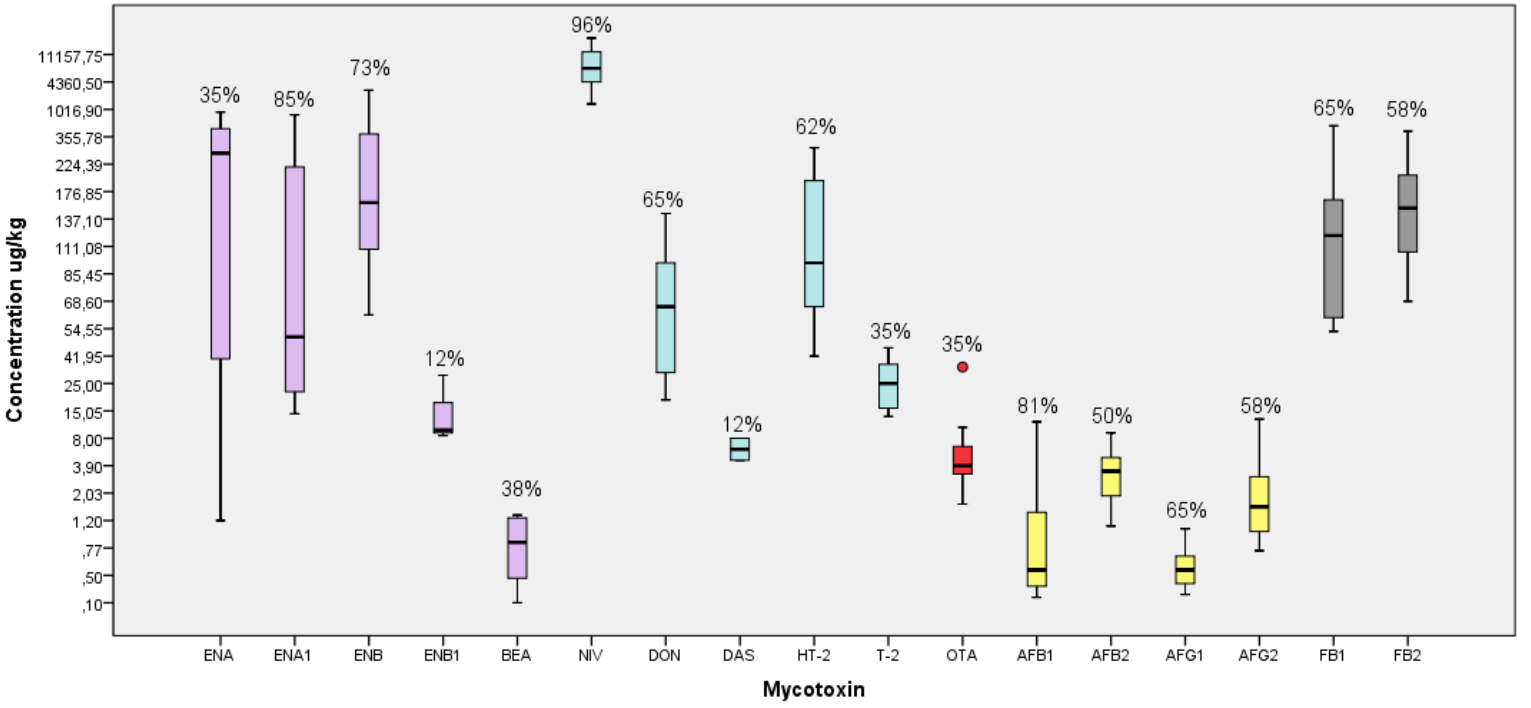

Fig. 2: Concentration (µg/kg) and occurrence (%) of mycotoxins in analyzed samples (n=103).

Contamination levels of aflatoxins, OTA and trichothecenes are similar in caffeinated and decaffeinated coffee (**Table 3a**). Unexpectedly, emerging mycotoxins and fumonisins show lower concentrations. As it is reported by Paterson et al. (2014), some strains of *Aspergillus spp*. produced higher amount of mycotoxin in presence of caffeine. It is possible that fumonisins and emerging mycotoxins producing fungi present the same behavior. Soliman (2002) shows the highest aflatoxins production by *Aspergillus flavus* on decaffeinated green and decaffeinated roasted coffee.

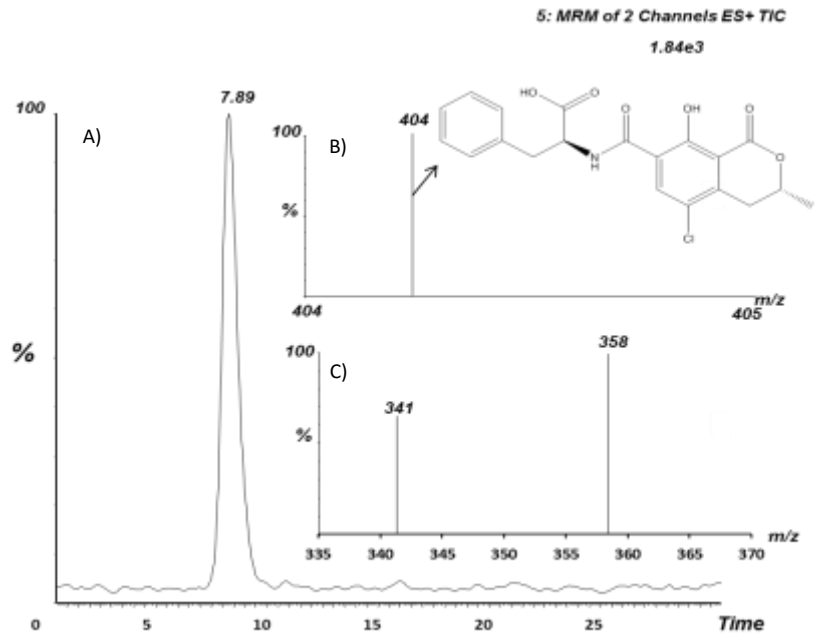

Fig. 3: Chromatogram and mass spectrum of a sample contaminated with OTA. A) HPLC-MS/MS QqQ chromatogram of an OTA contaminated sample. B) Mass spectrum of the precursor ion of OTA and the chemical structure. C) Mass spectrum of the product ion of OTA.

However, in the present study no differences were achieved on aflatoxins contamination levels between caffeinated and decaffeinated coffee. Differences between torrefacto and natural roasting were evaluated (**Table 3b**). The contents of aflatoxins, OTA, and fumonisins were similar. Trichothecenes and Emerging mycotoxins present higher concentration in torrefacto roasted coffee. Torrefacto coffee is usually associated with low quality coffee beans. The presence of trichothecenes and emerging mycotoxins in higher concentrations is probably the result of the use of low quality coffee beans previously contaminated.

Regarding the milk added coffee, samples had the lowest variety of mycotoxins, but these mycotoxins appear in similar or higher concentrations than roasted coffee (**Table 3c**). OTA were not detected in milk added coffee (composed by instant coffee and milk powder), in disagree with Lee, Saad, Khayoon, and Salleh (2012) that reported higher occurrence of OTA in instant coffee than in roasted coffee. The concentrations of the most mycotoxins occurring in this coffee type were higher than in roasted coffee. Reverberi et al., (2010) reported the enhancer of OTA production with the addition of lactose in the growth medium. As this coffee type is proportioned in isolated capsules the contamination is less probable, but according Lee et al., (2012) and

Table 3a
Occurrence (%), mean (µg/Kg) and range of mycotoxins (µg/kg) in samples classified according the decaffeination process

	Caffeinated				Decaffeinated			
Mycotoxin	Occurrence n= 52	Mean	Range		Occurrence n = 40	Mean	Range	
			Min	Max			Min	Max
AFB_1	69	0.55	0.25	12.48	100	0.71	0.25	2.33
AFB_2	60	3.60	1.65	7.27	40	3.41	1.50	6.00
AFG_1	69	0.54	0.28	1.41	70	0.57	0.25	1.00
AFG_2	56	1.05	0.75	3.15	60	1.55	0.84	13.12
OTA	13	4.12	1.91	11.43	73	4.35	1.56	32.40
NIV	94	6605.73	401.85	13071.24	98	8694.14	2641.95	25855.23
DON	71	67.40	20.65	148.52	75	73.56	20.60	129.86
DAS	23	6.50	5.70	8.56	0	-	-	-
HT-2	52	91.10	49.05	176.85	73	99.41	41.95	276.93
T-2	35	33.00	26.25	45.12	45	45.69	13.45	105.04
FB_1	87	130.00	58.62	537.45	55	75.32	58.81	182.62
FB_2	81	151.17	85.45	369.66	45	168.17	68.62	362.22
ENA	37	374.30	1.20	935.53	43	111.58	7.32	597.74
ENA_1	87	179.40	13.75	749.33	83	50.93	18.83	199.50
ENB	54	577.00	83.11	3569.92	90	128.56	59.15	205.30
ENB_1	8	10.20	10.03	12.46	13	12.25	10.20	15.61
BEA	35	0.67	0.10	1.23	48	0.87	0.14	1.34

Table 3b
Occurrence (%), mean (µg/Kg) and range of mycotoxins (µg/kg) in samples classified according the roasting process (Natural or Torrefacto)

Mycotoxin	Torrefacto				Natural			
	Occurrence n = 69	Mean	Range		Occurrence n = 23	Mean	Range	
			Min	Max			Min	Max
AFB_1	77	0.67	0.25	12.48	100	0.55	0.25	2.33
AFB_2	49	3.63	1.50	7.27	57	3.30	1.65	5.70
AFG_1	70	0.57	0.25	1.41	70	0.50	0.30	1.00
AFG_2	58	0.97	0.75	7.18	57	2.03	0.98	13.12
OTA	42	4.57	1.56	32.40	30	3.92	3.14	4.23
NIV	94	8370.97	401.85	25855.23	100	5215.50	1684.35	13071.24
DON	74	84.04	20.65	148.52	70	29.96	20.60	86.12
DAS	17	6.48	5.70	8.56	0	-	-	-
HT-2	67	94.90	41.95	225.68	43	99.40	67.45	276.90
T-2	42	43.13	13.45	105.04	30	21.25	16.25	26.25
FB_1	74	103.97	58.62	174.00	70	142.80	58.76	537.45
FB_2	68	155.99	85.40	369.66	57	154.60	68.62	201.60
ENA	42	288.41	1.20	935.53	30	176.89	7.32	346.45
ENA_1	84	136.84	13.75	749.33	87	115.24	19.01	355.78
ENB	64	423.33	59.15	2220.80	87	199.35	136.4	3569.92
ENB_1	13	11.51	10.03	15.61	0	-	-	-
BEA	35	0.82	0.47	1.34	57	0.68	0.10	1.23

Reverberi et al. (2010) when the fungi infect the food the production of mycotoxins is more intense.

Table 3c
Occurrence (%), mean (µg/Kg) and range of mycotoxins (µg/kg) in pre-portioned milk added samples (n=11)

	Occurrence (n=11)	Range	
		Min	Max
AFB_1	64	0.97	1.4
AFB_2	45	3.21	10.01
AFG_1	27	0.39	1.54
AFG_2	64	2.81	6.88
OTA	0		
NIV	100	3483.47	8353.47
DON	0		
DAS	0		
HT-2	73	107.52	325.68
T-2	0		
FB_1	0		
FB_2	0		
ENA	0		
ENA_1	91	57.54	224.39
ENB	100	290.22	659.27
ENB_1	27	14.82	29.54
BEA	0		

In green coffee a total of 11 mycotoxins were detected (ENA_1, ENB, NIV, DON, DAS, HT-2, T-2, AFG_1, AFG_2, FB_1, and FB_2); according Ventura et al., (2003) OTA has not been detected. The concentrations found for each mycotoxin were similar to the roasted samples, in disagreement with Soliman (2002) that report a reduction on AFs during roasting. It is not possible to make conclusions because only one green coffee sample was analyzed.

3.2.3. Occurrence and concentration levels in the different groups of mycotoxins

ENA_1 and ENB were the most common mycotoxins (85% and 73%, respectively). In the literature, no data are available for coffee samples. However, these results were consistent with Serrano et al. (2013) who found ENA_1 and ENB with frequencies of 81% and 76%, respectively, in conventional pasta. However, ENB_1 was lower for Serrano et al., (2013) (65%), but not as low as in this study. The concentrations of ENA ranged from 1.20 to 935.53 µg/kg, ENA_1 ranged from 13.75 to 749.33 µg/kg, ENB ranged from 0.06 to 3.57 mg/kg, ENB_1 ranged from 10.03 to 29.54 µg/kg, and BEA ranged from 0.10 to 1.34 µg/kg.

The concentrations of NIV ranged from 0.40 mg/kg in a sample of conventional caffeinated coffee to 25.86 mg/kg in a sample of decaffeinated coffee in pre-portioned capsules. DON and HT-2 were the two most common trichothecenes in occurrence (65% and 62%, respectively). The other trichothecenes were detected at low concentrations and low occurrences. The lowest was DAS with 12% of samples contaminated. Comparison with other studies is very difficult, even impossible, because a very limited number of publications are available.

OTA was not detected in the green coffee sample, although other studies (Batista et al., 2003; Bokhari, 2007; Imperato et al., 2011; Napolitano, Fogliano, Tafuri, & Ritieni, 2007; Pittet & Royer, 2002; Vanesa & Ana, 2013; Ventura et al., 2003; Visconti & De Girolamo, 2005) reported a natural occurrence in a range of concentrations (from 0.1 to 360 µg/kg).

OTA was detected in the 35% of the roasted coffee samples. **Fig. 3** shows a chromatogram and mass spectrum of the product ions of a sample contaminated with OTA. The concentration in positive samples ranged from 1.50 to 32.40 µg/kg. According to other studies (Bandeira, 2012; Batista et al., 2003; Casal, Vieira, Cruz, & Cunha, 2014; Coronel, Marin, Cano, Ramos, & Sanchis, 2011; Lee et al., 2012; Lobeau et al., 2005; Tozlovanu & Pfohl-Leszkowicz, 2010), a similar range of concentrations was obtained (from 0.15 to 23.70 µg/kg). Sibanda et al. (2002) did not detect OTA in coffee samples.

In the present study, some commercial coffee samples (5 of 103) exceeded the ML. Two samples were decaffeinated conventional coffee with concentrations of 9.30 and 6.20 µg/kg, two samples were pre-portioned caffeinated coffee with concentrations of 6.91 and 11.43 µg/kg, and one sample was pre-portioned decaffeinated coffee. In addition, the sample of decaffeinated pre-portioned coffee exceeded

six times (32.40 µg/kg) the ML proposed by EC legislation. Our results are similar to other studies. Some authors also detected OTA levels higher than the ML: 5.24 µg/kg by Coronel et al. (2011), 5.4 µg/kg by Lobeau et al. (2005), 9 µg/kg by Bandeira (2012), 15.08 µg/kg by Tozlovanu and Pfohl-Leszkowicz (2010), and 5.7 µg/kg in roasted coffee and 13.66 µg/kg in instant coffee by Vanesa and Ana (2013).

Aflatoxins had high occurrence but low concentrations (mean concentration: 1.95 µg/kg). No significant differences were found among the different coffee types. The concentrations ranged from 0.15 to 12.48 µg/kg Soliman (2002) obtained, for green coffee and roasted coffee, concentrations from 0.76 to 8.92 µg/kg, and Bokhari and Aly (2009) obtained concentrations from 5 to 23 µg/kg Khayoon et al. (2014) detected aflatoxins in canned coffee. Imperato et al. (2011) did not detect aflatoxins in green coffee.

The occurrence of fumonisins in the contaminated samples was 65 and 58% with concentrations from 0.06 to 0.54 mg/kg and from 0.07 mg/kg to 0.37 mg/kg for FB_1 and FB_2, respectively. The levels obtained for FB_2 were higher than those obtained by Noonim et al. (2009) in ground coffee (10 µg/kg).

4. Conclusion

A new method for the simultaneous analysis of NIV, DON, DAS, HT-2, T-2, OTA, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, ZEA, and the emerging mycotoxins ENA, ENA_1, ENB, ENB_1, and BEA in different coffee samples was validated. The analysis of the different commercial types of coffee showed the presence and coexistence of mycotoxins in every coffee type. The only legislated mycotoxin in coffee (OTA) showed a low incidence, but in some samples (5 of 26 samples), it exceeded the established ML. OTA was also detected over the ML, so it must be given special consideration. The absence of international legislation limits for the other mycotoxins in coffee, the toxicological effects, and the levels obtained in this study indicate that it is necessary to pay special attention to the evaluation of the presence of mycotoxins in coffee. It is important to evaluate the influence of technological and culinary practices to determine the population exposure.

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3.2. Development of a new method for the simultaneous determination of 21 mycotoxins in coffee beverages by liquid chromatography tandem mass spectrometry

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Development of a new method for the simultaneous determination of 21 mycotoxins in coffee beverages by liquid chromatography tandem mass spectrometry

*Ana García-Moraleja, Guillermina Font, Jordi Mañes, Emilia Ferrer**

Laboratory of Toxicology and Food Chemistry, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

* Corresponding author:
Emilia Ferrer
Tel.: 0034 963544950
fax: 0034 963544954.
E-mail address: emilia.ferrer@uv.es

Abstract

A newmethod for the simultaneous detection of 21mycotoxins (ochratoxin A, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, sterigmatocystin, nivalenol, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyl deoxynivalenol, diacetoxyscirpenol, neosolaniol, HT-2 toxin, T-2 toxin, fumonisin B_1, fumonisin B_2, enniatin A, enniatin A_1, enniatin B, enniatin B_1, and beauvericin) in coffee beverageswas internally validated. The method is based on liquid / liquid extractionwith a mixture of ethyl acetate/formic acid (95:5 v/v) and detection using triple quadrupole (QqQ) and ion trap (IT) liquid chromatography tandem mass spectrometry. The limits of detection and quantification were 0.02 to 39.64 µg/kg, respectively, and the correlation coefficients were optimal for all mycotoxins ($R^2 \geq 0.992$). The recovery values ranged from 72% to 97%. The developed method was demonstrated in six real samples of roasted and instant coffee, caffeinated and decaffeinated coffee, and coffee with sugar added. The analyses indicate the presence of the studied mycotoxins in coffee beverages at µg/kg concentrations. Ochratoxin A, a mycotoxin that is regulated in coffee, was detected in two samples under the maximum limit established by a European legislation (CE1881/2006).

Keywords: Aflatoxin Ochratoxin A Trichothecene Fumonisin B Emerging mycotoxin

1. Introduction

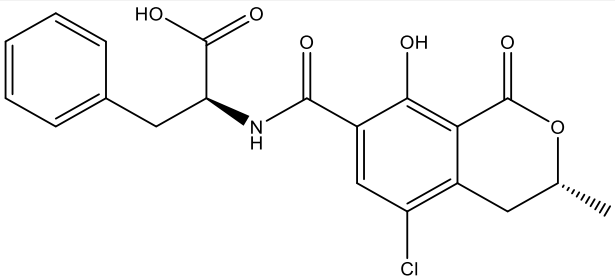
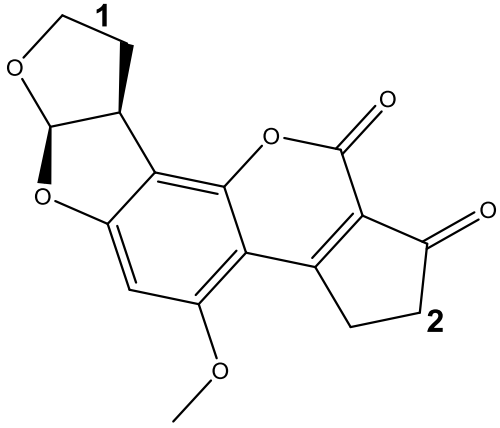
Coffee is a natural product that is rich in phenolic compounds, which are responsible for the astringency, flavor, antioxidant activity, and caffeine content of coffee; in moderate doses, coffee has a stimulating effect and reduces fatigue (Andrade et al., 2012; Cheong et al., 2013). These properties lead to the wide consumption of coffee beverages, with important economic and cultural roles (International Coffee Organization ICO, 2013).

Mycotoxins are secondary metabolites of filamentous fungi that are present in agricultural commodities (Schatzmayr & Streit, 2013). There are approximately 400 recognized mycotoxins, but only a few of them are present in food commodities (FAO, 2004; Sulyok, Krska, & Schuhmacher, 2010). Some mycotoxins are regulated in foods (e.g., aflatoxins (AFs), fumonisins B (FBs), and trichothecenes (TRs)) in most countries (Commission Regulation, EC1881/2006; U.S.F.D.A., 2005). In the case of coffee products, ochratoxin A (OTA) is the only regulated mycotoxin (Commission Regulation, EC1881/2006) and has maximum limits (MLs) of 5 μg/kg in roasted coffee beans and ground roasted coffee and 10 μg/kg in soluble coffee (IC). No specific MLs are established for mycotoxins in coffee in countries out of the European Union.

Mycotoxins are classified into different groups depending on their chemical structures (**Table 1**); these groups include OTA, AFs [aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2)], sterigmatocystin (STG), TRs [nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-aDON), 15-acetyldeoxynivalenol (15-aDON), diacetoxyscirpenol (DAS), neosolaniol (NEO), T-2 toxin (T-2) and HT-2 toxin (HT-2)], FBs [fumonisin B_1 (FB_1), fumonisin B_2 (FB_2)] and EMs [enniatin A (ENA), enniatin A_1 (ENA_1), enniatin B (ENB), enniatin B_1 (ENB_1), beauvericin (BEA)]. Different mycotoxins have different toxicological effects such as the production of nephropathies (OTA), carcinogenicity (AFs), hepatotoxicity (FBs), immunosuppressive properties (AFs), and estrogenic effects (ZEA) (Edite Bezerra da Rocha, Freire, Erlan Feitosa Maia, Izabel Florindo Guedes, & Rondina, 2014; I.A.R.C., 2014; Köppen et al., 2010).

The presence of toxigenic fungi in coffee plants, coffee beans, and roasted coffee have already been described. The fungi of *Fusarium spp., Aspergillus spp.*, and Ochratoxigenic species have been found in coffee plants and coffee beans (Gamboa-gaitán, 2012; Posada & Vega, 2006; Rezende et al., 2013; Serani, Taligoola, & Hakiza, 2007; Silva, Batista, & Schwan, 2008)

Table 1
Classification of mycotoxins in groups, their chemical structure, chemical formula, radicals and classification by IARC (international agency for research on cancer)

MYCOTOXIN	FORMULA	RADICALS	IARC[a] Clasiffication	CAS-RN[c]
Ochratoxin A				
				
OTA	$C_{20}H_{18}ClNO_6$	-	2B	303-47-9
Aflatoxins				
				
AFB_1	$C_{17}H_{12}O_6$	1_ Double bond 2_ C Alkane	1	1162-65-8
AFB_2	$C_{17}H_{14}O_6$	1_ Single bond 2_ C Alkane	1	7220-81-7
AFG_1	$C_{16}H_{10}O_7$	1_ Double bond 2_ O Carboxil	1	1165-39-5
AFG_2	$C_{16}H_{12}O_7$	1_ Single bond 2_ O Carboxil	1	7241-98-7

[a]IARC classification: Group 1: Carcinogenic to humans; Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans; Group 4: Probably not carcinogenic to humans (International Agency for Research in Cancer).
[b]NC: not classified by IARC.
[c]CAS-RN: Chemical Abstracts Service – Registry Number (American Chemical Society).

Table 1 (Continued)

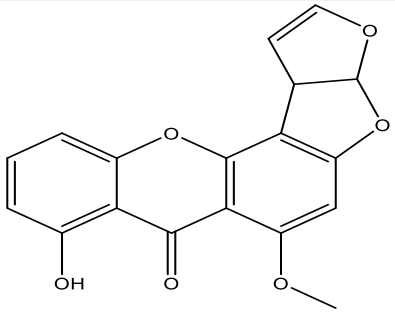
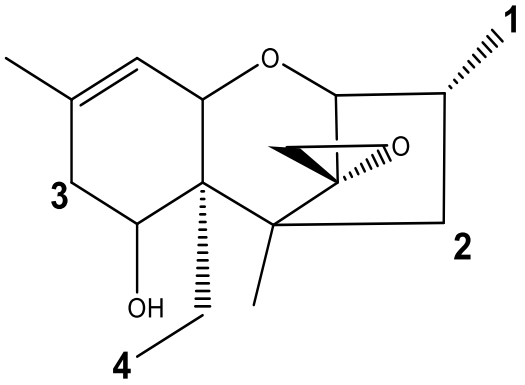
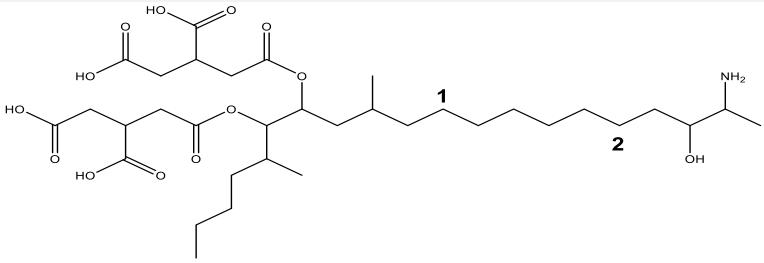
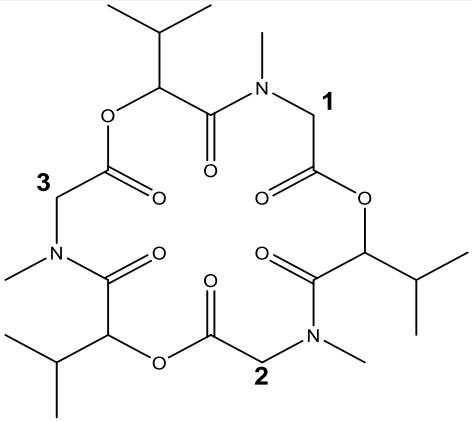
MYCOTOXIN	FORMULA	RADICALS	IARC[a] Clasiffication	CAS-RN[c]
Sterigmatocystin				
				
STG	$C_{18}H_{12}O_6$	-	2B	10048-13-2
Trichothecenes				
				
NIV	$C_{15}H_{20}O_7$	1_OH; 2_OH; 3_O Carbonyl; 4_OH	3	23282-20-4
DON	$C_{15}H_{20}O_6$	1_OH; 2_C Alkane; 3_O Carbonyl; 4_OH	3	51481-10-8
3aDON	$C_{17}H_{22}O_7$	1_OAc; 2_C Alkane; 3_O Carbonyl; 4_OH	3	50722-38-8
15aDON	$C_{17}H_{22}O_7$	1_OH; 2_OH; 3_O Carbonyl; 4_OAc	3	88337-96-6
DAS	$C_{19}H_{26}O_7$	1_OH; 2_OAc; 3_ C Alkane; 4_OAc	3	2270-40-8
NEO	$C_{19}H_{26}O_8$	1_OH; 2_OAc; 3_OH; 4_OAc	3	36519-25-2
HT-2	$C_{22}H_{32}O_8$	1_OH; 2_OH; 3_ 3-methylbutanoathyl; 4_OH	3	26934-87-2
T-2	$C_{24}H_{34}O_9$	1_OH; 2_OAc; 3_3-methylbutanoathyl; 4_OAc	3	21259-20-1

Table 1 (Continued)

MYCOTOXIN	FORMULA	RADICALS	IARC[a] Clasiffication	CAS-RN[c]
Fumonisins B				
				
FB_1	$C_{34}H_{59}NO_{15}$	1_OH 2_OH	2B	116355-83-0
FB_2	$C_{34}H_{59}NO_{14}$	1_ C Alkane 2_OH	2B	116355-84-1
Emerging Mycotoxins				
				
ENA	$C_{36}H_{63}N_3O_9$	1_*Sec*-butyl 2_*Sec*-butyl 3_*Sec*-butyl	NC[b]	2503-13-1
ENA_1	$C_{35}H_{61}N_3O_9$	1_*Sec*-butyl 2_*Sec*-butyl 3_*Iso*-propyl	NC	4530-21-6
ENB	$C_{33}H_{57}N_3O_9$	1_ *Iso*-propyl 2_ *Iso*-propyl 3_ *Iso*-propyl	NC	917-13-5
ENB_1	$C_{34}H_{59}N_3O_9$	1_ *Iso*-propyl 2_ *Iso*-propyl 3_ *Sec*-butyl	NC	19914-20-6
BEA	$C_{45}H_{57}N_3O_9$	1, 2 and 3_phenyl	NC	26048-05-5

Regarding the mycotoxin contents in coffee, OTA, AFs, STG, FB_2, and patulin have been studied in green and roasted coffees (Bandeira, 2012; Batista, Chalfoun, Prado, Schwand, & Wheals, 2003; Bokhari & Aly, 2009; Desmarchelier et al., 2014; Imperato, Campone, Piccinelli, Veneziano, & Rastrelli, 2011; Lobeau, De Saeger, Sibanda, Barna-Vetró, & Van Peteghem, 2005; Noonim, Mahakarnchanakul, Nielsen, Frisvad, & Samson, 2009; Rahmani, Jinap, & Soleimany, 2009; Sibanda, Saeger, & Van Peteghem, 2002 and Ventura et al., 2003). Moreover, some authors have studied how the different steps of the coffee production process (drying, fermentation, roasting, packing, and brewing) affect mycotoxin contents (La Pera et al., 2008; Romani, Pinnavaia, & Dalla Rosa, 2003; Santini et al., 2011; Soliman, 2002; Tozlovanu & Pfohl-Leszkowicz, 2010). Only OTA and AFs have been studied in coffee beverages (Casal, Vieira, Cruz, & Cunha, 2014; Khayoon, Saad, Salleh, Manaf, & Latiff, 2014; Tozlovanu & Pfohl-Leszkowicz, 2010; Noba, Uyama, & Mochizuki, 2009).

Different authors have developed analytical strategies for the determination of multi-mycotoxins in liquid foods. The presence of TRs, FBs, AFs, and EMs has been studied in malt, beer, milk, and water (Zöllner & Mayer-Helm, 2006). Multi-mycotoxin methods for the simultaneous analysis of different mycotoxins (TRs, FBs, AFs, STG, OTA and EMs) in cheese, food supplements, and wine have been developed

using extraction with methanol (MeOH), ethyl acetate, formic acid, and acetonitrile and detection with liquid chromatography tandem mass spectrometry (LC-MS/MS) (Kokkonen & Jestoi, 2009; Mavungu et al., 2009; Pizzutti et al., 2014). However, to our knowledge, there are no data regarding the occurrence of multi-mycotoxins in coffee beverages.

In this context, the aim of this study was to develop a new, rapid, sensitive, and reproducible analytical strategy to identify and quantify 21 mycotoxins (OTA, AFB_1, AFB_2, AFG_1, AFG_2, STG, NIV, DON, 3-aDON, 15-aDON, DAS, NEO, HT-2, T-2, FB_1, FB_2, ENA, ENA_1, ENB, ENB_1, and BEA) in coffee beverages. For this purpose, the extraction process and multi-mycotoxin determination by triple quadrupole (QqQ) and ion trap (IT) LC-MS/MS were optimized. In addition, the developed and optimized analytical method was applied to determine the mycotoxin contents in different coffee beverages.

2. Experimental

2.1. Chemicals

Acetonitrile, ethyl acetate, and MeOH were provided by Merck (Darmstadt, Germany). Formic acid and the mycotoxin and casein standards were provided by Sigma-Aldrich (St. Louis, MO, USA).

Ammonium formiate (99%) and $K_2Fe(CN)_6$ were supplied by Panreac Química (Barcelona, Spain). $ZnSO_4 \cdot 7H_2O$ and graphitized carbon black were provided by Alfa AESAR (Karlsruhe, Germany). The micro-solid phase dispersion sorbent was octadecyl (C_{18})-bonded silica from Analisis Vinicos (Ciudad Real, Spain). Deionized water (H_2O; <18MΩ cm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System from Millipore (Bedford, USA). All solvents were passed through a 0.45-μm cellulose filter from Scharlau (Barcelona, Spain) and degassed for 10 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT, USA) before use. Carrez solutions were prepared in the laboratory with $ZnSO_4 \cdot 7H_2O$ (1.0M) for solution (I) and $K_2Fe(CN)_6$ (0.3 M) for solution (II). A mixture of ethyl acetate:formic acid (95:5 v/v) was prepared daily with 25 ml of formic acid and 500 ml of ethyl acetate. Individual stock solutions of OTA, AFs (AFB_1, AFB_2, AFG_1, and AFG_2), STG, TRs (NIV, DON, 3-ADON, 15-ADON, DAS, T-2, HT-2, and NEO), FBs (FB_1 and FB_2), and EMs (ENA, ENA_1, ENB, ENB_1, and BEA) with concentrations of 500 mg/l were prepared in MeOH, and two 50-ml multi-mycotoxin standard solutions were prepared: one in methanol and one in methanol with the matrix from a mycotoxin-free sample (mycotoxin-free sample was selected from a previous study in non-processed roasted and instant coffee). The concentrations of the multi-mycotoxin standard solutions were 1 mg/l

of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, NIV, DAS, T-2, HT-2, ENA, ENA_1, BEA, and ENB, 5 mg/l of ENB_1, and 10 mg/l of DON. The solutions were stored in safe conditions at −20 °C. These stock solutions were diluted with MeOH to obtain the appropriate working concentrations immediately before use.

2.2. Samples

To optimize the analytic method for the determination of multimycotoxins in coffee beverages, different commercial coffee samples were selected based on their compositions. All samples were purchased at a local supermarket (Valencia, Spain). A total of six coffee samples were used: three samples of ground roasted coffee and three samples of instant coffee. The ground coffee samples were classified as follows: one sample of natural roasted coffee (NRC), one sample of torrefacto roasted coffee (TRC; special industrially roasted coffee with added sugar to enhance the flavor), and one sample of decaffeinated natural roasted coffee (DC). The instant coffee samples were classified as follows: one sample of instant coffee (IC), one sample of instant coffee with sugar (ICS), and one sample of instant coffee with sugar and milk (ICSM).

All coffee samples were stored at room temperature. After their packages had been opened, they were put into specific glass food containers at 4 °C and analyzed within three days.

2.3. Coffee preparation

Ground coffee samples (NRC, TRC, and DC) were processed to reproduce home brewing using an Italian coffeepot (Italian moka). This pot consists of three compartments: the first contains water (50 ml); the second is funnel shaped, separated from the first compartment by a metallic filter, and contains ground coffee (5 g); and the third is initially empty on the top of the coffeepot. When the water in the first compartment is heated, the temperature and pressure in this compartment increase; when the internal pressure overcomes the external pressure, the water flows through the coffee in the second compartment at approximately 98.6 °C, and the final coffee beverage then rises into the third compartment. The process starting with the heating of the water until the final coffee beverage is produced is 5 to 7 min long (Gianino, 2007).

Instant coffee beverages (IC, ICS, and ICSM) were prepared according to the brand recommendations. Water was heated to 85 °C on a hotplate and was added to a glass beaker with the measured instant coffee powder.

The beverages were kept in specific glass containers and analyzed immediately.

2.4. Extraction procedures

2.4.1. Clarification

The pigments of coffee samples increase the matrix effects during mass spectrometry detection. To improve the sensibility and accuracy, different cleanup procedures were studied. Cleanup methods that have been previously used in mycotoxin analysis, such as reverse phase silica C_{18} cartridges and graphitized carbon black (Rahmani et al., 2009), along with methods used previously in the extraction of different compounds from coffee samples, such as precipitation with Carrez solutions (Perrone, Farah, & Donangelo, 2012) and casein (Niseteo, Komes, Belščak-Cvitanović, Horžić, & Budeč, 2012) were studied.

The coffee beverages (roasted or instant) were each initially clarified with 1 ml of Carrez solution in 100 ml of distilled water. The solution was shaken vigorously and filtered after 30 min. A 20-ml aliquot of this filtered solution was referenced as a purified solution for the subsequent extraction.

2.4.2. Ultra-turrax extraction (UTE)

For the optimization, different analytical parameters were tested (solvent, volume, time, and cycles) varying one parameter at time. The optimum extraction solvent was chosen first because it is the most important extraction parameter (Ferrer et al., 2011). The aqueous coffee samples were preliminarily conditioned for extraction with polar solvents (MeOH and acetonitrile). The coffee beverage water was previously dried with a freeze drier. Different solvents or mixtures of them were tested: MeOH, acetonitrile, ethyl acetate, and a mixture of ethyl acetate/ formic acid (95:5 v/v). In addition, the volume of solvent (10– 30 ml), extraction time (1–6 min) and numbers of extractions (number of UTA times of the same aliquot with new solvent; 1–4 times) were studied to obtain the highest recoveries of the most mycotoxins. All experiments were carried out in triplicate using a standard sample spiked at concentrations of 100 LOQ for each mycotoxin. The purified solution (20 ml) was extracted with a mixture of ethyl acetate/formic acid (95:5 v/v; 20 ml × 3 cycles × 5 min) with an Ika T18 basic Ultra-Turrax (Staufen, Germany). The supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Flawil, Switzerland). The extract was dissolved with 5 ml of ethyl acetate/formic acid mixture and dried by nitrogen gas at 35 °C using a multi-sample Turbovap LV Evaporator from Zymark (Hoptikinton, USA). The extract was reconstituted with 1

ml of H_2O/MeOH (50:50 v/v) and filtered through a 0.22-µm nylon filter from Membrane Solutions (Dallas, TX, USA) prior to injection in the LC-MS/MS system.

2.5. Instrumentation and chromatographic conditions

2.5.1. LC-MS/MS-IT

An HPLC Agilent 1200 Chromatograph from Agilent Technologies (Palo Alto, CA, USA) in tandem with a 3200QTRAP mass spectrometer (Applied Bio-systems, AB Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionization interface was employed. Chromatography was performed using a Gemini-NX C_{18} column (3 µm, 110 A 150 mm × 2 mm). The run time was 30 min, and the injection volume was 20 µl including phase A (H_2O in 5 mM ammonium formate and 0.1% formic acid) and phase B (MeOH in 5 mM ammonium formate and 0.1% formic acid). A pre-run time of 3 min was used for equilibration with a flowrate of 0.25 ml/min (10% eluent B). The elution gradient (with a flow rate of 0.25 ml/min) began with 70% of eluent B, which remained constant for 3 min, and then the amount of eluent B increased to 80% and 90% at minute 6 and minute 14, respectively. At minute 18, the flow increased to 0.350 ml/min and 100% B, at minute 20, the flow increased further to 0.400 ml/min with 50% B, and from minute 21 to minute 30, the flow returned to 0.250 ml/min with 10% eluent B and 90% eluent A.

The mass spectrometer parameters were as follows: ion source turbo spray; multiple reaction monitoring (MRM); positive ionization polarity (ESI+); resolution = 12.0 (unit resolution) for the first and third quadrupoles; ion energy = 0.5 V; entrance and exit energies = −3 and 1 V, respectively; multiplier = 650; collision gas = argon, 99.995% purity; pressure = 3.83×10^{-3} mbar; inter channel delay = 0.02 s; total scan time = 1.0 s; dwell time = 0.1 ms. Analyst version 1.5.1 was used to control each component of the system and for data acquisition.

2.5.2. LC-MS/MS-QqQ

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK) equipped with an LC Alliance 2690 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, and a pneumatically assisted electrospray probe, a Z-spray interface, and Mass Lynx NT software version 4.1 was used for the MS/MS analyses. Separation was achieved using a Gemini-NX C18 (3 µm, 110A size: 150 mm × 2 mm) analytical column supplied by Phenomenex (Madrid, Spain). The mobile phase consisted of a gradient between phase A (H_2O in 5 mM ammonium formiate) and phase B (MeOH). The optimized gradient parameters were as follows: a constant flow rate of 0.20 ml/min was used; the gradient began with 0% eluent B and increased to 100% over 10 min, decreased to 80% in 5 min and to 70% in 6 min more, decreased to 0% in 1 min, and finally

remained constant at 0% eluent B and 100% eluent A for 4 min at a constant flow rate of 0.2 ml/min.

The analysis was performed in positive ion mode. The electrospray ionization source values were as follows: capillary voltage, 3.50 kV; extractor, 1 V; RF lens, 0.5 V; source temperature, 120 °C; desolvation temperature, 400 °C; and desolvation gas (nitrogen, 99.99% purity) flow, 200 l/h. The analyzer settings were as follows: resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, 5 and 3, respectively; multiplier, 650; collision gas: argon (99.995% purity) at a pressure of 0.279 Pa; inter-channel delay, 0.02 s; and total scan time, 1.0 s. The dwell time was 0.2 s for all compounds. The mass spectrometer was operated in scan, product ion scan, and MRM modes. The MRM transitions (precursor–product ion transitions) and ratios between quantification ion, cone voltages, and collision energies were optimized for each mycotoxin during the infusion of pure standard. Masslynx V4.1 software was used to control each component of the system and for data acquisition.

2.6. Method internal validation

The MS/MS analytic method was optimized according to the guidelines established by the European Commission, which establishes that a substance can be identified using LC-MS/MS in and

confirmation MRM mode by the specific retention time and at least two ion transitions while monitoring the ion ratio (Commission Decision, EC657/2002). The most abundant product ions were selected for quantification, and the second one was selected for confirmation. Therefore, the quantification of each mycotoxin was carried out with the primary transition (transition of quantification) and confirmed with the second transition (transition of confirmation). The method internal validation included the determination of linearity, limit of detection (LOD), limit of quantification (LOQ), recovery, repeatability (intra-day precision; RSD_r), and reproducibility (inter-day precision; RSD_{int}). To determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in MeOH and from the standards prepared in extract of a mycotoxin-free sample (ICS). Standards for all mycotoxins were prepared in triplicate at six concentration levels ranging from the LOD to 100 µg/l for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, NIV, DAS, T-2, HT-2, ENA, ENA_1, BEA, and ENB, from LOD to 500 µg/l for ENB_1, and from LOD to 750 µg/l for DON. The LODs were calculated using a signal-to-noise ratio of three. The LOQs were calculated using a signal-to-noise ratio of 10. Recovery experiments were carried out by spiking a sample (n = 6) on two nonconsecutive days at concentrations of 10 LOQ and 100 LOQ. Intra-day precision was assessed by six determinations in the same day,

while inter-day precision was assessed by six determinations on nonconsecutive days. The matrix effects for each mycotoxin were evaluated using matrix-assisted calibration curves; the slopes from the calibration curves prepared with known amounts of standards and extracts of mycotoxin-free samples (with matrix) were compared with those from the calibration curves prepared with the same amounts of standards and MeOH (without matrix). A sample where none of the studied mycotoxins were detected was used as a blank sample in order to ensure representative results. The signal suppression/enhancement (SSE) due to matrix effects was calculated as follows: SSE (%) = 100 (slope with matrix / slope without matrix).

3. Results and discussion

3.1. LC-MS/MS optimization

The analytical method was internally validated for coffee beverages using LC/MS-MS-QqQ and LC/MS-MS-QqQ-IT. **Table 2** shows the MS/MS transitions and the instrumental parameters optimized for each compound for both instruments.

Table 2
Optimized parameters: LC-MS/MS (IT) and LC-MS/MS (QqQ) for each mycotoxin.

	LC-MS/MS (IT)							
	DP[a]	Precursor Ion	Quantification [Q]			Confirmation [q]		
			CE[b]	Product ion	CXP[c]	CE	Product ion	CXP[c]
OTA	55	404.3	97	102.1	6	27	239.0	6
AFB_1	46	313.1	39	284.9	4	41	241.0	4
AFB_2	81	315.1	39	259.0	6	33	286.9	6
AFG_1	76	329.0	29	311.1	6	39	243.1	6
AFG_2	61	331.1	27	313.1	6	39	245.1	4
STG	106	325.0	51	281.0	18	50	310.0	3
NIV	50	313.4	80	115.1	3	27	175.1	3
DON	36	297.1	17	249.2	4	29	161.0	4
3aDON	44	339.2	20	203.1	3	20	231.1	3
15aDON	50	339.2	20	137.0	3	20	261.1	3
DAS	66	384.0	15	307.2	16	63	105.0	12
NEO	46	400.2	25	215.0	12	29	185.0	14
T-2	21	484.3	22	185.1	4	29	215.1	4
HT-2	21	442.2	19	215.4	8	19	267.8	4
FB_1	101	722.2	51	334.2	20	45	352.2	26
FB_2	131	706.2	50	336.3	16	50	318.3	18
ENA	76	699.4	59	228.2	16	35	210.1	14
ENA_1	66	685.4	59	214.2	10	37	210.2	8
ENB	51	657.3	39	196.1	8	59	214.0	10
ENB_1	66	671.2	61	214.1	10	57	228.1	12
BEA	116	801.2	27	784.1	10	39	244.1	6

[a]DP: Decluster potential (Volts)
[b]CE: Collision Energy (Volts)
[c]CXP: Cell Exit Potential (Volts)
[d]CV: Cone Voltage (Volts)
[Q] Quantification ion
[q] Confirmation ion

Table 2 (Continued)

	LC-MS/MS (QqQ)					
	DP[d]	Precursor ion	Quantification [Q]		Confirmation [q]	
			CE[b]	Product ion	CE[b]	Producto ion
OTA	24	404.3	30	358	30	341
AFB_1	47	313.2	30	241	30	269
AFB_2	50	315.2	30	243	30	259
AFG_1	43	329.2	40	200	30	215
AFG_2	46	331.2	45	189	25	217
STG	50	325.3	30	281	30	297
NIV	22	391.4	35	149	35	167
DON	20	297.2	10	249	10	231
3aDON	30	339.2	20	185	16	203
15aDON	30	339.1	26	165	26	148
DAS	15	384.0	15	307	45	105
NEO	20	400.2	14	258	14	285
T-2	10	484.5	15	215	45	305
HT-2	10	442.2	14	215	16	263
FB_1	50	722.2	30	334	30	352
FB_2	50	706.4	30	336	30	318
ENA	40	681.9	25	210	25	228
ENA_1	40	667.9	35	210	35	228
ENB	40	639.8	35	196	35	214
ENB_1	40	654.9	13	196	12	214
BEA	35	784.4	30	244	20	262

3.2. Method internal validation

The internalmethod validationwas performedwith coffee beverage samples for the simultaneous analysis of 21 mycotoxins. The analytical parameters of the method are shown in **Table 3**. The results showed good correlation coefficients for both instruments (R^2 ≥ 0.992). For all studied mycotoxins, the SSE values reflect high signal suppression ranging from 23 to 89%; the experimental values ranged from 72 to 97%. The relative standard deviation (RSD) values ranged between 4 and 12% for RSD_r and between 5 and 15% for RSD_{int}. The final LOD and LOQ ranged from 0.02 and 0.06 to 10.00 and 18.94 µg/kg, respectively, for IT and from 0.02 and 0.05 to 18.70 and 39.64, respectively, for the QqQ instrument. For OTA, the only regulated mycotoxin in coffee, the LOD and LOQ (0.24 and 0.42 µg/kg, respectively) were lower than MLs established by the European Commission (Commission Regulation, EC1881/2006). **Fig. 1** shows the chromatograms and mass spectrum of a selected mycotoxin (AFB_1) fromaspiked sample of ICS (themycotoxin-free sample). Differences between the instruments were found regarding sensibility; the IT spectrometer provides the best LOD and LOQ concentrations for most mycotoxins.

Table 3
Method validation

	LC-MS/MS (IT)					
	LOD[a]	LOQ[a]	SSE[b]	R^{2c}	Recovery[d]	
					RSD_r n=6	RSD_{int} n=6
OTA	0.24	0.42	78	0.999	94 ± 8	92 ± 8
AFB_1	0.05	0.07	64	0.998	73 ± 6	77 ± 8
AFB_2	0.04	0.15	60	0.992	76 ± 9	75 ± 11
AFG_1	0.04	0.14	73	0.997	75 ± 5	74 ± 6
AFG_2	0.05	0.27	74	0.999	82 ± 5	78 ± 10
STG	1.00	2.04	44	0.997	74 ± 10	73 ± 12
NIV	0.02	0.06	40	0.998	94 ± 7	89 ± 9
DON	8.23	16.57	13	0.994	78 ± 6	76 ± 7
3-aDON	1.00	5.05	30	0.995	95 ± 4	90 ± 9
15-aDON	10.00	18.94	28	0.995	73 ± 6	79 ± 8
DAS	1.38	5.99	87	0.993	88 ± 7	84 ± 9
NEO	1.22	3.81	89	0.993	73 ± 9	74 ± 7
HT-2	5.41	8.67	71	0.998	92 ± 4	89 ± 5
T-2	0.21	0.73	44	0.997	90 ± 7	83 ± 12
FB_1	2.78	3.30	23	0.997	84 ± 5	80 ± 12
FB_2	0.60	3.21	45	0.997	81 ± 8	84 ± 8
ENA	0.15	0.49	58	0.999	94 ± 4	93 ± 6
ENA_1	0.02	0.07	56	0.997	86 ± 9	88 ± 10
ENB	0.14	0.49	58	0.998	90 ± 4	84 ± 9
ENB_1	0.15	0.49	55	0.998	97 ± 11	95 ± 15
BEA	0.03	0.10	49	0.995	94 ± 4	94 ± 5

[a]LOD and LOQ Limits of Detection and Quantification, respectively (µg/kg)
[b]SSE: Signal Suppression – Enhancer // SSE = (slope with matrix/slope without matrix) x 100
[c] R^2: Correlation Coefficient (statistical parameter)
[d]: Recoveries ± RSD: experimental data from analysis performed at concentrations of 10LOQ (data from 100LOQ experiments not shown due to low relevance)

Table 3
(Continued)

	LC-MS/MS (QqQ)					
	LOD[a]	LOQ[a]	SSE[b]	R^{2c}	Recovery[d]	
					RSD_r n=6	RSD_{int} n=6
OTA	0.93	2.90	79	0.999	92 ± 5	90 ± 7
AFB_1	0.14	0.28	66	0.998	72 ± 6	67 ± 8
AFB_2	0.12	0.30	59	0.993	72 ± 3	75 ± 5
AFG_1	0.13	0.35	74	0.997	73 ± 4	71 ± 6
AFG_2	0.14	0.37	74	0.999	81 ± 3	78 ± 9
STG	1.05	2.94	45	0.997	73 ± 4	79 ± 7
NIV	6.07	20.48	43	0.997	92 ± 5	89 ± 10
DON	10.00	22.46	15	0.994	75 ± 1	74 ± 5
3-aDON	9.02	31.78	37	0.995	94 ± 3	89 ± 7
15-aDON	10.00	19.06	22	0.995	77 ± 7	73 ± 8
DAS	18.70	39.64	87	0.993	81 ± 7	74 ± 9
NEO	6.04	27.99	84	0.993	81 ± 5	77 ± 6
HT-2	5.52	9.05	34	0.998	92 ± 4	89 ± 7
T-2	1.65	5.52	49	0.997	82 ± 4	79 ± 5
FB_1	7.69	16.68	31	0.997	81 ± 2	80 ± 5
FB_2	4.77	8.42	46	0.997	80 ± 9	76 ± 11
ENA	0.15	0.50	60	0.999	91 ± 5	90 ± 6
ENA_1	0.08	0.25	57	0.997	87 ± 8	88 ± 11
ENB	0.15	0.50	65	0.998	88 ± 7	84 ± 8
ENB_1	0.02	0.05	55	0.997	96 ± 5	95 ± 10
BEA	0.10	0.39	48	0.995	93 ± 3	92± 5

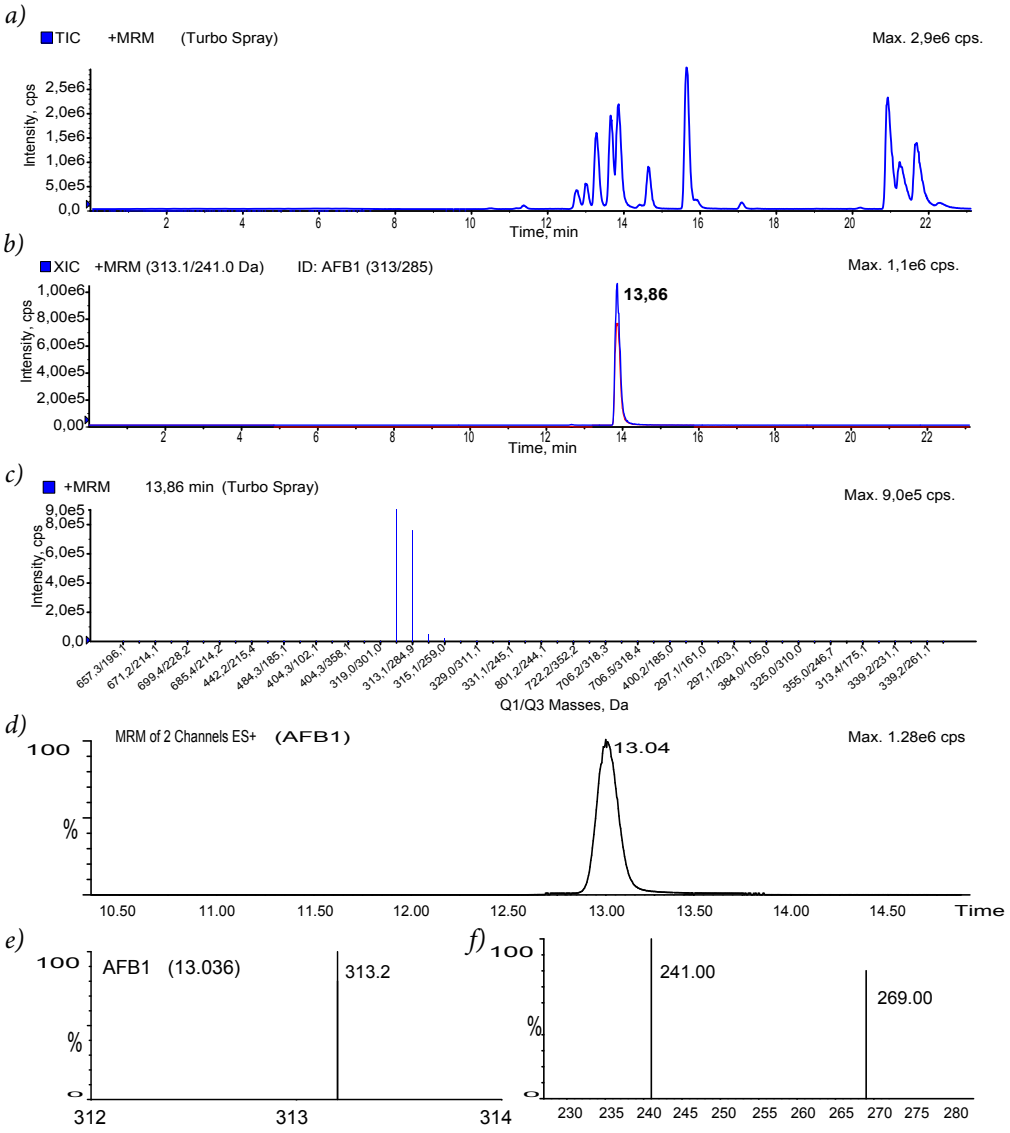


Fig. 1. AFB_1 in a matrix matched multi-mycotoxins standar solution report. a) Chromatogram in TIC with all studied mycotoxins (LC-MS/MS-IT); b) Chromatogram in XIC with two transitions of AFB_1 (313.1/241.0 and 313.1/284.9) (LCMS/MS-IT); c) Mass spectrum at 13.86 min of chromatogram "*b*"; d) Chromatogram of AFB_1 (LC-MS/MS-QqQ); e) Mass spectrum of precursor ion at 13.04 min of chromatogram "*d*"; f) Mass spectrum of products ions at 13.04 min of chromatogram "*d*".

3.3. Purification and extraction procedure

3.3.1. UTE optimization

Fig. 2 shows the effects of solvent type, solvent volume, extraction time, and number of cycles on the recoveries of the different analyzed mycotoxins. The mixture of ethyl acetate/formic acid (95:5 v/v) produced recoveries ranging from 72% to 97% (**Table 3**). Regarding solvent volume, the best recoverieswere obtainedwith 30 and 20 ml of solvent (**Fig. 2**). The extraction times affect recoveries for up to three cycles, but no significant differences were observed with larger cycle numbers. Thus, optimum recoveries without significant variations with respect to the waste of solvents were obtained using a solvent volume of 20 ml and three extraction cycles. The extraction time shows relevant differences in terms of recovery. Hence, the optimized parameters for mycotoxin extraction from coffee beverage samples using UTE with a mixture of ethyl acetate/formic acid (95:5 v/v) as the extraction solvent were a solvent volume of 20 ml, three extraction cycles, and an extraction time of 5 min. These optimized parameters are in agreement with the report of Mavungu et al. (2009), who developed a multimycotoxin method using a mixture of ethyl acetate/formic acid for the analysis of food supplements.

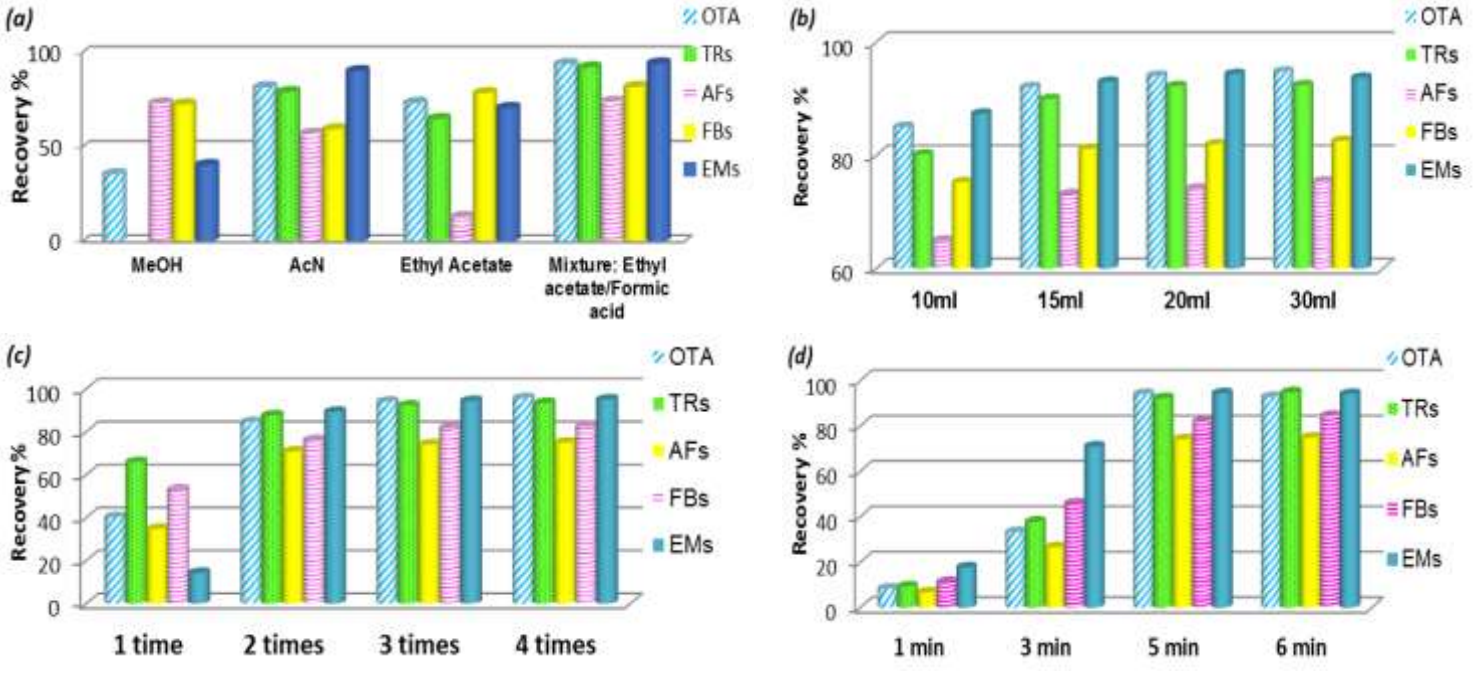


Fig. 2. UTE optimization. (a) Effect of solvents type on recovery: As it was the first step in the optimization, the other parameters were as optimum as possible (volume of solvent = 40ml; number of cycles = 5; time of extraction = 6 min). (b) Effect of solvent volume on recovery (solvent = mixture of ethyl acetate/formic acid (95/5), number of cycles = 5, time of extraction = 6 min). (c) Effect of number of cycles on recovery (solvent = mixture of ethyl acetate/formic acid (95/5), solvent volume = 20 ml, extraction time = 6 min). (d) Effect of extraction time on recovery (solvent = mixture of ethyl acetate/formic acid (95/5), solvent volume = 20ml, number of cycles = 3).

3.3.2. Cleanup optimization

Fig. 3 shows the effects of the different cleanup procedures used in each group of analyzed mycotoxins. The C_{18} cartridge and graphitized carbon blank methods were determined to be good cleanup methods in previous studies on roasted coffee. In this study, interferences (matrix substances that increase the SS or signal noise) are different with the same cleanup procedure because of the extremely acidic extraction medium and the non-polar nature of the solvent. The best recoveries for coffee samples were obtained by cleanup via clarification with Carrez solutions. This method was the best at preventing matrix effects, providing better recoveries and sensibilities.

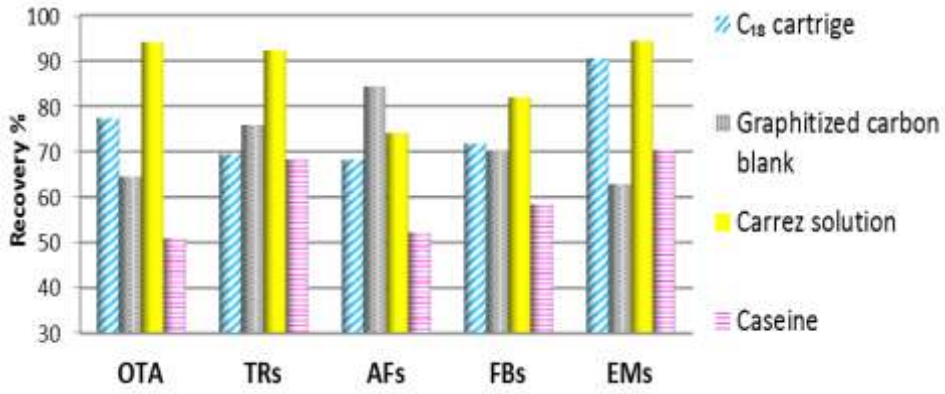


Fig. 3. Effect of cleanup on recovery (extraction conditions: solvent = mixture of ethyl acetate/formic acid (95/5), solvent volume = 20 ml, number of cycles = 3; extraction time = 5 min).

3.4. Analysis of real samples

The developed multi-residue method was applied to six commercial coffee samples. The samples were analyzed in triplicate, and the calculated RSD values were below 10%. The obtained results are shown in **Table 4**. Five mycotoxins (15-aDON, DAS, AFG_2, ENA, and FB_2) were not detected in any sample. Five samples were contaminated with mycotoxins, with the co-occurrence of at least six mycotoxins. The highest co-occurrence was nine of 21 mycotoxins in the DC sample.

OTA was present in two samples: DC (1.84 µg/kg) and ICSM (4.93 µg/kg); these values are lower than the established MLs (5.00 µg/kg in roasted coffee and 10.00 µg/kg in instant coffee; EC1881/2006). These results are in agreement with those of Casal et al. (2014), who detected OTA in instant coffee at concentrations ranging from0.15 to 11.80 µg/kg, Vanesa and Ana (2013), who detected OTA in instant coffee at concentrations ranging from0.22 to 13.66 µg/Kg, and Noba et al. (2009), who found OTA in ready-to-drink samples at insignificant concentrations.

In a previous study, Bokhari (2007) found AFB_1 at concentrations between 2.10 and 219.00 µg/kg in the study of coffee beans. In our study, AFs were found in one sample with concentrations of 3.66 and 6.65 µg/kg for AFB_1, and AFG_2, respectively. However, in

Table 4
Concentration of each mycotoxin in real samples expressed in µg/kg

	Groun coffee			Instant coffee		
	NRC[a]	TRC[b]	DC[c]	IC[d]	ICS[e]	ICSM[f]
OTA	-	-	1.84	-	-	4.93
AFB_1	-	-	-	3.66	-	-
AFB_2	-	-	-	5.64	-	-
AFG_1	-	-	-	6.65	-	-
AFG_2	-	-	-	-	-	-
STG	36.54	-	23.77	-	-	-
NIV	-	2.38	2.92	24.46	-	0.61
DON	-	-	-	-	-	18.34
3-aDON	-	-	5.17	-	-	-
15-aDON	-	-	-	-	-	-
DAS	-	-	-	-	-	-
NEO	-	3.82	30.24	-	-	12.37
T-2	0.89	2.14	7.84	3.57	-	-
HT-2	-	-	-	14.39	-	-
FB_1	5.18	-	-	-	-	-
FB_2	-	-	-	-	-	-
ENA	-	-	-	-	-	-
ENA_1	1.64	1.18	4.82	-	-	0.64
ENB	8.44	5.51	20.66	36.14	-	0.96
ENB_1	2.87	1.68	5.33	-	-	0.52
BEA	-	-	-	0.37	-	-
Co-presence	6/21	6/21	9/21	8/21	0/21	7/21

[a]NRC: Natural roasted coffee
[b]TRC: Torrefacto roasted coffee
[c]DC: Decaffeinated coffee
[d]IC: Instant coffee
[e]ICS: Instant coffee sugar added
[f]ICSM: Instant coffee sugar and milk added

contrast to the study by Bokhari (2007), no samples in the current study had high AF contents. The low incidence of AFs in the present study is in agreement with Khayoon et al. (2014), who did not detect AFs in coffee samples.

Liu et al. (2014) and Alkadri et al. (2014) detected STG in wheat and sesame butter at low concentrations under 5.10 µg/kg; in the present study, STG occurred in two samples at 23.77 and 36.54 µg/kg.

Yogendrarajah, Poucke, Meulenaer, and Saeger (2013) found STG at similar concentrations in white pepper.

The concentration of TRs ranged from 0.61 to 30.24 μg/kg, and their concentrations were low in the most cases. These contamination levels are lower than those found by other authors in different food products such as wheat, maize, and oat semola (Alkadri et al., 2014; Han et al., 2014; and Kirincic et al., 2015).

FB_1 occurred in one sample with a concentration 5.18 μg/kg. Only FB_2 has been studied by other authors in coffee beans, and low concentrations between 1.30 and 9.70 μg/kg were found, in agreement with this study (Noonim et al., 2009).

EMs were found in most samples at concentrations ranging from 0.29 to 36.14 μg/kg. No data on EMs in coffee are available, although studies on Ems in peanuts and pasta indicate higher concentrations (Serrano, Font, Mañes, & Ferrer, 2013; Tolosa, Font, Mañes, & Ferrer, 2013).

4. Conclusions

A new method for the simultaneous analysis of 21 mycotoxins in coffee beverages based on liquid/liquid extraction followed by

detection and quantification with LC-MS/MS (QqQ and QqQ-IT) has been optimized. The method was applied to the analysis of commercial samples.

The present study provides a multi-residue method suitable for the simultaneous detection and quantification of 21 mycotoxins in coffee beverages with LODs below the MLs established for other food commodities.

The application of this method to real samples shows the occurrence and co-occurrence of mycotoxins. No conclusion about the incidence or amount of mycotoxins in real samples can be established due to the low sample number. Further studies with more samples must be carried out. This method along with the subsequent study of commercial samples will facilitate the assessment of the safety of commercial products.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.foodres.2015.02.030.

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3.3. Analysis of mycotoxins in coffee and risk assessment in Spanish adolescents and adults

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Analysis of mycotoxins in coffee and risk assessment in Spanish adolescents and adults

*Ana García-Moraleja, Guillermina Font, Jordi Mañes, Emilia Ferrer**

Laboratory of Toxicology and Food Chemistry, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

* Corresponding author:
A. García-Moraleja
Tel.: 0034 963544950
fax: 0034 963544954.
E-mail address: ana.garcia-moraleja@uv.es

ABSTRACT

Mycotoxins are toxic compounds produced by fungal secondary metabolism that cause toxicological effects. Coffee is a highly popular beverage that is susceptible to contamination by mycotoxigenic fungi. The aim of the present study was to determine the presence of the following 21 mycotoxins in coffee using liquid chromatography tandem mass spectrometry (LC-MS/MS-IT): aflatoxin B_1, B_2, G_1 and G_2; ochratoxin A; nivalenol; deoxynivalenol; 3-acetyldeoxynivalenol; 15-acetyldeoxynivalenol; diacetoxyscirpenol; neosolaniol; T-2 and HT-2 toxin; sterigmatocystin; enniatin A, A_1, B, and B_1; beauvericin; and fumonisin B_1 and B_2. We aimed to determine differences by coffee process (coffee maker, electrical machine, soluble and traditional Turkish process) and to calculate the estimated daily intake (EDI) and risk assessment of mycotoxins from coffee consumption using deterministic approach at various scenarios of food consumption in Spanish adolescents and adults. The results demonstrate that all studied mycotoxins were detected in samples with mean concentrations ranging from 0.69 μg/kg to 282.89 μg/kg. Eleven percent of samples did not show contamination with legislated mycotoxins. Only 15-acetyldeoxynivalenol, deoxynivalenol, neosolaniol, fumonisin B_1, and ochratoxin A exhibited significant differences between methods of coffee brewing. The results show that

coffee intake does not represent a potential risk for consumers with respect to individual mycotoxin contamination.

Keywords: Mycotoxin, Coffee Risk assessment Brewing process

1. Introduction

Coffee is a drink obtained from ground roasted beans of the coffee plant (*Coffea spp*). The genus *Coffea* includes many species, though only the following three are cultivated for commercial purposes: *Coffea arabica, Coffea robusta* and *Coffea liberica*. Coffee is the second largest trade commodity in the world. Countries with high coffee consumption include the United States of America and Brazil (I.C.O, 2013). Coffee has become an irreplaceable product in the diet, and recent studies shows that while economic crises affect the quality of purchased coffee, they do not affect the level of coffee intake by consumers (Mussatto et al., 2011). Mycotoxins are toxic compounds that result from fungal secondary metabolism produced in different substrates under certain weather conditions (Rocha et al., 2014). Coffee is susceptible to contamination by mycotoxigenic fungi (Gamboa-Gait an, 2012; Serani et al., 2007). These mycotoxins cause different toxicological effects in humans (Afsah-Hejri et al., 2013). The most commonly studied mycotoxin in coffee is ochratoxin A (OTA) (Paterson et al., 2014), and this is the only mycotoxin subject to current legislation (European Commission (EC) 1881/2006); the maximum levels (ML) are 5 µg/kg in roasted coffee beans and ground roasted coffee and 10 µg/kg in soluble coffee (instant coffee). Nevertheless, available studies of green and roasted coffee beans demonstrate the presence of

different mycotoxins. Nielsen et al. (2015) studied OTA and fumonisins B (FBs) in green coffee, roasted coffee and soluble coffee and detected OTA in roasted coffee and FBs in green coffee. Bokhari and Ali (2009) detected OTA, aflatoxins (AFs), patulin and sterigmatocystin (STG) in coffee beans. Coffee is a very popular beverage, and its chemical composition is affected by the brewing process (Caporaso et al., 2014). It is likely that each brewing process affects themycotoxin content of coffee to a different degree. Studies have examined OTA and AFs as single mycotoxins in coffee, but studies of other mycotoxins such as trichothecenes (TRC) and enniatins have not been reported (Tozlovanu and Pfohl-Leszkowicz, 2010). In addition, several authors have studied the effect of different industrial and culinary processes on mycotoxin content; La Pera et al. (2008) reported a reduction in OTA (from 100% to 64%), and Napolitano et al. (2007) observed a reduction in OTA concentrations during different preparations of typical espresso. However, no data regarding the occurrence of multiple mycotoxins in a large sample of coffee beverages has been reported.

In this context, the aim of the present study was to determine the presence of 21 mycotoxins [aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), OTA, nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-aDON),15-acetyldeoxynivalenol (15-aDON), diacetoxyscirpenol (DAS),

neosolaniol (NEO), T-2 and HT-2 Toxin, STG, enniatin A (ENA), enniatin A_1 (ENA_1), enniatin B (ENB), enniatin B_1 (ENB_1), beauvericin (BEA), fumonisin B_1 (FB_1), and fumonisin B_2 (FB_2)] using liquid chromatography tandem mass spectrometry with triple quadrupole and ion trap (LC-MS/MS-IT) in different coffee beverages (caffeinated, decaffeinated, conventional, pre-portioned, soluble and Turkish coffee) to identify and assess the presence and possible coexistence of mycotoxins and to assess differences between commercial coffee types. We also calculated the EDI and performed a risk assessment for coffee consumption.

2. Materials and methods

2.1. Chemical and reagents

Ethyl acetate (HPLC grade) was supplied by Alfa Aesar (Karlsurhe), and acetonitrile and methanol (HPLC grade) were supplied by Merck (Damstadt, Germany). Ammonium formiate (99%), $ZnSO_4 \cdot 7H_2O$ and $K_2Fe(CN)_6$ were supplied by Panreac Quimica SAU (Barcelona, Spain). Formic acid (reagent grade 95%) was provided by SigmaeAldrich (Louis, USA), and deionized water (resistance <18MΩ cm^{-1}) was purified using the Milli-Q SP purification system from the

Millipore Corporation (Bedford, USA). Standards of the following mycotoxins were provided by Sigma (St. Louis, MO, USA): AFB_1, AFB_2, AFG_1, AFG_2, OTA, NIV, DON, 3-aDON, 15-aDON, DAS, NEO, T-2 and HT-2 toxin, STG, ENA, ENA_1, ENB, ENB_1, BEA, FB_1, and FB_2. Individual stock solutions with a concentration of 500 mg/l were prepared in acetonitrile. The appropriate working solutions were prepared and stored in darkness at 20ºC until LC-MS/MS-IT analysis. Carrez's Solutions were prepared within the laboratory with $ZnSO_4 \cdot 7H_2O$ (1.0 M) for Carrez's solution (I) and $K_2Fe(CN)_6$ (0.3 M) for Carrez's solution (II). A mixture of ethyl acetate/formic acid (95:5 v/v) was prepared daily with 25 ml of formic acid and up to 500 ml of ethyl acetate. Prior to injection, the samples were passed through a nylon filter of 13 mm/0.22 mm (Membrane Solutions, Texas, USA). The mobile phases were filtered through a cellulose filter with a 0.45-mm diameter from Scharlau (Barcelona, Spain).

2.2. Samples

A total of 169 samples of coffee representative of general consumption habits and according market share were purchased from supermarkets in Valencia, Spain. Total samples from 28 different brands representing all qualities (name brands and own brands) and within easy reach of consumers were collected, processed and

analyzed. The samples were classified according the brewing process as follows: conventional (coffee maker), soluble, pre-portioned (electric machine), and Turkish coffee. Within these types of coffee, the samples were classified as caffeinated and decaffeinated. Each coffee type was represented with as many brands as possible. All samples were analyzed before brewing according Gracía-Moraleja et al. (2015b) methodology; No significative differences in the mycotoxins contents were obtained between the different coffee type (excepting Turkish coffee).

2.3. Sample preparation and processing

To maximum the quality of the brewand minimize confounding between methods of coffee preparation, all coffee types were processed using mineral water (mycotoxin-free) of the same commercial brand. A coffee maker (Italian moka) was used to prepare ground conventional packed coffee samples similar to how coffee is prepared in the home. The coffee maker contained three compartments; the first compartment contained water, the second compartment was separated from the first by a metallic filter that holds the ground coffee and the third compartment is located on above the coffee pot and is initially empty. Water is boiled in the first compartment and flows through the coffee in the second

compartment; the water is approximately 98.6ºC (Gianino, 2007). Coffee then brews in the upper reservoir. For this preparation, approximately 5 g of ground coffee and 50 ml of water are used to obtain a final cup of coffee of approximately 45 ml. Soluble coffee samples were prepared according to the manufacturer's specifications regarding the ratio of grams of coffee per milliliters of water. In this case, water was heated to 85-90ºC in a hot plate and added to a glass beaker holding the soluble coffee; the mixture was continuously stirred to dissolve the coffee. Samples of Turkish coffee were processed using the traditional Turkish method. For this, 50 ml of water was heated on a hot plate to 95-100ºC and added to a glass beaker holding 5 g of coffee; this was mixed for 30 s. The upper phase was collected after allowing 10 min for sedimentation. Samples of pre-portioned coffee of approximately 5-7 g were prepared using corresponding electrical machines. The first 45 ml produced by the coffee machines were collected.

2.4. Extraction procedure

Briefly, samples were initially clarified with Carrez's solution. Then, the clarified solutionwas added to a mixture of ethyl acetate/ formic acid (95/5 v/v) and homogenized with Ultra-Turrax (T18 Basic Ika, Staufen, Germany) for 5 min; the supernatant with the organic

phase was then separated. This extraction was performed in triplicate using 20 ml of mixture (ethyl acetate/formic acid) each time, and the supernatants of each extraction were collected. The supernatant was evaporated in Büchi Rotavapor R-200 (Postfach, Switzerland) under conditions of 40º C and 140 mmHg. The extract obtained was dissolved with 10 ml of mixture (ethyl acetate/formic acid) and evaporated to dryness with a TurboVap LV Evaporator (Zymark, Hoptikinton, USA) under conditions of 50º C and nitrogen pressure of 5 psi. The residue was reconstituted with 1 ml of methanol/water (50/50 v/v), stirred with a vortex for 1 min, and filtered through a nylon filter of 0.22 mm (Membrane Solutions, Texas, USA) to remove impurities. The extract was put in specifics amber flaks for autosampler prior to LC-MS/MS-IT analysis (García- Moraleja et al., 2015a).

2.5. Instrumental parameters

Analyses were performed in a system equipped with an Agilent 1200 LC from Agilent Technologies (Palo Alto, CA, USA) coupled to a 3200 QTRAP mass spectrometer (Applied Biosystems, AB Sciex, Foster City, CA, USA) equipped with electrospray ionization interface Turbo Ion Spray (ESI). The QTRAP analyzer combines fully functional triple quadrupole and an ion trap mass spectrometer in the same instrument. The separation of the analytes was performed with a Gemini-NX C_{18}

column (3 mm, 110 A 150 mm 2 mm) (150mm 4.6 mm, 5 mm particle size) preceded by a guard column C_{18}. Run time was 30 min; injection volume was 20 ml; phase A (H_2O in 5 mM Ammonium formiate and 0.1% formic acid) and phase B (MeOH in 5mM Ammonium formiate and 0.1% formic acid) were completed; there was a pre-run time of 3 min for equilibration with flow of 0.25 ml/min (10% eluent B); the elution gradient started with flow of 0.25 ml/min with 70% of eluent B remaining constant for 3 min and increasing to 80% and 90% at minutes 6 and 14, respectively; at minute 18, the flow increased to 0.350 ml/min and 100% B; at minute 20, the flow increased to 0.400 ml/min and 50%; and from minute 21 to minute 30, the flow returned to 0.250 ml/ min and 10% eluent B and 90% eluent A.

Mass spectrometer parameters were as follows: Ion source Turbo spray, type Multiple Reaction Monitoring (MRM), and positive ionization polarity (ESI+) were used; the resolution was 12.0 (unit resolution) for the first and third quadrupoles, and the ion energy was 0.5 V; the entrance and exit energies were 3 and 1 V, respectively; the multiplier was 650; the collision gas (Argon 99.995% purity) pressure was 3.83 10^{-3} mbar, the inter-channel delay was 0.02 s, the total scan time was 1.0 s, and the dwell time was 0.1 ms. **Table 1** shows the specific ion transitions and energies applied for the analyses of each

Table 1
LC-MS/MS-QqQ-IT optimized instrumental parameters of the validated method

	DP [a]	Precursor ion	Quantification [Q]			Confirmation [q]		
			CE[b]	Product ion	CXP[c]	CE[b]	Product ion	CXP[c]
OTA	55	404.3	97	102.1	6	27	239.0	6
AFB_1	46	313.1	39	284.9	4	41	241.0	4
AFB_2	81	315.1	39	259.0	6	33	286.9	6
AFG_1	76	329.0	29	311.1	6	39	243.1	6
AFG_2	61	331.1	27	313.1	6	39	245.1	4
STG	106	325.0	51	281.0	18	50	310.0	3
NIV	50	313.4	80	115.1	3	27	175.1	3
DON	36	297.1	17	249.2	4	29	161.0	4
3aDON	44	339.2	20	203.1	3	20	231.1	3
15aDON	50	339.2	20	137.0	3	20	261.1	3
DAS	66	384.0	15	307.2	16	63	105.0	12
NEO	46	400.2	25	215.0	12	29	185.0	14
T-2	21	484.3	22	185.1	4	29	215.1	4
HT-2	21	442.2	19	215.4	8	19	267.8	4
FB_1	101	722.2	51	334.2	20	45	352.2	26
FB_2	131	706.2	50	336.3	16	50	318.3	18
ENA	76	699.4	59	228.2	16	35	210.1	14
ENA_1	66	685.4	59	214.2	10	37	210.2	8
ENB	51	657.3	39	196.1	8	59	214.0	10
ENB_1	66	671.2	61	214.1	10	57	228.1	12
BEA	116	801.2	27	784.1	10	39	244.1	6

[a] DP: Decluster potential (Volts)
[b]CE: Collision Energy (Volts)
[c]CXP: Cell Exit Potential (Volts)
[Q] Quantification ion
[q] Confirmation ion

mycotoxin. Analyst version 1.5.1 was used to control each component of the system and for data acquisition.

2.6. Statistical analysis

An observational descriptive analysis was performed to assess differences between coffee processes (traditional, pre-portioned, soluble, and Turkish coffee) and coffee types (caffeinated and decaffeinated). The analysis included 169 samples of coffee. A one-way ANOVA test was used to assess the coffee processes, and a multi comparative post hoc study with the honest significant difference Tukey's test was used with concentration (µg/kg) as the dependent variable and coffee process as an independent factor. For the coffee type (caffeinated or decaffeinated), a Student's T-test P ($T \leq t$) was used. For both tests, P-values of <0.05 were considered statistically significant. IBM SSPS statistics 22 software for Windows 7 was used for statistical analyses.

2.7. Dietary exposure assessment

Exposure assessment is an essential element for quantifying risk and is a tool for risk managers involved in food safety (Leblanc et al., 2005). The most common methodologies for exposure assessment are deterministic or point estimates of dietary exposure and probabilistic

assessments (WHO, 2009). The deterministic assessment is the simplest approach, as it is a single value of consumer exposure. The assessment is calculated as the product of food consumption and the average amount of the studied mycotoxin contained in the food. The advantage of deterministic assessments is that the simplicity of the results allows comparison between studies from different countries (Serrano et al., 2012), with tolerable daily intakes (TDI) proposed by organizations (Rodríguez- Carrasco et al., 2013) that is an estimate of the amount of a substance that can be taken in daily over a lifetime without appreciable health risk, and with total diet studies. Conversely, in the probabilistic approach, consumption is represented by a distribution instead of a single value (WHO, 2009). While coffee makes up a small part of the overall diet, on basis of its weight equivalence in percentage, many individuals drink coffee daily, and segments of the population consume large quantities of coffee. Calculating mycotoxins exposures from coffee consumption and including them in total diet studies would increases the precision of the estimates of coffee consumption in the total population. The estimated daily intake (EDI) of each group of mycotoxin was calculated based on the recommendations of the International Program on Chemical Safety: Principles and Methods for the Risk Assessment of Chemicals in Food (WHO, 2009). Data on mycotoxin concentrations in 169 coffee samples

purchased at supermarkets were obtained following coffee preparation using traditional consumer methods. Dietary exposure assessments (deterministic) use mean consumption data from the general population at different scenarious: mean, 95th 97.5th or 99th percentiles to define the population expected to have high exposure. The assessment of mycotoxin intake from coffee, abbreviated as EDI_{Coffee}, is calculated using the equation EDI_{Coffee} (ng/ kg bw day) = coffee consumption (g/kg bw day) x mycotoxin content (ng/g).

2.8. Food consumption data

The European Food Safety Authority (EFSA) has compiled food consumption data in diverse Member States (EFSA, 2015). This compilation classified food and beverages in a standardized way. In addition, consumption of a single food is specified for various segments of the population, and these segments represent the mean population of highly exposed consumers. As samples were purchased from Spain, which is included in the EFSA database, data from the Spanish Agency for Food Safety Survey for long-term exposure (chronic) food consumption statistics in grams per day per kilogram of body weight was selected. This survey from 2009 contains 1067 subjects, which include 86 adolescents and 981 adults. Children were

not included. The survey was conducted in accordance with a harmonised methodology approved by EFSA.

3. Results

3.1. Method validation

The analytic method that we used to assess the 21mycotoxins in coffee was previously validated in accordance with the European Commission (EC657/2002). For validation parameters such as linearity (R^2), the limit of detection (LOD), the limit of quantification (LOQ), recovery, signal suppression (SS) due to matrix effects, and intra- and inter-day precision were evaluated. **Table 2** shows the parameters of the method validation. The method showed good correlation coefficients (R^2 > 0.992). For all studied mycotoxins, the SS values reflect high signal suppression ranging from 13 to 89%; the experimental recovery values ranged from 72 to 96%. The relative standard deviation (RSD) values ranged between 1 and 9% for RSD_r and between 5 and 11% for RSD_{int}. The LOD and LOQ ranged from 0.02 to 10.00 and from 0.07 to 18.94 µg/kg, respectively.

Table 2
Analytical parameters of the validation method

	LOD (µg/kg)	LOQ (µg/kg)	SSE[a]	R^{2b}	Recovery			
					10LOQ		100LOQ	
					Intra day (n = 6)	Inter day (n = 6)	Intra day (n = 6)	Inter day (n = 6)
OTA	0.24	0.42	78	0.999	92 ± 5	90 ± 7	98 ± 4	96 ± 7
AFB_1	0.05	0.07	64	0.998	72 ± 6	67 ± 8	84 ± 5	84 ± 4
AFB_2	0.04	0.15	60	0.992	72 ± 3	75 ± 5	86 ± 3	87 ± 5
AFG_1	0.04	0.14	73	0.997	73 ± 4	71 ± 6	85 ± 4	89 ± 4
AFG_2	0.05	0.27	74	0.999	81 ± 3	78 ± 9	85 ± 3	85 ± 9
STG	1.00	2.04	44	0.997	73 ± 4	79 ± 7	79 ± 5	88 ± 5
NIV	0.02	0.06	40	0.998	92 ± 5	89 ± 10	95 ± 3	98 ± 9
DON	8.23	16.57	13	0.994	75 ± 1	74 ± 5	80 ± 2	80 ± 4
3aDON	1.00	5.05	30	0.995	94 ± 3	89 ± 7	100 ± 4	99 ± 4
15aDON	10.00	18.94	28	0.995	77 ± 7	73 ± 8	88 ± 4	88 ± 6
DAS	1.38	5.99	87	0.993	81 ± 7	74 ± 9	92 ± 7	92 ± 7
NEO	1.22	3.81	89	0.993	81 ± 5	77 ± 6	93 ± 5	93 ± 5
T-2	5.41	8.67	71	0.998	92 ± 4	89 ± 7	89 ± 3	89 ± 3
HT-2	0.21	0.73	44	0.997	82 ± 4	79 ± 5	95 ± 6	97 ± 6
FB_1	2.78	3.30	23	0.997	81 ± 2	80 ± 5	89 ± 4	89 ± 6
FB_2	0.60	3.21	45	0.997	80 ± 9	76 ± 11	90 ± 5	91 ± 8
ENA	0.15	0.49	58	0.999	91 ± 5	90 ± 6	96 ± 4	96 ± 4
ENA_1	0.02	0.07	56	0.997	87 ± 8	88 ± 11	95 ± 9	94 ± 9
ENB	0.14	0.49	58	0.998	88 ± 7	84 ± 8	91 ± 7	91 ± 6
ENB_1	0.15	0.49	55	0.998	96 ± 5	95 ± 10	97 ± 9	95 ± 7
BEA	0.03	0.10	49	0.995	93 ± 3	92± 5	98 ± 3	98 ± 5

[a]SSE: Signal Suppression – Enhancer // SSE = (slope with matrix/slope without matrix) x 100
[b] Linearity: expressed as R^2 from calibration curves matrix matched

3.2. Occurrence of mycotoxins in samples

The samples were analyzed in triplicate, and the calculated RSD values were below 10%. The obtained results are shown in **Table 3**. The results are expressed as mg of mycotoxins per kg of coffee (commercial roasted coffee) to standardize the data. All studied mycotoxins were detected in some samples. Nineteen samples did show contamination with the legislated mycotoxins, and only 5 samples did not show contamination with the emerging mycotoxins.

Table 3
Contamination levels in brewed coffee samples n = 169

	Occurrence (%)[b]	Mean (µg/kg)		Range[c]	
		Total samples	Positive samples	Min (µg/kg)	Max (µg/kg)
OTA	36	0.67	1.87	0.67	4.70
AFB_1	22	0.20	0.91	0.75	1.14
AFB_2	10	0.07	0.69	0.51	1.57
AFG_1	15	0.46	3.05	2.54	50.80
AFG_2	25	1.01	4.03	0.99	25.87
AFs_T[a]	53	2.70	5.07	0.59	26.93
STG	16	3.62	22.63	7.65	63.19
NIV	43	0.72	1.68	0.12	37.83
DON	43	43.95	102.22	16.58	954.91
3aDON	20	2.09	10.45	6.31	24.57
15aDON	41	38.89	94.86	61.04	884.25
DAS	7	13.73	196.20	195.91	402.23
NEO	13	11.27	86.73	29.06	313.84
T-2	29	0.69	2.37	0.74	21.17
HT-2	9	1.10	12.20	8.41	37.01
FB_1	4	0.62	15.55	15.52	18.25
FB_2	4	0.90	22.46	20.02	23.99
ENA	59	166.91	282.89	5.88	2875.57
ENA_1	66	162.82	246.69	2.22	12039.12
ENB	93	163.92	176.26	4.68	4147.96
ENB_1	75	103.93	138.57	5.16	3932.47
BEA	5	0.05	0.97	0.52	3.07

[a] AFS_T: total aflatoxins (AFB_1 + AFB_2 + AFG_1 + AFG_2)
[b]Occurrence in total samples n = 169
[c]Range of concentration in positive samples

Aflatoxins were present in 53% of the studied samples. As mentioned above, there are no regulations for AFs in coffee. In the present study, no samples exceeded 2 µg/kg of AFB_1, but 15% of samples had a concentration of total AFs above 5 µg/kg. Samples with concentrations of total AFs above 5 µg/kg are due to AFG_2 contamination; AFG_2 was present in the samples in greater concentrations but is the less toxic aflatoxin. In most cases of contamination, the samples were contaminated with one or of two AFs, and 3 of the 169 samples were contaminated with three aflatoxins; no samples were contaminated with four of the studied aflatoxins. In comparison, Khayoon et al. (2014) analyzed 21 samples of canned coffee but did not detect AFs; Bokhari (2007) detected AFB_1 in green coffee with contamination levels of 110.5 µg/kg. In a previous study, García-Moraleja et al. (2015b) detected AFB_1 and AFB_2 in roasted coffee and soluble coffee samples with a mean concentration of 1.95 µg/kg. This shows that AFs concentrations are higher in green coffee than in roasted coffee; during the brewing process, water liberates few AFs.

Thirty-six percent of the samples were contaminated with OTA, but none exceeded the limit established by ML for OTA in coffee (EC/1881/2006). The results from the present study are in accordance with Noba et al. (2009), who detected OTA in all samples of ready-to-

drink coffee, which had concentrations ranging from 0.01 µg/l to 0.04 mg/l; Casal et al. (2014) detected OTA in soluble coffee samples with a mean concentration of 2.50 µg/kg. In a previous study, García-Moraleja et al. (2015b) found concentrations of 1.50-32.40 µg/kg in roasted coffee, and 35% of samples were positive. As reported by Santini et al. (2011), a reduction in OTA concentration has been observed. According to previous studies, coffee beverages have less OTA contamination than roasted coffee. Nevertheless, the brewing process does not completely remove OTA from samples, according to Perez De Obanos et al. (2005), who studied the effects of the brewing process on the OTA content in nine positive samples; reductions in the concentrations were achieved in all cases, and only a single case had a reduction to a level below the limit of detection.

STG was present in few samples (16%). The results from the present study are in accordance with those of Bokhari and Aly (2009), who detected STG in two of 13 analyzed samples of coffee beans with concentrations of 13 and 11 µg/kg.

The trichothecenes analyzed in the present study (NIV, DON, 3-aDON, 15-aDON, DAS, NEO, HT-2 and T-2) presented variable concentrations from 1.68 µg/kg in the case of NIV to 196.20 µg/kg in the case of DAS, and the occurrence of contamination was low. García-

Moraleja et al. (2015b) analyzed trichothecenes in 103 samples of roasted coffee and detected NIV, DON, DAS, HT-2, and T- 2 with mean concentrations of 6605.73, 67.40, 6.50, 91.10 and 33.00 µg/kg, respectively. The results showed degradation of most mycotoxins (NIV, DAS, HT-2, and T-2). Concentration of DON was higher in beverages than in roasted coffee. The mycotoxin that showed the most degradation was NIV.

Fumonisins were analyzed in the present study, and FB_1 and FB_2 had a low occurrence and low contamination levels. García- Moraleja et al. (2015b) analyzed FBs in roasted coffee samples and detected mean concentrations of 58.62 and 85.45 µg/kg, respectively; the FBs were present in 87% and 81% of samples, respectively. Noonim et al. (2009) detected FB_2 in concentrationsfrom 1.3 to 9.7 µg/kg in roasted coffee samples. Fumonisins that consist of carbonated chains are susceptible to heat processes, particularly during brewing. These results are in accordance with those of Nielsen et al. (2015), who detected concentrations of up to 164 µg/kg in samples of green coffee beans and demonstrated degradation of fumonisins with a thermal process (roasting). The low incidence and concentrations in the present study show that the brewing process reduces and in the most cases removes fumonisins contamination.

The following emerging mycotoxins were analyzed in the present study and were detected in samples: ENA, ENA_1, ENB, ENB_1 and BEA. Enniatins were the most concentrated mycotoxins and were present in the most samples. BEA presented low contamination levels. García-Moraleja et al. (2015b) analyzed the samemycotoxins in roasted coffee samples and detected mean concentrations of 374.30,179.40, 577.00,10.20 and 0.6 7 μg/kg, respectively, and these mycotoxins were present in 37%, 87%, 54%, 8% and 35% of samples, respectively. These results suggest that the brewing process does not affect the concentrations of emerging mycotoxins.

3.3. Effects of processing for mycotoxin concentration

According to **Fig. 1**, each brewing process shows different levels of degradation of the mycotoxins; however, statistical analysis shows that in most cases, mycotoxins exhibited the same behavior in each process. Sixteen of the studied mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, NIV, 3a-DON, DAS, HT-2, T-2, STG, ENA, ENA_1, ENB, ENB_1, BEA and FB_2) did not differ according to the brewing process.

15-aDON, DON, NEO, and FB_1 showed extensive degradation after pre-portioned electric machine brewing, and the level of degradation was next highest in soluble coffee.

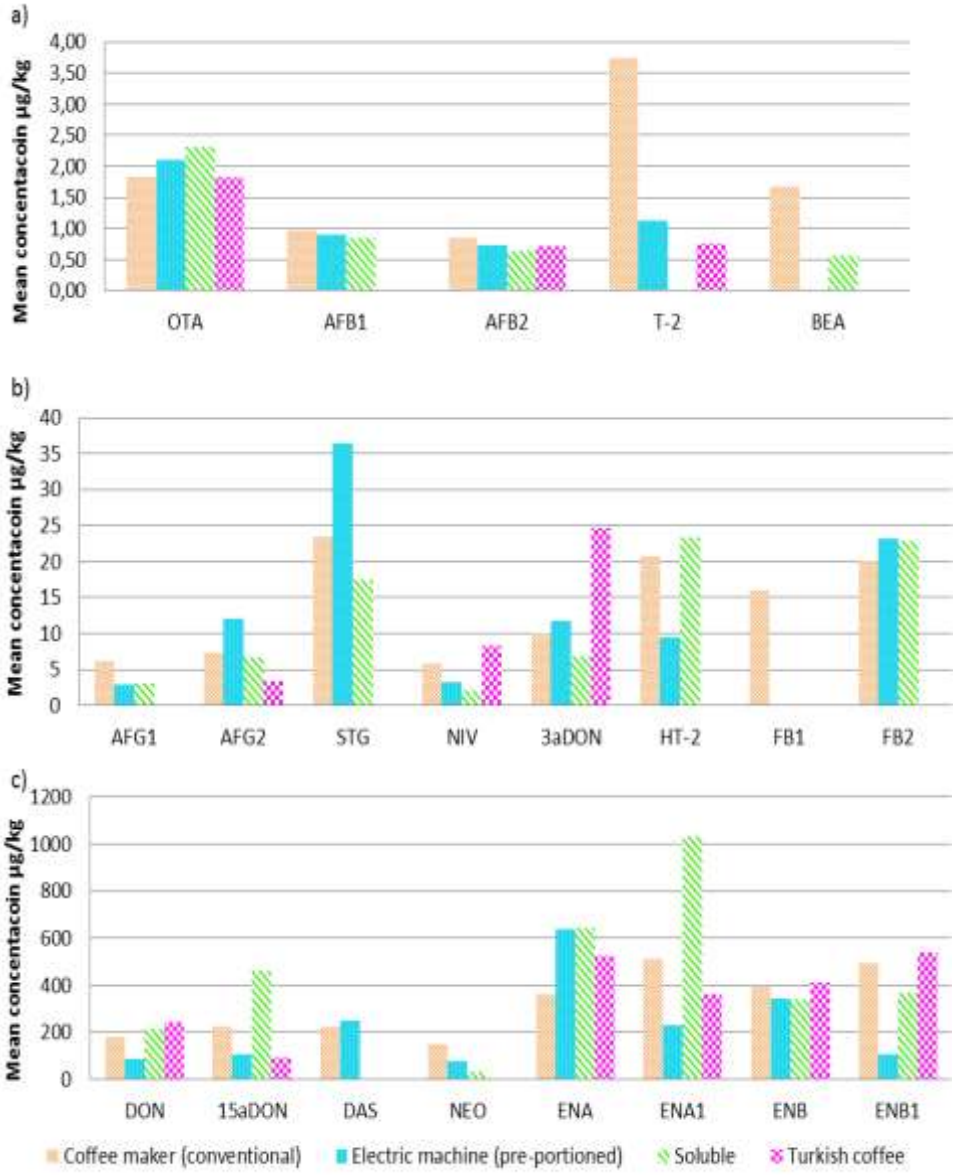

Fig 1. Mean concentration of mycotoxins in positive samples using different brewing processes. a) Mycotoxins with low levels of mean concentration. b) Mycotoxins with medium level of mean concentration. c) Mycotoxins with high level of mean concentration.

The traditional process with the moka machine showed the least degradation. However, degradation of OTA was greatest with the conventional process compared to the pre-portioned and soluble processes.

Turkish coffee had a high incidence of AFG_2 contamination (80%), but it was not contaminated with other aflatoxins or STG, and contamination levels were similar to other coffee types. The Turkish coffee preparation probably affects aflatoxin contamination levels in the same way. However, Turkish coffee samples were produced in countries in North Africa, and this geographical locale or this climate could stimulate AFG_2 production over the production of other AFs.

3.4. Difference between caffeinated and decaffeinated coffee beverages

Two sub-groups of caffeinated coffee (n = 109) and decaffeinated coffee (n = 60) were compared. Mean concentrations are shown in **Fig. 2**. The figures show that decaffeinated coffee had higher concentrations than caffeinated coffee, though the difference was not significant ($p \geq 0.05$) for OTA, AFs, NIV, DON, DAS, or the emerging mycotoxins. There were significantly lower concentrations in caffeinated coffee for STG, 3-aDON, 15-aDON, and NEO. Differences between caffeinated and decaffeinated samples do not result from the

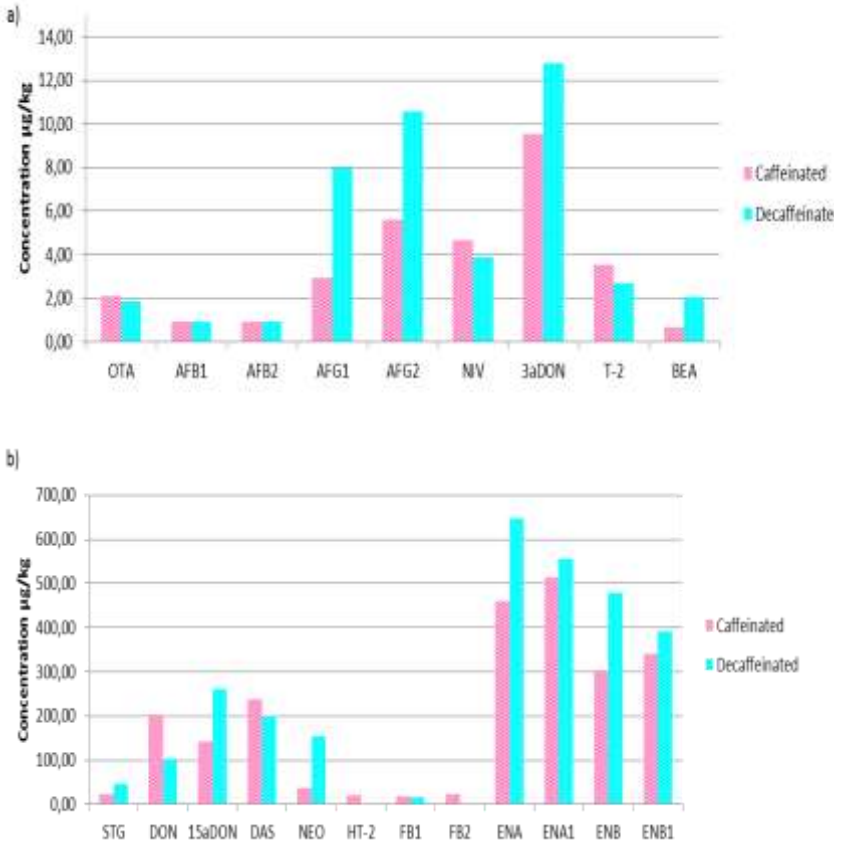

Fig. 2. Mean concentration of mycotoxin in positive caffeinated and decaffeinated samples. a) Mycotoxins with mean concentrations under 15 µg/kg; b) Mycotoxins with mean concentration above 15 µg/kg.

brewing process. It is likely that caffeinated commercial roasted coffee samples have lower contamination levels of STG, 3-aDON, 15-aDON, and NEO than decaffeinated samples. Soliman (2002) detected higher production of mycotoxins in decaffeinated coffee than in caffeinated coffee, but in this study, differences between caffeinated and

decaffeinated samples were detected for few mycotoxins and not for the same mycotoxin found by Soliman (2002).

3.5. Risk assessment

The dietary exposure assessment characterizes the population exposure. The results can be used for comparison with the TDI. All samples of the present study have been used for the statistical analysis. Samples with contamination levels under the LOD and LOQ were considered to be not contaminated (0 µg/kg).

The estimated daily intake (**Table 4**) of mycotoxins in coffee was calculated among diverse segments population; they were calculated in adults and adolescents and in different scenarios corresponding to population consumption habits, including the mean population and in highly exposed consumers (95th, 97.5th and 99th percentiles). The EDI in the mean population is not alarming with the exception of emerging mycotoxins, whose toxicology in humans is unknown; nevertheless, in highly exposed segments, the increased values of EDI are considerable. For example, for trichothecenes, specifically for DON and its metabolites, the mean consumption of the 99th population is not so concerning, though the effects of high doses of mycotoxins are uncertain. In a study of dietary exposure to OTA, higher levels of OTA exposure due to coffee consumption were observed in two segments

Table 4
Results of the dietary intake assessment of the studied mycotoxins for various scenarios expressed as (ng/g bw d)

	Adults				Adolescent			
	EDI_{mean}[b]	EDI_{P95}[b]	$EDI_{P97.5}$[b]	EDI_{P99}[b]	EDI_{mean}[b]	EDI_{P95}[b]	$EDI_{P97.5}$[b]	EDI_{P99}[b]
OTA	0.009	0.042	0.052	0.061	0.002	0.018	0.023	0.031
AFB_1	0.003	0.013	0.016	0.018	0.001	0.005	0.007	0.009
AFB_2	0.001	0.004	0.005	0.006	0.000	0.002	0.002	0.003
AFG_1	0.006	0.029	0.036	0.042	0.001	0.013	0.016	0.021
AFG_2	0.014	0.063	0.079	0.091	0.003	0.028	0.035	0.047
AFs_T[a]	0.036	0.169	0.210	0.244	0.008	0.074	0.093	0.125
STG	0.049	0.226	0.282	0.327	0.011	0.099	0.125	0.167
NIV	0.010	0.045	0.056	0.065	0.002	0.020	0.025	0.033
DON	0.593	2.747	3.418	3.973	0.138	1.204	1.516	2.028
3aDON	0.028	0.131	0.163	0.189	0.007	0.057	0.072	0.096
15aDON	0.524	2.431	3.025	3.515	0.122	1.065	1.341	1.795
DAS	0.185	0.858	1.068	1.241	0.043	0.376	0.473	0.634
NEO	0.152	0.704	0.877	1.019	0.035	0.309	0.389	0.520
T-2	0.009	0.043	0.054	0.062	0.002	0.019	0.024	0.032
HT-2	0.015	0.069	0.086	0.099	0.003	0.030	0.038	0.051
FB_1	0.008	0.039	0.048	0.056	0.002	0.017	0.021	0.029
FB_2	0.012	0.056	0.070	0.081	0.003	0.025	0.031	0.042
ENA	2.251	10.432	12.982	15.088	0.523	4.573	5.756	7.704
ENA_1	2.196	10.176	12.664	14.718	0.510	4.461	5.614	7.515
ENB	2.211	10.245	12.749	14.818	0.513	4.491	5.652	7.566
ENB_1	1.402	6.496	8.083	9.395	0.325	2.847	3.584	4.797
BEA	0.001	0.003	0.004	0.005	0.000	0.001	0.002	0.002
Intake[c]	0.013	0.063	0.078	0.090	0.003	0.027	0.034	0.046

[a]Mean concentration in µg/kg in all samples; those samples under the LOD are assigned as 0 µg/kg and LOQ as LOD.
[b]EDI Estimated Daily intake in the different exposed consumers (mean, 95th,97.5th, 99th) expressed as ng / kg bw day.
[c]Consumption: expressed as g / kg bw day

of the population; the of the mean level was 0.05 ng/kg bw d, and the 95th percentile was 0.22 ng/kg bw d (Leblanc et al., 2005).

For the risk assessment (**Table 5**) the mean and less favorable scenario were chosen for the study. Adults and adolescent in the mean and 99th percentile of consumption and an average sample were assumed. The risk was characterized via comparison with the TDI or the probable tolerable weekly intake (PTWI) proposed by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and with available studies of total diet from Spain, France, Japan and Libano (Coronel et al., 2011; Raad et al., 2014; Sirot et al., 2013; Sugita et al., 2013). As **Table 5** shows, any mycotoxins in coffee represent a risk to human health. The higher EDI was the result of emerging mycotoxins, and 15 ng of each enniatin per kg of body weight day was estimated. The EDI of DON was higher than the EDI of the other mycotoxins; however, DON toxicity is lower than that of the other mycotoxins. The risk characterization shows that DON and its metabolites (sum of DON, 3-aDON, and 15-aDON) represent 0.77% of the TDI; DON levels in coffee do not represent a risk to consumers. These results are in agreement with those of Rodríguez-Carrasco et al. (2013), who studied 10 mycotoxins in wheat rice and maize. Only total aflatoxins presented a considerable risk (15.68% of the TDI), and the other mycotoxins showed low EDI. It is important to consider that synergism and/or

Table 5
Risk characterization for each mycotoxin and groups of mycotoxins, and total mycotoxins

		Adults				Adolescents			
	TDI[a]	RA_{Mean} (%TDI)[b]	RAg_{Mean} (%TDI)[b]	RA_{P99} (%TDI)[b]	RAg_{P99} (%TDI)[b]	RA_{Mean} (%TDI)[b]	RAg_{Mean} (%TDI)[b]	RA_{P99} (%TDI)[b]	RAg_{P99} (%TDI)[b]
OTA	16	0.06		0.38		0.01		0.19	
AFB_1	1	0.27		1.81		0.06		0.92	
AFB_2	1	0.09	2.35	0.62	15.68	0.02	0.54	0.32	8.03
AFG_1	1	0.62		4.14		0.14		2.12	
AFG_2	1	1.36		9.11		0.32		4.66	
STG		0.05[c]		0.33[c]		0.01[c]		0.17[c]	
NIV	700	0.00		0.01		0.00		0.00	
DON	1000	0.06		0.40		0.01		0.20	
3aDON		0.00[d]	0.11	0.02[d]	0.77	0.00[d]	0.03	0.01[d]	0.39
15aDON		0.05[d]		0.35[d]		0.01[d]		0.18[d]	
DAS	2000	0.01		0.06		0.00		0.03	
NEO		0.15[c]		1.02[c]		0.04[c]		0.52[c]	
T-2	100	0.01	0.02	0.06	0.16	0.00	0.01	0.03	0.08
HT-2	100	0.01		0.10		0.00		0.05	
FB1	2000	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
FB2	2000	0.00		0.00		0.00		0.00	
ENA		2.25[c]		15.09[c]		0.52[c]		7.70[c]	
ENA_1		2.20[c]		14.72[c]		0.51[c]		7.51[c]	
ENB		2.21[c]	8.06	14.82[c]	54.02	0.51[c]	1.87	7.57[c]	27.57
ENB_1		1.40[c]		9.39[c]		0.33[c]		4.80[c]	
BEA		0.00[c]		0.00[c]		0.00		0.00	
Total		14.46		72.43		3.34		49.44	

[a]TDI: tolerable daily intake.
[b] Risk assessment and risk assessment of the groups of mycotoxins. RA = EDI/TDIx100.
[c] Risk assessment with respect to HT-2 TDI: 100 ng/kg bw d
[d] Risk assessment with respect to DON TDI: 1000 ng/kg bw d

additive effects of mycotoxins by co-occurrence have been described (Wan et al., 2013) unfortunately no TDI or similar value has been stablished and comparisons are not available. **Table 6** shows the comparison of the EDI between coffee consumption among the detailed percentile of the population and that from total diet examined in recent studies. This comparison shows that coffee is a low contributor food to the overall exposure to most mycotoxins.

Table 6
Assessment of mycotoxin exposure by coffee consumption according to studies from the literature that assessed total diet

Studies of total diet	Mycotoxin	Intake from total diet in mean population (ng/ kg bw d)	Intake from total diet high exposed population (ng/ kg bw d)	Share from coffee[a] (%)	Share from coffee in high exposed population[a] (%)
Raad et al., 2014	AFB_1	0.63		0.48	
	OTA	4.28		0.21	
	DON	1560.00		0.06	
Sugita et al., 2013	OTA		1.49 (95th)		2.82
	FB		5.30 (99th)		2.58
Sirot et al., 2013	OTA		3.23 (95th)		1.30
	AFB_1		0.39 (95th)		3.33
	AFB_2		0.30 (95th)		1.33
	AFG_1		0.30 (95th)		9.67
	AFG_2		0.30 (95th)		21.00
	DON		722.00 (95th)		0.38
	3-aDON		28.50 (95th)		0.46
	15-aDON		26.90 (95th)		9.04
	NIV		66.60 (95th)		0.07
	T-2		36.50 (95th)		0.12
	HT-2		58.90 (95th)		0.12
	FB_1		65.60 (95th)		0.06
	FB_2		42.20 (95th)		0.13
Coronel et al., 2012	OTA	2.91		0.31	

[a]Share from coffee calculated as: EDI_{coffee} / Intake from total diet * 100

4. Conclusions

A study of multi-mycotoxins in ready-to-drink coffee demonstrated the presence and coexistence of mycotoxins in coffee beverages. The study of coffee brewing shows similar effects by different coffee processes for most mycotoxins. In a few cases, as with the pre-portioned electric machine, there was greater degradation of 15-aDON, DON, NEO, and FB_1, and with the traditional process, there was significantly greater degradation of OTA. The risk assessment has been performed for Spanish adults and adolescents; population exposure was estimated at various scenarios. The results show that coffee intake does not represent a potential risk for consumers with respect to individual mycotoxin contamination. However, contamination of coffee by mycotoxins likely affects the EDI of the total diet, especially in highly exposed segments of the population. New parameters which take into account the co-occurrence and co-intake of mycotoxins are required to ensure a suitable understanding of this health hazard.

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4. GENERAL DISCUSSION

4. DISCUSIÓN GENERAL

4.1. Desarrollo y validación de los métodos analíticos

La metodología analítica para la detección de multimicotoxinas en café tostado y café preparado ha sido optimizada y validada de acuerdo con las guías recomendadas por la CE, donde se establece que una sustancia puede ser identificada usando CL-EM/EM en modo MRM por su tiempo de retención y con al menos dos iones de transición con una ratio constante entre sus áreas (Commission Decision, EC657/2002).

La validación del método se ha llevado a cabo con soluciones multimicotoxina preparadas en extracto de café procedente de una muestra libre de contaminación; y ha incluido: el cálculo del LOD mediante la ratio señal / ruido de 3; el LOQ mediante la ratio señal / ruido de 10; la linealidad, evaluada con rectas de calibrado a niveles que oscilan desde el LOQ hasta 1 mg/kg; evaluación del efecto matriz; pruebas de recuperación con ensayos (n=6) a dos niveles de concentración: 10LOQ y 100LOQ para cada una de las micotoxinas; cálculo de la repetibilidad (inter-día) y reproducibilidad (intra-día).

4.1.1. Optimización del método por CL-EM/EM

La optimización de la técnica CL-EM/EM se ha llevado a cabo en dos instrumentos, un triple cuadrupolo (QqQ) y un triple cuadrupolo con trampa de iones (IT). Para ello se ha procedido a la infusión de solución patrón en metanol de cada una de las micotoxinas objeto de estudio. Inicialmente con el modo "*full SCAN*" para conocer el ion precursor y el voltaje óptimo de entrada ("*Decluster Potential*" y "*Cone Coltaje*" para CL-EM/EN-IT y CL-EM/EM-QqQ respectivamente), obteniendo los valores menores para HT-2 de 21V y 10V para CL-EM/EN-IT y CL-EM/EM-QqQ, respectivamente, y los valores más altos para FB_2 con voltajes de 131V y 50V, respectivamente. Los iones precursores corresponden a la micotoxina protonada $[M + H]^+$ con excepción de los TRCs tipo A que presentan aductos de amonio $[M + NH_4]^+$. Para la optimización de los iones producto y de las energías de colisión se procede en modo "*selected ion monitoring*" (SIM) y "full SCAN". El ion más abundante ha sido seleccionado para la cuantificación, y el menos abundante para la confirmación. Las energías de colisión oscilan entre 15V (DAS) y 97V (OTA) en el CL-EM/EM-IT; y 10V (DON) y 45V (AFG_2) en CL-EM/EM-QqQ.

Tras la optimización del método para la EM, se procede a la optimización de los parámetros cromatográficos. Para ello se ha

realizado la inyección en la columna cromatografía de una solución multimicotoxinas preparada en el extracto de una muestra de café libre de contaminación. La columna cromatografía consiste en un polímero de C_{18}, y la fase móvil se optimiza con diferentes gradientes de fase móvil, siendo las fases para el intrumento CL-EM/EM-IT: fase A (H_2O con 5mM de formiato de amonio y 0,1% de ácido fórmico) y fase B (metanol), y para el intrumento CL-EM/EM-QqQ: A (H2O con 5 mM de formiato de amonio) y fase B (metanol). Los tiempos de retención fueron para CL-EM/EM-IT desde 11 min (DON) a 22 min (ENA) y para CL-EM/EM-QqQ desde 8 min (OTA) a 18 min (ZEA). Con estos datos se procede a la conformación final para la CL-EM/EM en modo "*multiple reaction monitorin*" (MRM) (García-Moraleja et al. 2015a) (García-Moraleja et al. 2015b).

4.1.2. Clarificación y extracción

Los pigmentos que se encuentran el café interfieren en la detección por EM. Para mejorar la sensibilidad y la precisión se han comparado diferentes métodos de purificación como columnas de C_{18}, carbón activo, precipitación con Solución Carrez y caseína (Niseteo et al. 2012; Perrone et al. 2012; Rahmani et al., 2009), utilizando diferentes disolventes para la extracción: metanol, acetonitrilo y acetato de etilo, y mezclas de acetato de etilo con ácido fórmico (Kokkonen y Jestoi,

2009; Mavungu et al. 2009; Pizzutti et al. 2014). Se ha optimizado el volumen de extractante, tiempos de extracción, y número de extracciones. Todos los ensayos han sido elaborados por triplicado.

4.1.2.1. Análisis de multimicotoxinas en café tostado.

El método optimizado consiste en extracción con Ultra-Turrax con mezcla de acetonitrilo y agua (80:20) y purificación con columna de C_{18}, y carbón activo. El método analítico está validado para 18 micotoxinas: AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, DON, NIV, DAS, T-2, HT-2, ZEA, ENA, ENA_1, ENB, ENB_1, y BEA. El análisis presenta efecto matriz para todas las micotoxinas, pero en diferentes grados, encontrándose efecto matriz entre el 21% en DON al 47% en AFB_1. Para todas las micotoxinas los coeficientes de correlación (R^2) son superiores a 0.992 y las desviaciones estándar relativas (DSR) son inferiores al 12% para la repetibilidad (intra-dia) y menores al 15% para la reproducibilidad (intrer-día). Y los valores de recuperación oscilan entre 71 para HT-2 y el 112% para ENB, como se puede ver en la **Tabla 4** (García-Moraleja et al. 2015a).

Table 4.
Metodo para el análisis de micotoxinas en café tostado. Valores de recuperacion experimentales ± la deviación estándar relativa (intra-day e inter- day)

	10 LOQ					100 LOQ				
Mycotoxin	Concentration[a]	R^b	DSR_r^c	R^b	DSR_R^d	Concentration	R^b	DSR_r^c	R^b	DSR_R^d
ENA	5.00	91	4	90	6	50.00	92	5	91	5
ENA_1	2.50	86	9	88	8	25.00	89	4	89	5
ENB	5.00	109	9	112	5	50.00	104	7	106	10
ENB_1	5.00	97	11	95	15	50.00	98	5	98	5
BEA	1.00	94	4	94	5	10.00	95	3	95	9
NIV	850.00	72	4	76	5	8500.00	73	4	73	9
DON	200.00	86	8	85	7	2000.00	89	2	88	4
DAS	50.00	82	6	80	9	500.00	84	4	83	6
HT-2	350.00	73	4	73	7	3500.00	71	6	74	6
T-2	125.00	82	12	84	11	1250.00	85	10	84	11
OTA	15.00	84	8	78	12	150.00	85	4	83	5
AFB_1	2.50	83	7	81	11	25.00	83	5	84	5
AFB_2	15.00	76	9	75	11	150.00	77	3	76	4
AFG_1	2.50	75	5	74	6	250.00	76	4	76	4
AFG_2	7.50	82	5	78	10	75.00	83	3	79	9
FB_1	500.00	84	5	80	12	5000.00	86	4	85	6
FB_2	500.00	88	8	88	7	5000.00	89	4	88	6
ZEA	125.00	77	6	77	7	1250.00	78	4	76	5

[a] Concentración, expresada como µg/kg
[b] Recuperación, expresada como % de patrón que es añadido a una muestra en blanco y es detectado en el CL-MS/MS (R = µg/kg detectados * 100 / µg/kg añadidos)
[C] DSR_r: Desviación Estándar Relativa intra-día (repetibilidad)
[d] DSR_R: Desviación Estándar Relativa inter-día (reproducibilidad)

4.1.2.2. Análisis de multimicotoxinas en café preparado.

Las muestras de café comerciales son procesadas según las diferentes técnicas (cafetera tradicional italiana, máquina eléctrica para cápsulas monodosis, café turco, y café soluble). Se procede a la clarificación con solución de Carrez, extracción en Ultra-Turrax con acetato de etilo con un 5% de ácido fórmico. La validación del método se ha realizado para el análisis simultáneo de 21 micotoxinas: OTA, AFB_1, AFB_2, AFG_1, AFG_2, STG, NIV, DON, 3-aDON, 15-aDON, DAS, NEO, HT-2, T-2, FB_1, FB_2, ENA, ENA_1, ENB, ENB_1, y BEA. El método muestra efecto matriz con una elevada supresión de señal desde el 13 en DON al 89% en el caso de NEO; buenas R^2, superiores a 0,992 para todas las micotoxinas estudiadas; La DSR inferiores al 9% en la repetibilidad, e inferiores al 11% en la reproducibilidad como se puede ver en la **Tabla 5**; las recuperaciones son buenas, entre el 72% para AFB_1 y el 97% para ENB_1; Los LOD y LOQ van desde 0,02 y 0,06 hasta 10,00 y 18,94 µg/kg respectivamente. El LOQ para la OTA es de 0,42 µg/kg, inferior al LMP: 5,0 µg/kg (CE, 1881/2006) (García-Moraleja et al. 2015b).

Table 5.
Metodo para el análisis de micotoxinas en bebida de café. Valores de recuperacion experimentales ± la deviación estándar relativa (intra-day e inter- day)

	10 LOQ					100 LOQ				
Mycotoxin	Concentration[a]	R[b]	DSR_r[c]	R[b]	DSR_R[d]	Concentration	R[b]	DSR_r[c]	R[b]	DSR_R[d]
OTA	5.00	94	8	92	8	50.0	98	4	96	7
AFB_1	0.75	73	6	77	8	7.50	84	5	84	4
AFB_2	1.50	76	9	75	11	15.00	86	3	87	5
AFG_1	1.50	75	5	74	6	15.00	85	4	89	4
AFG_2	2.50	82	5	78	10	25.00	85	3	85	9
STG	20.00	74	10	73	12	200.00	79	5	88	5
NIV	0.60	94	7	89	9	6.00	95	3	98	9
DON	175.00	78	6	76	7	1750.00	80	2	80	4
3-aDON	50.00	95	4	90	9	500.00	100	4	99	4
15-aDON	175.00	73	6	79	8	1750.00	88	4	88	6
DAS	60.00	88	7	84	9	600.00	92	7	92	7
NEO	40.00	73	9	74	7	400.00	93	5	93	5
HT-2	90.00	92	4	89	5	900.00	95	6	97	6
T-2	7.50	90	7	83	12	75.00	89	3	89	3
FB_1	33.00	84	5	80	12	300.00	89	4	89	6
FB_2	33.00	81	8	84	8	300.00	90	5	91	8
ENA	5.00	94	4	93	6	50.00	96	4	96	4
ENA_1	1.00	86	9	88	10	10.00	95	9	94	9
ENB	5.00	90	4	84	9	50.00	91	7	91	6
ENB_1	5.00	97	11	95	15	50.00	97	9	95	7
BEA	1.00	94	4	94	5	10.00	98	3	98	5

[a] Concentración, expresada como µg/kg
[b] Recuperación, expresada como % de patrón que es añadido a una muestra en blanco y es detectado en el CL-MS/MS (R = µg/kg detectados * 100 / µg/kg añadidos)
[C] DSR_r: Desviación Estándar Relativa intra-día (repetibilidad)
[d] DSR_R: Desviación Estándar Relativa inter-día (reproducibilidad)

4.2. Presencia de micotoxinas en café

4.2.1. Presencia de micotoxinas en muestras de café tostado

Se han analizado 103 muestras de café tostado comercializadas en tiendas y supermercados de Valencia. Los resultados obtenidos tras el análisis de AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, DON, NIV, DAS, T-2, HT-2, ZEA, ENA, ENA_1, ENB, ENB_1 y BEA muestran contaminación de al menos 6 micotoxinas simultáneamente, excepto 4 muestras de café (3 café con cafeína, 1 descafeinado) que no presentan contaminación por micotoxinas. La mayor parte de las muestras 61% presentan contaminación simultánea de entre 10 y 12 micotoxinas, como se puede observar en la **figura 3**.

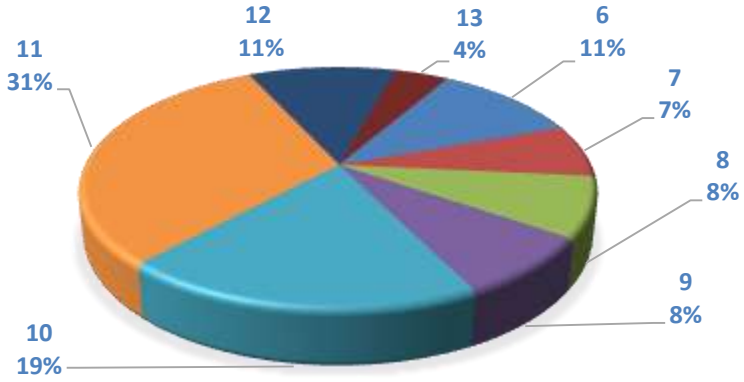

Figura 3: representación gráfica de la coexistencia de diferentes micotoxinas, siendo el número superior el número simultáneo de micotoxinas y el inferior el porcentaje de muestra que lo contenía.

El NIV es la micotoxina que presenta una mayor incidencia (96%) y niveles de concentración. Por el contrario, la ZEA no es detectada en ninguna de las muestras analizadas. Las micotoxinas que presentan menores niveles de concentración son ENB_1 y DAS, detectándose en el 12% de las muestras de café tostado analizadas. El resto de micotoxinas se detectan en más del 50% de las muestras con una distribución variable.

Respecto a las ME, las ENA_1 y ENB son las micotoxinas que presentan mayor incidencia en muestras de café tostado (85% y 73%, respectivamente). En cuanto a los TRCs, el NIV oscila entre 0,4 a 25,86 mg/kg. DON y HT-2 son los TRCs con mayor presencia detrás de NIV. El resto de TRCs presentan bajas incidencias y concentraciones. El que presenta menor incidencia es el DAS que se detecta en el 12% de las muestras. En la bibliografía revisada no hay datos disponibles a cerca de ME ni TRCs en muestras de café.

Se detecta OTA en el 35% de las muestras. La concentración en las muestras positivas oscila entre 1,50 a 32,40 µg/kg, estas concentraciones están de acuerdo con otros estudios (Bandeira, 2012; Batista et al. 2003; Casal et al. 2014; Coronel et al. 2011; Lee et al. 2012; Lobeau et al., 2005; Tozlovanu y Pfohl-Leszkowicz, 2010) en los que se

cuantifican concentraciones similares (desde 0,15 a 23,70 µg/kg). Por el contrario, Sibanda et al (2002) no detectan OTA en café tostado. Cabe destacar que se detectan 5 muestras con concentraciones superiores a los LMP por la UE (5 de 103 muestras). Dos de ellas son café descafeinado, con concentraciones de 9,3 y 6,2 µg/kg; dos muestras de café con cafeína en cápsulas monodosis con concentraciones de 6,91 y 11,43 µg/kg; y una muestra de café descafeinado en cápsulas monodosis 32,4 µg/kg. Estos resultados son similares a otros estudios que detectan contaminación por OTA en muestras de café por encima de los LMP: Coronel et al. (2011) detectan OTA a una concentración de 5,24 µg/kg, Lobeau et al. (2005) a 5,4 µg/kg; Bandeira (2012) a 9 µg/kg; Tozlovanu y Pfohl-Leszkowicz (2010) a 15,08 µg/kg; Vanesa y Ana (2013) 5,7 µg/kg en café tostado y 13,66 µg/kg en café soluble.

Las AFs presentan una alta incidencia, pero bajas concentraciones. La concentración media es de 1,95 µg/kg (rango de 0,15 hasta 12,45 µg/kg). Soliman (2002) en café verde y café tostado detecta concentraciones de 0,76 a 8,92 µg/kg; Bokhari y Aly (2009) detectan concentraciones entre 5 y 23 µg/kg.

La incidencia y rangos de concentración de FB en las muestras contaminadas son del 65 y 58%, y de 0,06 - 0.54 y de 0.07 - 0.37 mg/kg

en FB_1 y FB_2 respectivamente. Estas concentraciones son superiores a las encontradas por Noonim et al, (2009) en el estudio de café tostado y molido, donde detectan niveles de 10 µg/kg.

Además, se ha realizado el análisis de una muestra de café verde, en ella se detectan 11 micotoxinas: ENA_1, ENB, NIV, DON, DAS, HT-2, T-2, AFG_1, AFG_2, FB_1, and FB_2. De acuerdo con Ventura et al. (2003) la OTA no es detectada, no obstante, otros estudios disponibles (Batista et al., 2003; Bokhari, 2007; Imperato et al., 2011; Napolitano et al, 2007; Pittet y Royer, 2002; Vanesa y Ana, 2013; Ventura et al., 2003; Visconti y De Girolamo, 2005) reportan concentraciones variables, de entre 0,1 a 360 mg/kg de OTA (García-Moraleja et al. 2015a).

4.2.2. Presencia de micotoxinas en café procesado

En el análisis de micotoxinas en café precesado con diferentes tratamientos (cafetera tradicional italiana, máquina eléctrica para cápsulas monodosis, café turco, y café soluble). Todas las micotoxinas estudiadas se detectan en muestras comerciales con incidencias entre el 4 % para la FB_2 al 93% para la ENB. De las 169 muestras analizadas 19 no presentan contaminación por ninguna de las micotoxinas contempladas en la legislación alimentaria actual, y solo 5 muestras no

presentan contaminación por ninguna micotoxina estudiada (García.Moraleja et al. 2015c).

Las AFs están presentes en el 53% de las muestras estudiadas, y ninguna muestra presenta concentraciones superiores a 2 µg/kg de AFB_1, pero sí que en algunos casos se superaron unos niveles de AFs superiores a 5 µg/kg. Los casos en los que las AFs superan los LMP es debido a las concentraciones de AFG_2, cabe señalar que las AFG presentan menor toxicidad. En la mayoría de las muestras en las que se detectan AFs las muestras están contaminadas con una o dos AFs simultáneamente y en raros casos con tres AFs simultáneamente; ninguna muestra presenta contaminación simultánea por las 4 AFs. En comparación, Khayoon et al. (2014) analizan 21 muestras de café en lata, y no detectan AFs; Bokhari et al (2007) detectan AFB_1 en café verde con concentraciones de 110,5 µg/kg. Comparando estos datos con las concentraciones en café tostado, podemos ver que durante el proceso de percolación en la preparación del café, el agua arrastra solo una parte de las AFs contenidas en el café tostado.

El 36% de las muestras están contaminadas por OTA, pero en ningún caso se exceden los LMP establecidos (EC/1881/2006), además se puede ver como las concentraciones son inferiores que las concnetraciones detectadas en las muestras de café tostado. Los

resultados de la presente tesis están de acuerdo con Noba et al. (2009) que detecta OTA en todas las muestras de café listo para beber analizadas con concentraciones que oscilan entre el 0,01 y 0,04 µg/L; Casal et al. (2014) detecta OTA en café soluble con concentraciones medias de 2,50 µg/kg. Como reporta Santini et al. (2011) se puede observar una reducción de los niveles de contaminación por OTA durante la preparación del café. De acuerdo con estudios previos, la bebida de café contiene niveles de contaminación inferiores a los que se muestran en café tostado, no obstante, la infusión de la bebida no produce la eliminación total de esta micotoxina. Perez De Obanos et al. (2005), estudian los efectos del procesado en los contenidos de OTA de 9 muestras positivas; registrando reducciones de las concentraciones en todos los casos, solo en uno de los casos se observa una reducción hasta concentraciones inferiores al LOD.

La STG está presente en un 16% de las muestras. Los resultados del presente estudio están de acuerdo con estudios de Bokhari y Aly (2009), quienes detectan STG en 2 de 13 muestras de café en grano analizadas con concentraciones de 13 y 11 µg/kg.

Los TRCs analizados (NIV, DON, 3-aDON, 15-aDON, DAS, NEO, HT-2 and T-2) presentan contaminaciones variables. Con concentraciones desde 1,68 µg/kg de NIV hasta 196,2 µg/kg en el caso

de DAS. Las incidencias son bajas en todos los casos. En el estudio del café tostado se encuentran concentraciones muy superiores de hasta unidades de mg/kg, lo que implica una fuerte reducción de las concentraciones en la mayoría de los casos (NIV, DAS, HT-2, and T-2) no obstante el DON presenta concentraciones superiores en la bebida de café que en las muestras de café tostado. La micotoxina que muestra una mayor degradación o una menor solubilización y consecuentemente una migración inferior desde el café tostado a la bebida es NIV.

Las FB_1 y FB_2 presentan una baja incidencia 4% y bajos niveles de contaminación 15,55 y 22,46 µg/kg respectivamente (García.Moraleja et al. 2015c). En el estudio del café tostado se detectan niveles de contaminación superiores, con concentraciones mínimas de FB_1 y FB_2 detectadas de 58,62 y 85,45 µg/kg respectivamente (García-Moraleja et al. 2015a) indicando una clara reducción de los contenidos. Si bien, siguen siendo superiores a los reportados por Noonim et al. (2009), quienes detectan niveles de FB_2 en café verde con concentraciones de 1,3 y 9,7 µg/kg. Los resultados de la presente tesis están de acuerdo con los encontrados con Nielsen et al. (2015) que detectan concentraciones de 164 µg/kg en café verde, y una reducción de FBs tras el tostado. Las FBs consisten en una cadena de carbonos susceptibles de degradación en tratamientos térmicos, lo

que explica la degradiación durante la preparación del café para su consumo. La baja incidencia de estas micotoxinas demuestra que el procesado del café no solo reduce, sino que incluso elimina en muchos casos su presencia.

En cuanto a las ME, las ENs son las que presentan una mayor contaminación. Mientras que la BEA presenta bajos niveles de contaminación. La comparación con los resultados de ME en café tostado sugiere que el procesado del café no afecta al contenido de ME.

4.2.3. Diferencias entre tipos de café y procesado

4.2.3.1. Café con cafeína y descafeinado

La mayor parte de las micotoxinas (AFs, OTA y TRCs) no presentan diferencias significativas entre las muestras de café con cafeína y descafeinado en las muestras de café tostado. Unicamente las ME y FB presentan una diferencia entre las muestras con cafeína y descafeinado, presentando mayores concentraciones en las muestras de café con cafeína (García.Moraleja et al. 2015a). Según Paterson et al. (2014), algunas cepas de *Aspergillus spp*. producen mayores cantidades de micotoxinas en medios de cultivo con contenidos de

cafeína, posiblemente los hongos productores de FBs y ME en café muestran el mismo comportamiento. Por otra parte, Soliman (2002) observa un aumento de la producción de AFs por *Aspergillus flavus* en café descafeinado. Sin embargo, en el presente estudio no se encuentran diferencias en los niveles de AFs entre los dos tipos de café.

En café preparado, el descafeinado en general muestra mayores niveles de contaminación que el café con cafeína. No obstante, las diferencias estadísticas no son significativas para OTA, AFs, NIV, DON, DAS, ni ME. Sí que se observan diferencias significativas con niveles inferiores en muestras de café con cafeína en STG, 3a-DON, 15a-DON, y NEO. Las diferencias entre café con cafeína y descafeinado no son resultado del procesado, sino que radican en el tipo de materia prima usada. Soliman (2002) detecta niveles más altos de contaminación por micotoxinas en café descafeinado que en café con cafeína.

4.2.3.2. Tipo de tostado

Los contenidos de AFs, OTA, y FBs son similares en los diferentes tipos de tostado, en cambio los TRCs y las ME presentan mayores concentraciones en el café torrefacto. El café torrefacto normalmente está asociado a semillas de café de baja calidad. La presencia de concentraciones más elevadas de micotoxina en muestras de café torrefacto puede ser la consecuencia del uso de cafés de peor calidad.

4.2.3.3. Tipo de procesado

Se han evaluado diferentes tipos de procesado: convencional, mediante la máquina de café tradicional italiana; soluble, siguiendo las recomendaciones del fabricante; café en cápsulas monodosis, utilizando las respectivas maquinas eléctricas específicas de preparación del café; y café turco, siguiendo la receta tradicional.

Cada tipo de procesado muestra un efecto diverso en las diferentes muestras. No obstante, no se observan diferencias estadísticamente significativas en 16 de las 21 micotoxinas estudiadas (AFB_1, AFB_2, AFG_1, AFG_2, NIV, 3a-DON, DAS, HT-2, T-2, STG, ENA, ENA_1, ENB, ENB_1, BEA and FB_2) entre los diferentes tipos de procesado. En cambio 15a-DON, DON, NEO, y FB_1 muestran una degradación superior tras el procesado con máquina eléctrica para cápsulas monodosis seguido del café soluble, mientras que el procesado con máquina de café tradicional italiana es el que muestra menores reducciones. Por otro lado, la OTA presenta mayores degradaciones tras el procesado con cafetera tradicional italiana comparada con el café en máquina eléctrica para cápsulas monodosis y café soluble. Esto indica que probablemente 15a-DON, DON, NEO y FB_1 son mas sensibles al aumento de la presión y al tiempo de contacto, mientra que OTA es mas sensible al aumento de temperatura. El café turco es el que presenta mayor cantidad de AFG_2 (80%), pero no presenta

contaminación por otras AFs o STG que es un derivado de las AFs, y los niveles de contaminación son similares a otros tipos de café. Probablemente, la influencia del procesado de café turco en la contaminación por AFs no presenta diferencias con el resto de preparaciones, no obstante, el café turco producido en países del norte de África, en una zona geográfica localizada con un clima específico, puede favorecer la producción de AFG_2 frente a la producción de AFB_1, AFB_2 o AFG_1).

4.2.4. Café soluble con leche en polvo

Las muestras de café soluble con adición de leche en polvo presentan un número más reducido de micotoxinas diferentes, pero los niveles de contaminación son más elevados que las muestras de café tostado (García-Moraleja et al., 2015a). La OTA no es detectada en muestras de café soluble, al contrario que en estudios previos de otros autores, como Lee et al. (2012) quienes reportan mayores incidencias en muestras de café soluble con respecto a café tostado. Reverberi et al. (2010) observan un aumento de la producción de OTA en medios con presencia de lactosa. Como se puede observar en la descripción de las muestras en el presente estudio, las muestras de café soluble con adición de leche en polvo presentan envases monodosis, siendo más complicada la contaminación con hongos productores, esto explicaría la baja incidencia, por otro lado, de

acuerdo con Lee et al. (2012) y Reverbery et al. (2010), una vez se ha producido la contaminación por el hongo, los niveles de producción de micotoxinas son más elevados, lo que explicaría las altas concentraciones.

4.3. Ingesta diaria estimada y evaluación del riesgo

Con el fin de evaluar si la exposición a micotoxinas del café supone un riesgo para la población, se ha realizado el calculo de la IDE y la posterior estimación del riesgo, de acuerdo con los principios y métodos para la estimación del riesgo de químicos en alimentos publucada conjuntamente por la OMS y la FAO (WHO, 2009).

Para el cálculo de la IDE se usan los datos de contaminación por micotoxinas en muestras de café preparado para su consumo. Las muestras con niveles de concentración menores al LOD y LOQ se han considerado no contaminadas. La IDE se ha calculado para diferentes segmentos de la población: adolescentes y adultos, y en diferentes escenarios correspondientes a los diferentes hábitos de consumos: población general y consumidores con elevada exposición (percentiles: p95, p97.5 y p99). La IDE para la población media en la mayoría de los casos es inferior a 0,5 ng/kg pc/día, con excepción de

las ME (2,2 ng/kg pc/día). En grupos de población que presentan un elevado consumo de café, la mayoría de las IDE de micotoxinas siguen siendo bajas (inferiores a 0,5 ng/kg pc/día); sin embargo, los valores de IDE de TRCs aumentan considerablemente, DON llega hasta 3,9 ng/kg pc/día en consumidores adultos del percentil 99, y en el caso de las ME la ENA llegan a 15,0 ng/kg pc/día. En el estudio de la exposición a OTA los valores de IDE son de 0,009 ng/kg pc/día en la población general y de 0,042 ng/kg pc/día en población del percentil 95. Leblanck et al. (2005) reporta exposiciones superiores en ambos segmentos de la población, con IDE en la población general de 0,05ng/kg pc/día y de 0,22 ng/kg pc/día en el percentil 95.

La evaluación del riesgo se realiza para adultos y adolescentes considerando dos niveles de exposición: la exposición en población general y el escenario más desfavorable (percentil 99). El riesgo se ha evaluado por medio de la comparación con la IDT o con el IST (según disponibilidad) que proporciona la CE (EFSA, 2006, EFSA 2014a, EFSA, 2015); y con otros estudios disponibles de dieta total de España, Francia, Japón, y Líbano (Coronel et al. 2011; Raad et al. 2014; Sirot et al. 2013; Sugita-Konishi et al. 2013). Los resultados muestran que ninguna micotoxina representa un riesgo para la salud humana. DON y sus metabolitos, suma de DON, 15-aDON, 3-aDON, representan el 0,77% de la IDT, de forma que los altos niveles de DON en el café no

representan un riesgo apreciable para los consumidores. Cabe destacar que la suma de AFs al tener un valor bajo de IDT (1 ng/kg pc/día) sí que supone un porcentaje relativamente significativo en la población general (2,4 ng/kg pc/día) y en el percentil 99 (15,7 ng/kg pc/día). Estos resultados están de acuerdo con Rodríguez-Carrasco et al. (2013), quienes estudian 10 micotoxinas en trigo, maíz y arroz, y la mayoría de las micotoxinas muestran bajos niveles de IDE y únicamente las AFs muestran unos niveles de riesgo relativamente altos (16% del IDT). La comparación de la IDE con los datos de IDE en dieta total disponibles, muestra que el café representa una baja contribución a la IDE en la mayoría de las micotoxinas, siendo inferior al 0,5% en la población media e inferior al 4% en la mayoría de micotoxinas en sectores de la población con alta exposición (excepto AFG y 15-aDON) (García-Moraleja et al. 2015c). Si bien, el contenido en algunas micotoxinas como AFs y TRCs debería ser tenido en cuenta en futuros estudios de IDE en dieta total. Además, la presencia de micotoxinas en café, que es un producto consumido a diario por gran parte de la población, indica la necesidad de una legislación específica.

5. CONCLUSIONS

5. CONCLUSIONES

I. Se ha desarrollado y validado la metodología analítica para el análisis de multimicotoxinas en muestras de café tostado y de bebida de café. La CL-EM/EM previa extracción con Ultra-Turrax, muestra ser un método que cumple con los estándares de calidad exigidos por la Unión Europea y, por lo tanto, es apto para el control de multimicotoxinas en café.

II. El análisis de muestras de café comercializadas muestra la presencia y la coexistencia de las micotoxinas en las dos etapas de la post-comercialización, tostado y preparación de la bebida.

III. La única micotoxina legislada en café, la ocratoxina A, muestra una baja incidencia. Si bien, algunas muestras exceden los límites máximos permitidos. Estos niveles indican la necesidad de controles más rigurosos del producto en cuanto al contenido en ocratoxina A.

IV. Respecto a la presencia de ocratoxina A, aflatoxinas, nivalenol, deoxinivalenol, diacetoxiscirpenol y micotoxinas emergentes no existe una diferencia significativa entre el café con cafeína y café descafeinado, sin embargo, el café con cafeína muestra menores concentraciones de esterigmatocistina, 3-acetoxideoxinivalenol, 15-acetideoxinivalenol, y neosolaniol.

V. El café torrefacto presenta mayores niveles de Tricotecenos y de micotoxinas emergentes que el café de tostado natural, debido

seguramente a una calidad inferior de la materia prima empleada.

VI. El diferente procesado de las muestras en la preparación de la bebida (cafetera tradicional italiana, máquina eléctrica para cápsulas monodosis, café turco, y café soluble) causan reducciones en los contenidos de todas las micotoxinas.

VII. Los tipos de preparación del café líquido muestran efectos similares en el contenido de micotoxinas. Con excepción de 15-acetideoxinivalenol, deoxinivalenol, neosolaniol, y fumonisina B1 que tienen una mayor degradación tras el procesado con la máquina eléctrica para cápsulas monodosis; y ocratoxina A que presenta una mayor degradación con la cafetera tradicional italiana.

VIII. Tras la evaluación del riesgo en adultos y adolescentes españoles, se concluye que el consumo de café no representa un riesgo potencial por su contenido en micotoxinas. No obstante, la contaminación del café por micotoxinas afecta a la ingesta diaria estimada en la dieta total, especialmente en sectores de la población con alto consumo de café.

IX. La presencia y coexistencia de micotoxinas en café, los efectos toxicológicos de las mismas y la legislación actual del café, que solo contempla la ocratoxina A, muestran la necesidad de una legislación más completa, que regule un mayor número de micotoxinas en este producto.

CONCLUSIONS

I. New analytical methodology for the simultaneous analysis of mycotoxins in roasted coffee and in coffee beverages has been developed and validated. The LC-MS/MS perform over a Ultra-Turrax extraction provides a multi-mycotoxins method suitable for the simultaneous detection and quantification according the quality standards required by European Union.

II. The analysis of the commercial coffee samples shows the presence and coexistence of mycotoxins in both post-commercialized phases (roasted coffee and coffee beverages).

III. The only legislated mycotoxin in coffee ochratoxin A shows a low occurrence, but in some samples it exceeded the established maximum limits. This levels indicate that it is necessary to increase the controls of ochratoxin A contamination in coffee.

IV. There are not significant differences between decaffeinated and caffeinated coffee regarding the presence of Ochratoxin A, aflatoxins, nivalenol, deoxynivalenol, diacetoxyscirpenol and emerging mycotoxins. Nevertheless, caffeinated coffee shows lower concentration levels for sterigmatocystin, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol and neosolaniol.

V. Trichothecenes and Emerging mycotoxins present higher concentration in torrefacto roasted coffee than in natural roasted coffee. Probably, because of torrefacto coffee is usually associated with low quality coffee beans.

VI. The different brewing process of coffee (Italian conventional coffee, electric machine for pre-portioned coffee capsules, Turkish coffee and soluble coffee) reduce the contents of all the studied mycotoxins.

VI. The different coffee brewing shows similar effects in the mycotoxins contents. In a few cases, as pre-portioned electric machine, there is a greater degradation of 15-acetyl deoxynivalenol, deoxynivalenol, neosolaniol, and fumonisin B_1, and as Italian traditional process where is significantly greater the degradation of ochratoxin A.

VII. The risk assessment has been performed for Spanish adults and adolescents. The results show that coffee intake does not represent a potential risk for consumers with respect to mycotoxin contamination. However, contamination of coffee by mycotoxins likely affects the estimated daily intake of the total diet, especially in highly exposed segments of the population.

VIII. The presence and co-occurrence of mycotoxins in coffee, their toxic effects and current coffee legislation, that only include ochratoxin A, reaffirm the necessity of new coffee regulations which include a greater number of mycotoxins.

6. REFERENCES

6. REFERENCIAS

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ANEXO I. OUTREACH OF RESULTS

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Simultaneous determination of mycotoxin in commercial coffee

Ana García-Moraleja, Guillermina Font, Jordi Mañes, Emilia Ferrer*

Laboratory of Toxicology and Food Chemistry, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

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Aflatoxin B_2 (PubChem CID: 2724360)
Sterigmatocystin (PubChem CID: 5284457)
Nivalenol (PubChem CID: 5284433)
T-2 toxin (PubChem CID: 40024)
Deoxynivalenol (PubChem CID: 5284461)
Enniatin A_1 (PubChem CID: 57369704)
Enniatin B (PubChem CID: 164754)
Fumonisin B_1 (PubChem CID: 3431)

ABSTRACT

Mycotoxins are secondary metabolites produced by filamentous fungi that usually contaminate food products. Coffee is a natural product susceptible to mycotoxin contamination. The present study evaluates the presence of nivalenol, deoxynivalenol, T-2 and HT-2 Toxin, diacetoxyscirpenol, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, fumonisin B_1, fumonisin B_2, ochratoxin A, zearalenone, enniatin A, enniatin A_1, enniatin B, enniatin B_1, and beauvericin in coffee samples, using liquid chromatography tandem mass spectrometry (LC-MS/MS). The results show that zearalenone was not present in any sample. In the positive samples the contents of fumonisins ranged from 58.62 to 537.45 μg/kg, emerging mycotoxins ranged from 0.10 to 3569.92 μg/kg, aflatoxins ranged from 0.25 to 13.12 μg/kg, and trichothecenes, excepting nivalenol, ranged from 5.70 to 325.68 μg/kg. Nivalenol presented the highest concentrations, from 0.40 to 25.86 mg/kg. Ochratoxin A ranged from 1.56 to 32.40 μg/kg, and five samples exceeded the maximum limit established by the European Commission.

1. Introduction

Mycotoxins are secondary metabolites produced by filamentous fungi, such as *Aspergillus* spp., *Penicillium spp.*, and *Fusarium* spp., in the matrix where they grow (Rocha, Freire, Erlan Feitosa Maia, Izabel Florindo Guedes, & Rondina, 2014). Hypotheses about their functions on the producer fungus are still being studied, but they probably perform ecological functions against other organisms and protect fungi from oxidative stress (Reverberi, Ricelli, Zjalic, Fabbri, & Fanelli, 2010). Mycotoxins are classified into different groups depending on their molecular structure, producer fungus, and toxicity: Trichothecenes, which comprise nivalenol (NIV), deoxynivalenol (DON), diacetoxyscirpenol (DAS) and T-2 and HT-2 Toxin,[1] are molecules that produce the inhibition of protein synthesis (Rocha, Freire, Erlan Feitosa Maia, Izabel Florindo Guedes, & Rondina, 2014). Aflatoxins are produced by *Aspergillus* spp. and are classified as aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2),[2] and aflatoxin M_1 (AFM_1). The International Agency for Research on Cancer (IARC, 2012) classified AFB_1, AFB_2, AFG_1, and AFG_2 as carcinogenic to humans, mostly in liver cells. Fumonisins B are classified as fumonisin B_1 (FB_1) and fumonisin B_2 (FB_2)[3] and are hepatotoxic and immunotoxic molecules (Köppen et al., 2010). Ochratoxin A (OTA)[4] produces nephropathies and urothelial tumors in humans (Amézqueta et al., 2012). Zearalenone (ZEA)[5] has estrogenic effects (Afsah-Hejri, Jinap, Hajeb, Radu, & Shakibazadeh, 2013). Emerging mycotoxins, such as

* Corresponding author. Tel.: +34 963544950; fax: +34 963544954.
E-mail address: emilia.ferrer@uv.es (E. Ferrer).

[1] NIV: nivalenol; DON: deoxynivalenol; DAS: diacetoxyscirpenol; T-2: T-2 toxin; HT-2: HT-2 toxin.

[2] AFB_1: aflatoxin B_1; AFB_2: aflatoxin B_2; AFG_1: aflatoxin G_1; AFG_2: aflatoxin G_2.

[3] FB_1: fumonisin B_1; FB_2: fumonisin B_2.

[4] OTA: Ochratoxin A.

[5] ZEA: Zearalenone.

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enniatin A, enniatin A_1, enniatin B, enniatin B_1, and beauvericin (ENA, ENA_1, ENB, ENB_1, and BEA, respectively)[6] have only recently been studied (Serrano, Font, Mañes, & Ferrer, 2013), and some studies show that they can represent a potential risk for the human health because of their toxic effects in cell lines (Prosperini, Meca, Font, & Ruiz, 2012).

The stimulant properties of coffee, probably because of its caffeine content and the lifestyle trends of the population, have led to increased coffee beverage consumption each year (International Coffee Organization ICO, 2013). In Spain, a total of 80,000.00 tons of coffee (ground and instant coffee) are consumed annually (Ministry of Agriculture Food and Environment, 2014). Coffee is one of the most consumed food products, with an important economic and cultural role. It is cultivated in a total of 75 countries on four continents, with Brazil the largest manufacturer of coffee, with approximately one third of the world production (Cabrera & Gimenez, 2010). Fungi from *Fusarium* genera cause diseases in coffee plants (*Coffea arabica* and *Coffea canephora*). They penetrate through the aerial portions of the plants, but the first symptoms start on the roots (Nina, Smeltekop, Almanza, & Loza-Murguia, 2011). The contamination of coffee by filamentous fungi can occur at various stages, harvesting, preparation, transportation, or storage, and in fermentation and drying, especially where the water activity is lower (Silva, Batista, & Schwan, 2008).

Some studies have reported mycotoxin contamination in coffee. The coffee legislation of mycotoxins (European commission (EC)[7] 1881/2006) sets maximum limits (ML)[8] of OTA: 5.0 μg/kg (in roasted coffee beans and ground roasted coffee, excluding soluble coffee), and 10.0 μg/kg (in soluble coffee-instant coffee). In fact the most studied mycotoxin in coffee is OTA (Paterson, Lima, & Taniwaki, 2014). The presence of non-regulated mycotoxins in coffee as aflatoxins, fumonisins, patulin and sterigmatocystin has been studied (Rahmani, Jinap, & Soleimany, 2009). A study of green coffee reported concentrations of FB_2 ranging from 9.70 to 13.00 μg/kg (Noonim, Mahakarnchanakul, Nielsen, Frisvad, & Samson, 2009). Nielsen, Ngemela, Jensen, Medeiros, and Rasmussen (2015) studied green, roasted and instant coffee ($n = 57$) and reported concentrations of OTA and FB_2 (mean: 2.83 μg/kg and maximum: 8.30 μg/kg; mean: 25 μg/kg and maximum: 136 μg/kg respectively). A qualitatively study has been conducted in 30 green coffee samples, results showed incidences of 10, 17, 23, 7, 17, and 7% of OTA, ($AFB_1 + AFB_2$), AFG_1, PAT and STG respectively (Bokhari & Aly, 2009). Other studies reporting non contamination of coffee samples with OTA and AFs or not reporting data from real samples has been published (Desmarchelier et al., 2014; Khayoon, Saad, Salleh, Manaf, & Latiff, 2014; Sibanda, De Saeger, Barna-Vetro, & Van Peteghem, 2002; Ventura et al., 2003). Publications about method validations or multi matrix studies reporting concentrations of OTA in green and roasted coffee show contamination levels ranging from 0.09 to 9.00 μg/kg (Bandeira, 2012); ranging from 0.64 to 4.14 μg/kg (Batista, Chalfoun, Prado, Schwan, & Wheals, 2003); of 23.70 μg/kg (Imperato, Campone, Piccinelli, Veneziano, & Rastrelli, 2011); ranging from 0.80 to 5.40 μg/kg (Lobeau, De Saeger, Sibanda, Barna-Vetró, & Van Peteghem, 2005).

A recent study on mycotoxins and climate change (Paterson et al., 2014) advised the necessity of urgent consideration of mycotoxins in coffee. As far as we know, no data have been published to evaluate the simultaneous presence of different groups of mycotoxins produced by *Fusarium* and/or *Aspergillus* (aflatoxins, OTA, ZEA, fumonisins, trichothecenes, and emerging mycotoxins) in coffee.

In this context, the aim of the present study was to evaluate the contamination levels and co-occurrence of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, DON, NIV, DAS, T-2, HT-2, ZEA, ENA, ENA_1, ENB, ENB_1, and BEA in different coffee samples using Ultra Turrax liquid extraction and Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)[9] with triple quadrupole (QqQ)[10] mass analyzer determination.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (AcN)[11] and methanol (MeOH)[12] were provided by Merck (Darmstadt, Germany). Ammonium formate (99%) was supplied by Panreac Quimica (Barcelona, Spain) (Madrid, Spain). Deionized water (<18 MΩ cm^{-1} resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System (Millipore, Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter from Scharlau (Barcelona, Spain). The standards were purchased from Sigma–Aldrich (St. Louis, MO, USA). Individual stock solutions of ENA_1, ENB, ENB_1, and BEA with a concentration of 1000 mg/L, and ZEA, NIV, DON, FB_1, FB_2 and ENA with a concentration of 500 mg/L, were prepared in MeOH. Aflatoxins and OTA were prepared at 500 mg/L, and DAS, T-2 and HT-2 at 100 mg/L were prepared in AcN. The solutions were stored in glass-stoppered bottles in the dark in secure conditions at −20 °C. These stock solutions were diluted with AcN/MeOH [50:50] to obtain the appropriate working concentrations before LC-MS/MS analysis.

2.2. Sample collection

Commercial samples of coffee (103 samples) were purchased from different supermarkets located in Valencia (Spain). One sample of green coffee beans was kindly provided from a coffee roaster (Asturias, Spain). All samples were collected during 2013–2014. The method of sampling was accomplished according to the Commission Regulation (EC401/2006) for the official control of the ML established for OTA in roasted coffee beans, ground roasted coffee and soluble coffee. The samples were classified according roasting process: 28 samples of natural roasted coffee and 75 samples of torrefacto roasted coffee (special industrial roasted coffee with additional sugar to increase the flavor). Also, the samples were classified according the caffeine content: 52 samples of caffeinated coffee and 40 samples of decaffeinated coffee. These samples were traditional packing and pre-portioned capsules. In addition, 11 milk and coffee pre-portioned coffee capsules (composed by soluble coffee and milk powder) were monitored.

2.3. LC-MS/MS analysis

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK), equipped with an LC Alliance 2695 system (Waters, Milford, MA) consisting of an autosampler, a quaternary pump, a pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Version 4.1, was used for the MS/MS analyses. The separation was achieved by a Gemini-NX C_{18} (150 mm × 4.6 mm I.D., 5 μm particle size)

[6] ENA: enniatin A; ENA_1 enniatin A_1; ENB: enniatin B; ENB_1: enniatin B_1; BEA: beauvericin.
[7] EC: European Commission.
[8] ML: maximum limit.
[9] LC-MS/MS: Liquid Chromatography tandem Mass Spectrometry.
[10] QqQ: triple quadrupole.
[11] AcN: acetonitrile.
[12] MeOH: methanol.

analytical column supplied by Phenomenex (Barcelona, Spain), preceded by a C_{18} guard column (4 mm × 2 mm I.D.), using a gradient that started at 80% A (5 mM ammonium formate in H_2O) and 20% B (MeOH), increased linearly to 95% B in 10 min, followed by a linear decrease to 80% B in 5 min, then to 70% B in 10 min. Afterward, the initial conditions were kept constant for 5 min. The flow rate was 0.2 ml/min Fig 1 shows the chromatograms from a spiked sample during validation of the method as a proof of the method is suitable and the separation accurate enough. Chromatograms include the mass-to-charge ratio (m/z) of precursors and product ions and specify the retention time of each mycotoxin. The analysis was performed in positive and negative ion mode. The electrospray ionization source values were as follows: capillary voltage, 3.50 kV; extractor, 5 V; RF lens, 0.5 V; source temperature, 100 °C; desolvation temperature, 300 °C; desolvation gas (nitrogen 99.99%

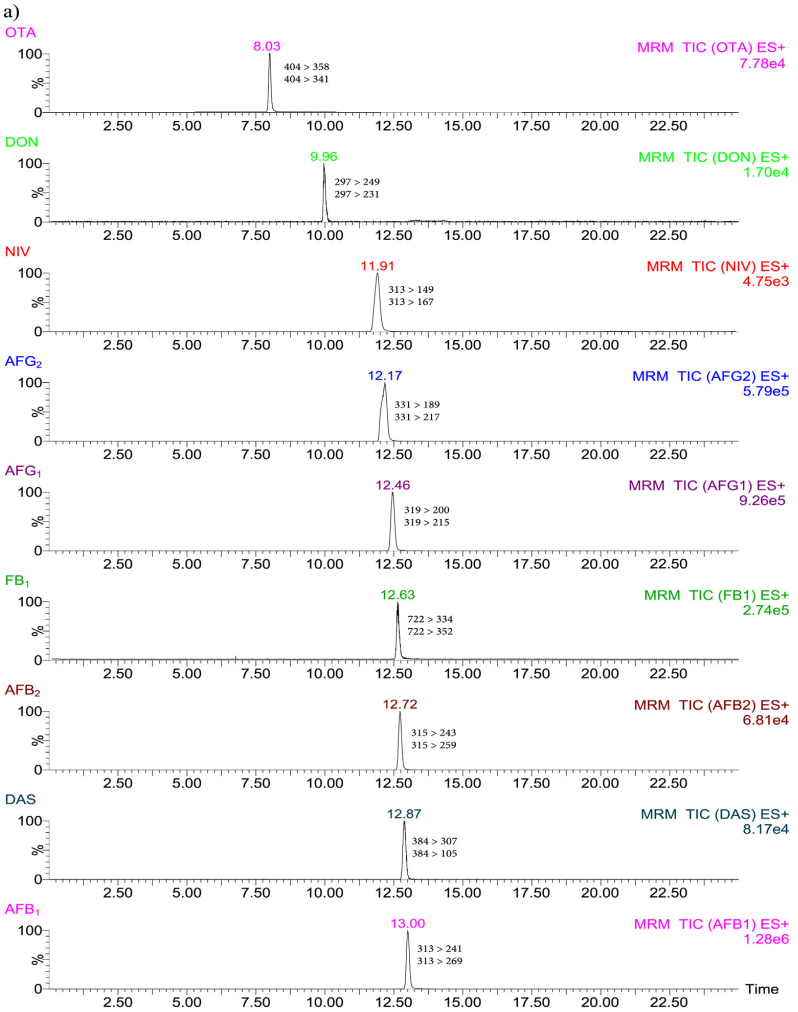

Fig. 1. a) Chromatogram of a 1 mg/kg spiked sample of each mycotoxin (OTA, DON, NIV, AFG2, AFG1, FB1, AFG2, DAS, AFB1). b) Chromatogram of a 1 mg/kg spiked sample of each mycotoxin (HT-2, FB2, T-2, ENB, ENB1, BEA, ENA1, ENA, ZEA). c) Mass spectrum with precursor ion (left) and products ions (right) of each mycotoxin (OTA, DON, NIV, AFG2, AFG1, FB1, AFG2, DAS, AFB1). d) Mass spectrum with precursor ion (left) and products ions (right) of each mycotoxin (HT-2, FB2, T-2, ENB, ENB1, BEA, ENA1, ENA, ZEA).

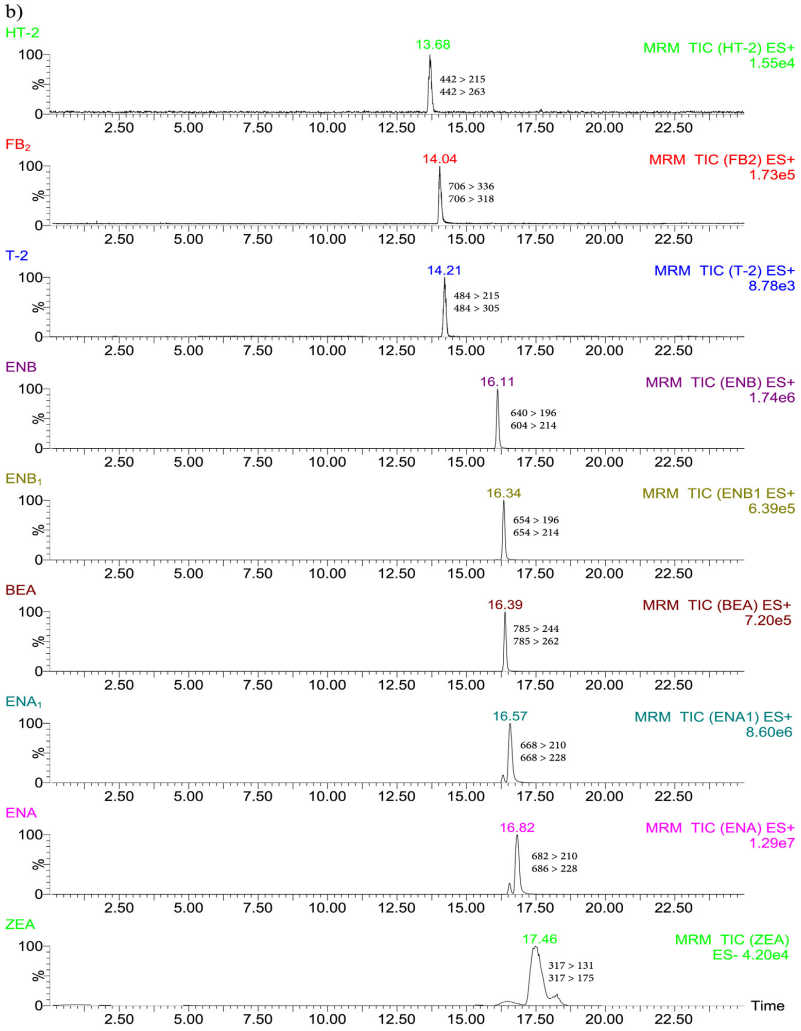

Fig. 1. (*continued*).

purity) flow, 800 L/h; cone gas 50 L/h (nitrogen 99.99% purity). For the instrument parameters, full scans and daughter scans under positive and negative modes were used. In addition, each compound was also characterized by the retention time. The criteria adopted for accepting the analysis was a retention time deviation lower than 2.5% compared to the standard. Ideal fragmentation conditions were determined by testing different cone voltages and collision energies for each compound during infusion of pure standard. The selected parameters that gave the most abundant fragment ion (collision energies (eV) and cone voltages (V)) are shown in Table 1. The analyzer settings were as follows: resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, −3 and 1; multiplier, 650; collision gas (Argon 99.995% purity) pressure, 3.83×10^{-3} mbar; interchanel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass spectrometer was operated in Multi Reaction Monitoring (MRM)[13] mode using two transitions

[13] MRM: Multi Reaction Monitoring.

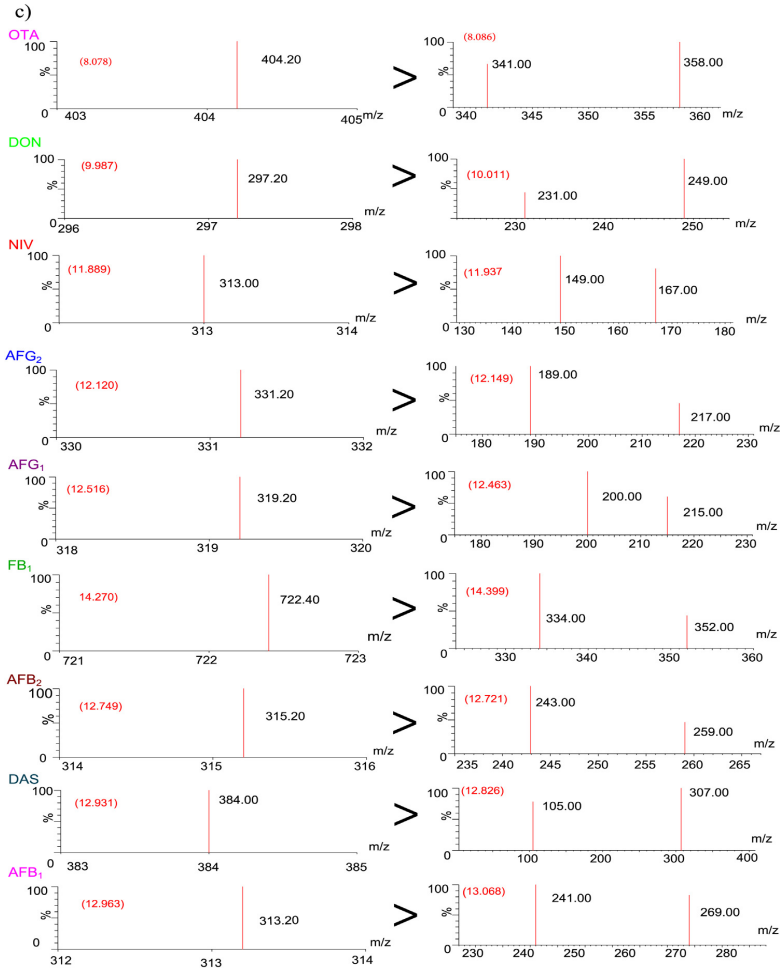

Fig. 1. (*continued*).

(Quantification and Confirmation) and the ion ratio between transitions, according with the European criteria (EC657/2002). The selected precursor and product ions are shown in Table 1. The precursor ion was the protonated form in most cases, excepting DAS, HT-2 and T-2, which were ammonium adducts.

2.4. Sample preparation and extraction procedure

Conventional coffee samples were ground with a blender to obtain ≤0.1 cm particles. For pre-portioned coffee, 15 capsules of each sample were opened and collected until the aliquots weighed 100 g. All aliquots were shaken vigorously.

For the extraction, 5 g of sample was extracted with 50 ml of AcN/H_2O [80:20] in Ultra Turrax (Ika T18 basic, Staufen, Germany) for 3 min. The extract was centrifuged for 15 min at 5 °C and 4500 rpm. The supernatant was evaporated to dryness with a Büchi Rotavapor (R-200 Flawil, Switzerland). The reconstituted extract with 10 ml of MeOH was filtered and purified using C_{18} columns (Waters, Milford, Massachusetts) and powdered activated carbon by applying a slight vacuum. Then, the solvent was evaporated again with a Turbovap LV Evaporator (Zymark, Hoptikinton, USA). The extract was reconstituted with 1 ml of AcN/MeOH [50:50] and filtered with a 0.22 μm nylon filter (Membrane Solutions, Texas, USA) before injection on the LC-MS/MS (QqQ). All samples were processed in triplicate.

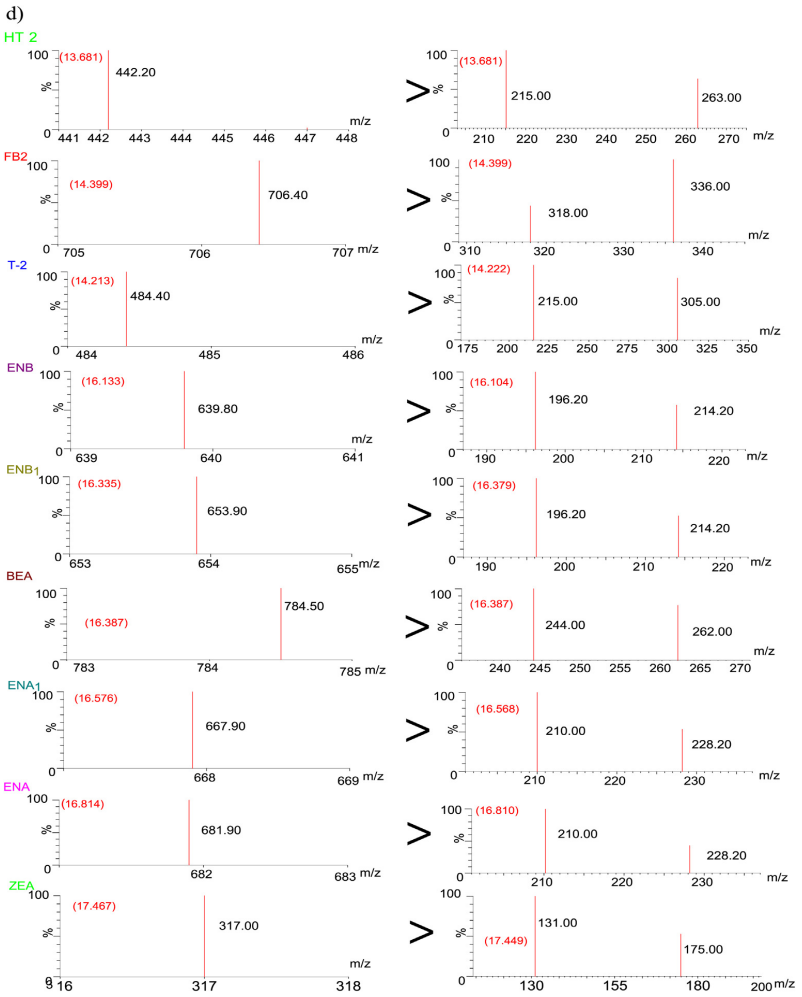

Fig. 1. (*continued*).

3. Results and discussion

3.1. Method validation

The analytical method was validated for coffee samples with a blank sample (mycotoxin-free coffee sample). The analytical pa-rameters are shown in Table 1. The evaluation of the matrix effects was performed using matrix-assisted calibration curves; the suppression of the signal (SS)[14] was obtained for all mycotoxins (between 21 and 47%). The detection limit (LOD)[15] for each myco-toxin was calculated using a signal-to-noise ratio of 3. The limit of quantification (LOQ)[16] was calculated using a signal-to-noise ratio of 10. The accuracy was evaluated through recovery studies (n = 6) at two concentration levels: 10 LOQ and 100 LOQ (Table 2). For the evaluation of the linearity, calibration curves were constructed for

[14] SS: suppression of the signal.

[15] LOD: limit of detection.

[16] LOQ: limit of quantification.

Table 1
LC-MS/MS (QqQ) optimized parameters and analytical parameters of the validated method.

	Cone (V)	Collision energy (eV)		Precursor ion (*m/z*)		Product ion		SS[c]	LOD (μg/kg)	LOQ (μg/kg)	Linearity (R^2)
		Q[a]	q[b]			Q[a]	q[b]				
ENA	25	25	25	682	$[M+H]^+$	210	228	40	0.15	0.50	0.999
ENA_1	35	35	35	668	$[M+H]^+$	210	228	43	0.08	0.25	0.997
ENB	35	35	35	640	$[M+H]^+$	196	214	33	0.15	0.50	0.999
ENB_1	13	13	12	654	$[M+H]^+$	196	214	40	0.02	0.50	0.998
BEA	30	30	20	785	$[M+H]^+$	244	262	46	0.03	0.10	0.998
NIV	22	35	35	313	$[M+H]^+$	149	167	45	75.00	85.24	0.997
DON	20	10	10	297	$[M+H]^+$	249	231	21	13.12	20.50	0.995
DAS	15	15	45	384	$[M+NH_4]^+$	307	105	22	3.00	5.00	0.999
HT-2	10	14	16	442	$[M+NH_4]^+$	215	263	34	10.00	35.50	0.995
T-2	10	15	45	484	$[M+NH_4]^+$	215	305	47	5.00	12.50	0.998
OTA	24	30	30	404	$[M+H]^+$	358	341	21	1.13	1.45	0.996
AFB_1	47	30	30	313	$[M+H]^+$	241	269	47	0.10	0.25	0.998
AFB_2	50	30	30	315	$[M+H]^+$	243	259	40	1.00	1.50	0.996
AFG_1	43	40	30	319	$[M+H]^+$	200	215	35	0.10	0.25	0.999
AFG_2	46	45	25	331	$[M+H]^+$	189	217	35	0.50	0.75	0.998
FB_1	30	30	30	722	$[M+H]^+$	334	352	31	16.00	53.33	0.993
FB_2	30	30	30	706	$[M+H]^+$	336	318	44	16.13	53.75	0.992
ZEA	25	25	25	317	$[M+H]^-$	131	175	22	6.00	12.50	0.993

[a] *Q*: Quantification ion.
[b] *q*: Confirmation ion.
[c] SS: Suppression of signal (Matrix effect) = (slope matrix-matched/slope standard in solvent) × 100.

all mycotoxins at six concentration levels from LOQ to 1 mg/kg for all mycotoxins. Special calibration curves for highly contaminated samples were made for ENB (from 1 to 10 mg/kg) and for NIV (from 5 to 50 mg/kg). The results showed good correlation coefficients (R^2 > 0.992). The intra-day precision was assessed by six determinations at each addition level on the same day, whereas inter-day precision was assessed by one determination at each addition level for three days. The relative standard deviations ranged between 4 and 12% for the intra-day precision and between 5 and 15% for the inter-day precision. The recovery values ranged from 72 to 112%. Therefore, the results were in accordance with the limits established by the EC (EC657/2002).

3.2. Monitoring study

3.2.1. Occurrence of mycotoxins

The samples analyzed in the present study were contaminated with at least six mycotoxins, excepting four samples (three caffeinated coffee samples and one decaffeinated coffee sample) that were mycotoxins-free. Most samples (61%) showed co-occurrence ranging from 10 to 12 mycotoxins.

The occurrence and levels of the concentrations of mycotoxins in the samples are reported in Fig. 2. The MRM chromatogram and the product ions spectrum corresponding to a sample of pre-portioned caffeinated coffee naturally contaminated with OTA (11.43 μg/kg) is shown in Fig. 3.

NIV was detected in most samples (96%). ZEA was not detected in any sample. ENB_1 and DAS were detected in a lower number of samples (12%). ENA_1, ENB, DON, HT-2, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, and FB_2 were detected in more than 50% of the analyzed samples.

3.2.2. Influence of coffee type

Incidence, mean, minimum and maximum concentration of each mycotoxin are shown according the different classification in Table 3a (caffeinated and decaffeinated), Table 3b (natural and torrefacto roasting), and Table 3c (milk added coffee).

Contamination levels of aflatoxins, OTA and trichothecenes are similar in caffeinated and decaffeinated coffee (Table 3a).

Table 2
Experimental values of recovery ± intra-day and inter-day relative standard deviation.

Mycotoxin	10 LOQ			100 LOQ		
	Concentration (μg/kg)	Intra-day (n = 6)	Inter-day (n = 6)	Concentration (μg/kg)	Intra-day (n = 6)	Inter-day (n = 6)
ENA	5.00	91 ± 4	90 ± 6	50.00	92 ± 5	91 ± 5
ENA_1	2.50	86 ± 9	88 ± 8	25.00	89 ± 4	89 ± 5
ENB	5.00	109 ± 9	112 ± 5	50.00	104 ± 7	106 ± 10
ENB_1	5.00	97 ± 11	95 ± 15	50.00	98 ± 5	98 ± 5
BEA	1.00	94 ± 4	94 ± 5	10.00	95 ± 3	95 ± 9
NIV	850.00	72 ± 4	76 ± 5	8500.00	73 ± 4	73 ± 9
DON	200.00	86 ± 8	85 ± 7	2000.00	89 ± 2	88 ± 4
DAS	50.00	82 ± 6	80 ± 9	500.00	84 ± 4	83 ± 6
HT-2	350.00	73 ± 4	73 ± 7	3500.00	71 ± 6	74 ± 6
T-2	125.00	82 ± 12	84 ± 11	1250.00	85 ± 10	84 ± 11
OTA	15.00	84 ± 8	78 ± 12	150.00	85 ± 4	83 ± 5
AFB_1	2.50	83 ± 7	81 ± 11	25.00	83 ± 5	84 ± 5
AFB_2	15.00	76 ± 9	75 ± 11	150.00	77 ± 3	76 ± 4
AFG_1	2.50	75 ± 5	74 ± 6	250.00	76 ± 4	76 ± 4
AFG_2	7.50	82 ± 5	78 ± 10	75.00	83 ± 3	79 ± 9
FB_1	500.00	84 ± 5	80 ± 12	5000.00	86 ± 4	85 ± 6
FB_2	500.00	88 ± 8	88 ± 7	5000.00	89 ± 4	88 ± 6
ZEA	125.00	77 ± 6	77 ± 7	1250.00	78 ± 4	76 ± 5

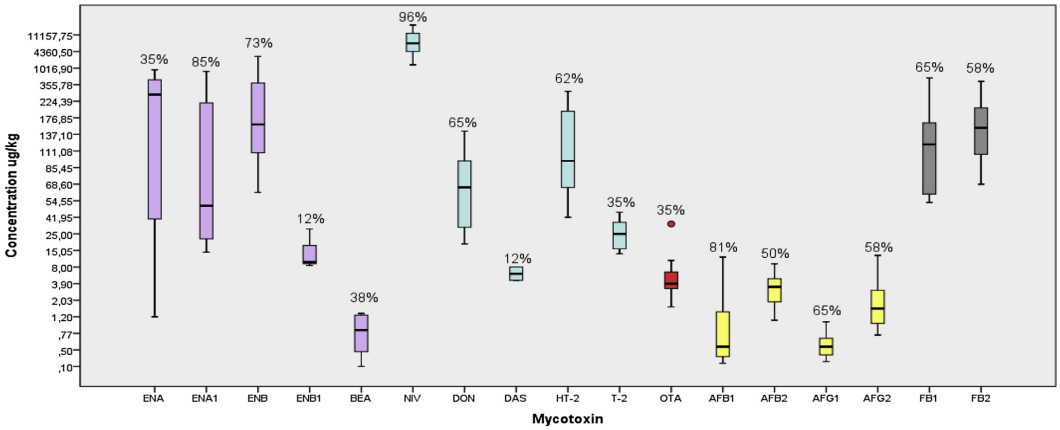

Fig. 2. Concentration (μg/kg) and occurrence (%) of mycotoxins in analyzed samples (n = 103).

Unexpectedly, emerging mycotoxins and fumonisins show lower concentrations. As it is reported by Paterson et al. (2014), some strains of *Aspergillus* spp. produced higher amount of mycotoxin in presence of caffeine. It is possible that fumonisins and emerging mycotoxins producing fungi present the same behavior. Soliman (2002) shows the highest aflatoxins production by *Aspergillus fla-vus* on decaffeinated green and decaffeinated roasted coffee. However, in the present study no differences were achieved on aflatoxins contamination levels between caffeinated and decaf-feinated coffee.

Differences between torrefacto and natural roasting were eval-uated (Table 3b). The contents of aflatoxins, OTA, and fumonisins were similar. Trichothecenes and Emerging mycotoxins present higher concentration in torrefacto roasted coffee. Torrefacto coffee is usually associated with low quality coffee beans. The presence of trichothecenes and emerging mycotoxins in higher concentrations

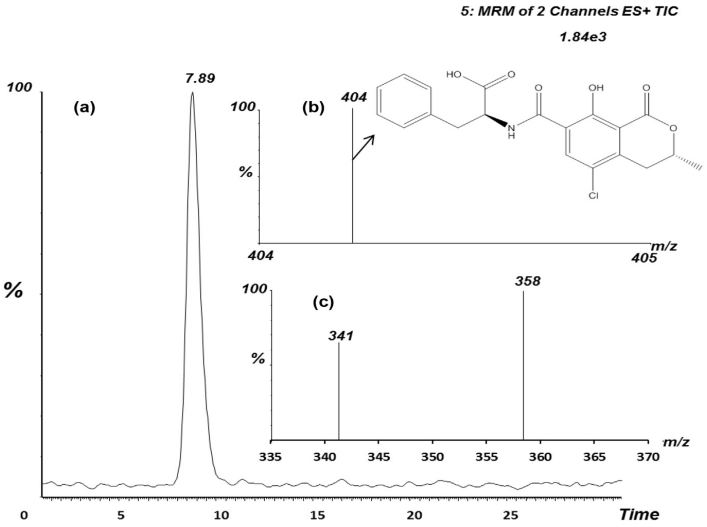

Fig. 3. Chromatogram and mass spectrum of a sample contaminated with OTA. a) HPLC-MS/MS QqQ chromatogram of an OTA contaminated sample. b) Mass spectrum of the precursor ion of OTA and the chemical structure. c) Mass spectrum of the product ion of OTA.

Table 3a
Occurrence (%), mean (μg/kg) and range of mycotoxins (μg/kg) in samples classified according the decaffeination process.

Mycotoxin	Caffeinated				Decaffeinated			
	Occurrence n = 52	Mean	Range		Occurrence n = 40	Mean	Range	
			Min	Max			Min	Max
AFB_1	69	0.55	0.25	12.48	100	0.71	0.25	2.33
AFB_2	60	3.60	1.65	7.27	40	3.41	1.50	6.00
AFG_1	69	0.54	0.28	1.41	70	0.57	0.25	1.00
AFG_2	56	1.05	0.75	3.15	60	1.55	0.84	13.12
OTA	13	4.12	1.91	11.43	73	4.35	1.56	32.40
NIV	94	6605.73	401.85	13071.24	98	8694.14	2641.95	25855.23
DON	71	67.40	20.65	148.52	75	73.56	20.60	129.86
DAS	23	6.50	5.70	8.56	0	–	–	
HT-2	52	91.10	49.05	176.85	73	99.41	41.95	276.93
T-2	35	33.00	26.25	45.12	45	45.69	13.45	105.04
FB_1	87	130.00	58.62	537.45	55	75.32	58.81	182.62
FB_2	81	151.17	85.45	369.66	45	168.17	68.62	362.22
ENA	37	374.30	1.20	935.53	43	111.58	7.32	597.74
ENA_1	87	179.40	13.75	749.33	83	50.93	18.83	199.50
ENB	54	577.00	83.11	3569.92	90	128.56	59.15	205.30
ENB_1	8	10.20	10.03	12.46	13	12.25	10.20	15.61
BEA	35	0.67	0.10	1.23	48	0.87	0.14	1.34

Table 3b
Occurrence (%), mean (μg/kg) and range of mycotoxins (μg/kg) in samples classified according the roasting process (natural or torrefacto).

Mycotoxin	Torrefacto				Natural			
	Occurrence n = 69	Mean	Range		Occurrence n = 23	Mean	Range	
			Min	Max			Min	Max
AFB_1	77	0.67	0.25	12.48	100	0.55	0.25	2.33
AFB_2	49	3.63	1.50	7.27	57	3.30	1.65	5.70
AFG_1	70	0.57	0.25	1.41	70	0.50	0.30	1.00
AFG_2	58	0.97	0.75	7.18	57	2.03	0.98	13.12
OTA	42	4.57	1.56	32.40	30	3.92	3.14	4.23
NIV	94	8370.97	401.85	25855.23	100	5215.50	1684.35	13071.24
DON	74	84.04	20.65	148.52	70	29.96	20.60	86.12
DAS	17	6.48	5.70	8.56	0	–	–	
HT-2	67	94.90	41.95	225.68	43	99.40	67.45	276.90
T-2	42	43.13	13.45	105.04	30	21.25	16.25	26.25
FB_1	74	103.97	58.62	174.00	70	142.80	58.76	537.45
FB_2	68	155.99	85.40	369.66	57	154.60	68.62	201.60
ENA	42	288.41	1.20	935.53	30	176.89	7.32	346.45
ENA_1	84	136.84	13.75	749.33	87	115.24	19.01	355.78
ENB	64	423.33	59.15	2220.80	87	199.35	136.4	3569.92
ENB_1	13	11.51	10.03	15.61	0	–	–	
BEA	35	0.82	0.47	1.34	57	0.68	0.10	1.23

Table 3c
Occurrence (%), mean (μg/kg) and range of mycotoxins (μg/kg) in pre-portioned milk added samples (n = 11).

	Occurrence	Range	
		Min	Max
AFB_1	64	0.97	1.4
AFB_2	45	3.21	10.01
AFG_1	27	0.39	1.54
AFG_2	64	2.81	6.88
OTA	0		
NIV	100	3483.47	8353.47
DON	0		
DAS	0		
HT-2	73	107.52	325.68
T-2	0		
FB_1	0		
FB_2	0		
ENA	0		
ENA_1	91	57.54	224.39
ENB	100	290.22	659.27
ENB_1	27	14.82	29.54
BEA	0		

is probably the result of the use of low quality coffee beans previously contaminated.

Regarding the milk added coffee, samples had the lowest variety of mycotoxins, but these mycotoxins appear in similar or higher concentrations than roasted coffee (Table 3c). OTA were not detected in milk added coffee (composed by instant coffee and milk powder), in disagree with Lee, Saad, Khayoon, and Salleh (2012) that reported higher occurrence of OTA in instant coffee than in roasted coffee. The concentrations of the most mycotoxins occurring in this coffee type were higher than in roasted coffee. Reverberi et al., (2010) reported the enhancer of OTA production with the addition of lactose in the growth medium. As this coffee type is preportioned in isolated capsules the contamination is less probable, but according Lee et al., (2012) and Reverberi et al. (2010) when the fungi infect the food the production of mycotoxins is more intense.

In green coffee a total of 11 mycotoxins were detected (ENA_1, ENB, NIV, DON, DAS, HT-2, T-2, AFG_1, AFG_2, FB_1, and FB_2); according Ventura et al., (2003) OTA has not been detected. The concentrations found for each mycotoxin were similar to the roasted samples, in disagreement with Soliman (2002) that report a reduction on

AFs during roasting. It is not possible to make conclusions because only one green coffee sample was analyzed.

3.2.3. Occurrence and concentration levels in the different groups of mycotoxins

ENA_1 and ENB were the most common mycotoxins (85% and 73%, respectively). In the literature, no data are available for coffee samples. However, these results were consistent with Serrano et al. (2013) who found ENA_1 and ENB with frequencies of 81% and 76%, respectively, in conventional pasta. However, ENB_1 was lower for Serrano et al., (2013) (65%), but not as low as in this study. The concentrations of ENA ranged from 1.20 to 935.53 μg/kg, ENA_1 ranged from 13.75 to 749.33 μg/kg, ENB ranged from 0.06 to 3.57 mg/kg, ENB_1 ranged from 10.03 to 29.54 μg/kg, and BEA ranged from 0.10 to 1.34 μg/kg.

The concentrations of NIV ranged from 0.40 mg/kg in a sample of conventional caffeinated coffee to 25.86 mg/kg in a sample of decaffeinated coffee in pre-portioned capsules. DON and HT-2 were the two most common trichothecenes in occurrence (65% and 62%, respectively). The other trichothecenes were detected at low concentrations and low occurrences. The lowest was DAS with 12% of samples contaminated. Comparison with other studies is very difficult, even impossible, because a very limited number of publications are available.

OTA was not detected in the green coffee sample, although other studies (Batista et al., 2003; Bokhari, 2007; Imperato et al., 2011; Napolitano, Fogliano, Tafuri, & Ritieni, 2007; Pittet & Royer, 2002; Vanesa & Ana, 2013; Ventura et al., 2003; Visconti & De Girolamo, 2005) reported a natural occurrence in a range of concentrations (from 0.1 to 360 μg/kg).

OTA was detected in the 35% of the roasted coffee samples. Fig. 3 shows a chromatogram and mass spectrum of the product ions of a sample contaminated with OTA. The concentration in positive samples ranged from 1.50 to 32.40 μg/kg. According to other studies (Bandeira, 2012; Batista et al., 2003; Casal, Vieira, Cruz, & Cunha, 2014; Coronel, Marin, Cano, Ramos, & Sanchis, 2011; Lee et al., 2012; Lobeau et al., 2005; Tozlovanu & Pfohl-Leszkowicz, 2010), a similar range of concentrations was obtained (from 0.15 to 23.70 μg/kg). Sibanda et al. (2002) did not detect OTA in coffee samples.

In the present study, some commercial coffee samples (5 of 103) exceeded the ML. Two samples were decaffeinated conventional coffee with concentrations of 9.30 and 6.20 μg/kg, two samples were pre-portioned caffeinated coffee with concentrations of 6.91 and 11.43 μg/kg, and one sample was pre-portioned decaffeinated coffee. In addition, the sample of decaffeinated pre-portioned coffee exceeded six times (32.40 μg/kg) the ML proposed by EC legislation. Our results are similar to other studies. Some authors also detected OTA levels higher than the ML: 5.24 μg/kg by Coronel et al. (2011), 5.4 μg/kg by Lobeau et al. (2005), 9 μg/kg by Bandeira (2012), 15.08 μg/kg by Tozlovanu and Pfohl-Leszkowicz (2010), and 5.7 μg/kg in roasted coffee and 13.66 μg/kg in instant coffee by Vanesa and Ana (2013).

Aflatoxins had high occurrence but low concentrations (mean concentration: 1.95 μg/kg). No significant differences were found among the different coffee types. The concentrations ranged from 0.15 to 12.48 μg/kg Soliman (2002) obtained, for green coffee and roasted coffee, concentrations from 0.76 to 8.92 μg/kg, and Bokhari and Aly (2009) obtained concentrations from 5 to 23 μg/kg Khayoon et al. (2014) detected aflatoxins in canned coffee. Imperato et al. (2011) did not detect aflatoxins in green coffee.

The occurrence of fumonisins in the contaminated samples was 65 and 58% with concentrations from 0.06 to 0.54 mg/kg and from 0.07 mg/kg to 0.37 mg/kg for FB_1 and FB_2, respectively. The levels obtained for FB_2 were higher than those obtained by Noonim et al. (2009) in ground coffee (10 μg/kg).

4. Conclusion

A new method for the simultaneous analysis of NIV, DON, DAS, HT-2, T-2, OTA, AFB_1, AFB_2, AFG_1, AFG_2, FB1, FB_2, ZEA, and the emerging mycotoxins ENA, ENA_1, ENB, ENB_1, and BEA in different coffee samples was validated. The analysis of the different commercial types of coffee showed the presence and coexistence of mycotoxins in every coffee type. The only legislated mycotoxin in coffee (OTA) showed a low incidence, but in some samples (5 of 26 samples), it exceeded the established ML. OTA was also detected over the ML, so it must be given special consideration. The absence of international legislation limits for the other mycotoxins in coffee, the toxicological effects, and the levels obtained in this study indicate that it is necessary to pay special attention to the evaluation of the presence of mycotoxins in coffee. It is important to evaluate the influence of technological and culinary practices to determine the population exposure.

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Development of a new method for the simultaneous determination of 21 mycotoxins in coffee beverages by liquid chromatography tandem mass spectrometry

Ana García-Moraleja, Guillermina Font, Jordi Mañes, Emilia Ferrer *

Laboratory of Toxicology, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

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T-2 toxin (PubChem CID: 40024)
Deoxynivalenol (PubChem CID: 5284461)
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ABSTRACT

A new method for the simultaneous detection of 21 mycotoxins (ochratoxin A, aflatoxin B_1, aflatoxin B_2, aflatoxin G_1, aflatoxin G_2, sterigmatocystin, nivalenol, deoxynivalenol, 3-acetyl deoxynivalenol, 15-acetyl deoxynivalenol, diacetoxyscirpenol, neosolaniol, HT-2 toxin, T-2 toxin, fumonisin B_1, fumonisin B_2, enniatin A, enniatin A_1, enniatin B, enniatin B_1, and beauvericin) in coffee beverages was internally validated. The method is based on liquid/liquid extraction with a mixture of ethyl acetate/formic acid (95:5 v/v) and detection using triple quadrupole (QqQ) and ion trap (IT) liquid chromatography tandem mass spectrometry. The limits of detection and quantification were 0.02 to 39.64 μg/kg, respectively, and the correlation coefficients were optimal for all mycotoxins ($R^2 \geq 0.992$). The recovery values ranged from 72% to 97%. The developed method was demonstrated in six real samples of roasted and instant coffee, caffeinated and decaffeinated coffee, and coffee with sugar added. The analyses indicate the presence of the studied mycotoxins in coffee beverages at μg/kg concentrations. Ochratoxin A, a mycotoxin that is regulated in coffee, was detected in two samples under the maximum limit established by a European legislation (CE1881/2006).

1. Introduction

Coffee is a natural product that is rich in phenolic compounds, which are responsible for the astringency, flavor, antioxidant activity, and caffeine content of coffee; in moderate doses, coffee has a stimulating effect and reduces fatigue (Andrade et al., 2012; Cheong et al., 2013). These properties lead to the wide consumption of coffee beverages, with important economic and cultural roles (International Coffee Organization ICO, 2013).

Mycotoxins are secondary metabolites of filamentous fungi that are present in agricultural commodities (Schatzmayr & Streit, 2013). There are approximately 400 recognized mycotoxins, but only a few of them are present in food commodities (FAO, 2004; Sulyok, Krska, & Schuhmacher, 2010). Some mycotoxins are regulated in foods (e.g., aflatoxins (AFs),[1] fumonisins B (FBs),[2] and trichothecenes (TRs)[3]) in most countries (Commission Regulation, EC1881/2006; U.S.F.D.A., 2005). In the case of coffee products, ochratoxin A (OTA)[4] is the only regulated mycotoxin (Commission Regulation, EC1881/2006) and has maximum limits (MLs)[5] of 5 μg/kg in roasted coffee beans and ground roasted coffee and 10 μg/kg in soluble coffee (IC). No specific MLs are established for mycotoxins in coffee in countries out of the European Union.

* Corresponding author. Tel.: + 34 963544950; fax: + 34 963544954.
E-mail address: emilia.ferrer@uv.es (E. Ferrer).

[1] AFs: aflatoxins.
[2] FBs: fumonisins B.
[3] TRs: trichothecenes.
[4] OTA: ochratoxin A.
[5] MLs: maximum limits.

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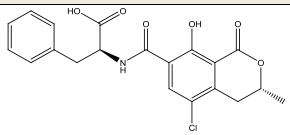
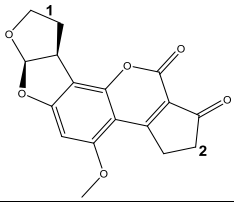
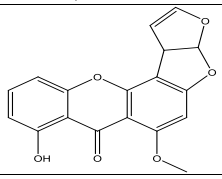
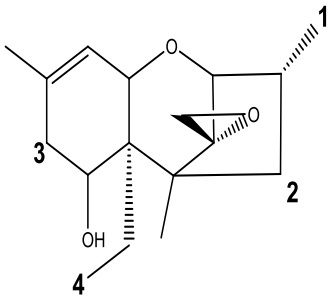
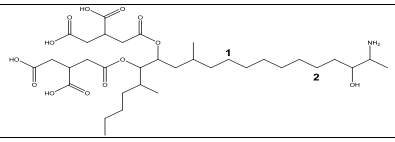
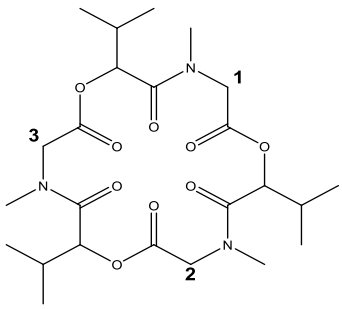
Table 1
(A) Classification of mycotoxins in groups, their chemical structure, chemical formula, radicals and classification by IARC (international agency for research on cancer).

GROUP	STRUCTURE	MYCOTOXIN	FORMULA	RADICALS	IARC[a] Clasiffication	CAS-RN[c]
Ochratoxin A		OTA	$C_{20}H_{18}ClNO_6$		2B	303-47-9
AFs		AFB_1	$C_{17}H_{12}O_6$	1_ Double bond 2_ C Alkane	1	1162-65-8
		AFB_2	$C_{17}H_{14}O_6$	1_ Single bond 2_ C Alkane	1	7220-81-7
		AFG_1	$C_{16}H_{10}O_7$	1_ Double bond 2_ O Carboxil	1	1165-39-5
		AFG_2	$C_{16}H_{12}O_7$	1_ Single bond 2_ O Carboxil	1	7241-98-7
Sterigmatocystin		STG	$C_{18}H_{12}O_6$	-	2B	10048-13-2
TRs		NIV	$C_{15}H_{20}O_7$	1_OH; 2_OH; 3_O Carbonyl; 4_OH	3	23282-20-4
		DON	$C_{15}H_{20}O_6$	1_OH; 2_C Alkane; 3_O Carbonyl; 4_OH	3	51481-10-8
		3aDON	$C_{17}H_{22}O_7$	1_OAc; 2_C Alkane; 3_O Carbonyl; 4_OH	3	50722-38-8
		15aDON	$C_{17}H_{22}O_7$	1_OH; 2_OH; 3_O Carbonyl; 4_OAc	3	88337-96-6
		DAS	$C_{19}H_{26}O_7$	1_OH; 2_OAc; 3_ C Alkane; 4_OAc	3	2270-40-8
		NEO	$C_{19}H_{26}O_8$	1_OH; 2_OAc; 3_OH; 4_OAc	3	36519-25-2
		HT-2	$C_{22}H_{32}O_8$	1_OH; 2_OH; 3_ 3-methylbutanoathyl; 4_OH	3	26934-87-2
		T-2	$C_{24}H_{34}O_9$	1_OH; 2_OAc; 3_3-methylbutanoathyl; 4_OAc	3	21259-20-1
FBs		FB_1	$C_{34}H_{59}NO_{15}$	1_OH 2_OH	2B	116355-83-0
		FB_2	$C_{34}H_{59}NO_{14}$	1_ C Alkane 2_OH	2B	116355-84-1
EMs		ENA	$C_{36}H_{63}N_3O_9$	1_*Sec*-butyl 2_*Sec*-butyl 3_*Sec*-butyl	NC[b]	2503-13-1
		ENA_1	$C_{35}H_{61}N_3O_9$	1_*Sec*-butyl 2_*Sec*-butyl 3_*Iso*-propyl	NC	4530-21-6
		ENB	$C_{33}H_{57}N_3O_9$	1_ *Iso*-propyl 2_ *Iso*-propyl 3_ *Iso*-propyl	NC	917-13-5
		ENB_1	$C_{34}H_{59}N_3O_9$	1_ *Iso*-propyl 2_ *Iso*-propyl 3_ *Sec*-butyl	NC	19914-20-6
		BEA	$C_{45}H_{57}N_3O_9$	1_phenyl 2_phenyl 3_phenyl	NC	26048-05-5

[a]IARC classification: group 1: carcinogenic to humans; group 2A: probably carcinogenic to humans; group 2B: possibly carcinogenic to humans; group 3: not classifiable as to its carcinogenicity to humans; group 4: probably not carcinogenic to humans (International Agency for Research in Cancer).
[b]NC: not classified by IARC.
[c]CAS-RN: Chemical Abstracts Service – Registry Number (American Chemical Society).

Mycotoxins are classified into different groups depending on their chemical structures (Table 1); these groups include OTA, AFs [aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2)],[6] sterigmatocystin (STG),[7] TRs [nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-aDON), 15-acetyldeoxynivalenol (15-aDON), diacetoxyscirpenol (DAS), neosolaniol (NEO), T-2 toxin (T-2) and HT-2 toxin (HT-2)],[8] FBs [fumonisin B_1 (FB_1), fumonisin B_2 (FB_2)][9] and EMs [enniatin A (ENA), enniatin A_1 (ENA_1), enniatin B (ENB), enniatin B_1 (ENB_1), beauvericin (BEA)].[10] Different mycotoxins have different toxicological effects such as the production of nephropathies (OTA), carcinogenicity (AFs), hepatotoxicity (FBs), immunosuppressive properties (AFs), and estrogenic effects (ZEA) (Edite Bezerra da Rocha, Freire, Erlan Feitosa Maia, Izabel Florindo Guedes, & Rondina, 2014; I.A.R.C., 2014; Köppen et al., 2010).

The presence of toxigenic fungi in coffee plants, coffee beans, and roasted coffee have already been described. The fungi of *Fusarium* spp., *Aspergillus* spp., and Ochratoxigenic species have been found in coffee plants and coffee beans (Gamboa-gaitán, 2012; Posada & Vega, 2006; Rezende et al., 2013; Serani, Taligoola, & Hakiza, 2007; Silva, Batista, & Schwan, 2008). Regarding the mycotoxin contents in coffee, OTA, AFs, STG, FB_2, and patulin have been studied in green and roasted coffees (Bandeira, 2012; Batista, Chalfoun, Prado, Schwand, & Wheals, 2003; Bokhari & Aly, 2009; Desmarchelier et al., 2014; Imperato, Campone, Piccinelli, Veneziano, & Rastrelli, 2011; Lobeau, De Saeger, Sibanda, Barna-Vetró, & Van Peteghem, 2005; Noonim, Mahakarnchanakul, Nielsen, Frisvad, & Samson, 2009; Rahmani, Jinap, & Soleimany, 2009; Sibanda, Saeger, & Van Peteghem, 2002 and Ventura et al., 2003). Moreover, some authors have studied how the different steps of the coffee production process (drying, fermentation, roasting, packing, and brewing) affect mycotoxin contents (La Pera et al., 2008; Romani, Pinnavaia, & Dalla Rosa, 2003; Santini et al., 2011; Soliman, 2002; Tozlovanu & Pfohl-Leszkowicz, 2010). Only OTA and AFs have been studied in coffee beverages (Casal, Vieira, Cruz, & Cunha, 2014; Khayoon, Saad, Salleh, Manaf, & Latiff, 2014; Tozlovanu & Pfohl-Leszkowicz, 2010; Noba, Uyama, & Mochizuki, 2009).

Different authors have developed analytical strategies for the determination of multi-mycotoxins in liquid foods. The presence of TRs, FBs, AFs, and EMs has been studied in malt, beer, milk, and water (Zöllner & Mayer-Helm, 2006). Multi-mycotoxin methods for the simultaneous analysis of different mycotoxins (TRs, FBs, AFs, STG, OTA and EMs) in cheese, food supplements, and wine have been developed using extraction with methanol (MeOH),[11] ethyl acetate, formic acid, and acetonitrile and detection with liquid chromatography tandem mass spectrometry (LC-MS/MS)[12] (Kokkonen & Jestoi, 2009; Mavungu et al., 2009; Pizzutti et al., 2014). However, to our knowledge, there are no data regarding the occurrence of multi-mycotoxins in coffee beverages.

In this context, the aim of this study was to develop a new, rapid, sensitive, and reproducible analytical strategy to identify and quantify 21 mycotoxins (OTA, AFB_1, AFB_2, AFG_1, AFG_2, STG, NIV, DON, 3-aDON, 15-aDON, DAS, NEO, HT-2, T-2, FB_1, FB_2, ENA, ENA_1, ENB, ENB_1, and BEA) in coffee beverages. For this purpose, the extraction process and multi-mycotoxin determination by triple quadrupole (QqQ)[13] and ion trap (IT)[14] LC-MS/MS were optimized. In addition, the developed and optimized analytical method was applied to determine the mycotoxin contents in different coffee beverages.

2. Experimental

2.1. Chemicals

Acetonitrile, ethyl acetate, and MeOH were provided by Merck (Darmstadt, Germany). Formic acid and the mycotoxin and casein standards were provided by Sigma-Aldrich (St. Louis, MO, USA). Ammonium formate (99%) and $K_2Fe(CN)_6$ were supplied by Panreac Química (Barcelona, Spain). $ZnSO_4 \cdot 7H_2O$ and graphitized carbon black were provided by Alfa AESAR (Karlsruhe, Germany). The micro-solid phase dispersion sorbent was octadecyl (C_{18})-bonded silica from Analisis Vinicos (Ciudad Real, Spain). Deionized water (H_2O; $<18\ M\Omega\ cm^{-1}$ resistivity) was obtained in the laboratory using a Milli-Q SP® Reagent Water System from Millipore (Bedford, USA). All solvents were passed through a 0.45-μm cellulose filter from Scharlau (Barcelona, Spain) and degassed for 10 min using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corporation, Danbury, CT, USA) before use. Carrez solutions were prepared in the laboratory with $ZnSO_4 \cdot 7H_2O$ (1.0 M) for solution (I) and $K_2Fe(CN)_6$ (0.3 M) for solution (II). A mixture of ethyl acetate:formic acid (95:5 v/v) was prepared daily with 25 ml of formic acid and 500 ml of ethyl acetate. Individual stock solutions of OTA, AFs (AFB_1, AFB_2, AFG_1, and AFG_2), STG, TRs (NIV, DON, 3-ADON, 15-ADON, DAS, T-2, HT-2, and NEO), FBs (FB_1 and FB_2), and EMs (ENA, ENA_1, ENB, ENB_1, and BEA) with concentrations of 500 mg/l were prepared in MeOH, and two 50-ml multi-mycotoxin standard solutions were prepared: one in methanol and one in methanol with the matrix from a mycotoxin-free sample (mycotoxin-free sample was selected from a previous study in non-processed roasted and instant coffee). The concentrations of the multi-mycotoxin standard solutions were 1 mg/l of AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, NIV, DAS, T-2, HT-2, ENA, ENA_1, BEA, and ENB, 5 mg/l of ENB_1, and 10 mg/l of DON. The solutions were stored in safe conditions at −20 °C. These stock solutions were diluted with MeOH to obtain the appropriate working concentrations immediately before use.

2.2. Samples

To optimize the analytic method for the determination of multi-mycotoxins in coffee beverages, different commercial coffee samples were selected based on their compositions. All samples were purchased at a local supermarket (Valencia, Spain). A total of six coffee samples were used: three samples of ground roasted coffee and three samples of instant coffee. The ground coffee samples were classified as follows: one sample of natural roasted coffee (NRC),[15] one sample of torrefacto roasted coffee (TRC[16]; special industrially roasted coffee with added sugar to enhance the flavor), and one sample of decaffeinated natural roasted coffee (DC).[17] The instant coffee samples were classified as follows: one sample of instant coffee (IC),[18] one sample of instant coffee with sugar (ICS),[19] and one sample of instant coffee with sugar and milk (ICSM).[20]

All coffee samples were stored at room temperature. After their packages had been opened, they were put into specific glass food containers at 4 °C and analyzed within three days.

[6] AFB_1: aflatoxin B_1; AFB_2: aflatoxin B_2; AFG_1: aflatoxin G_1; AFG_2: aflatoxin G_2.
[7] STG: sterigmatocystin.
[8] NIV: nivalenol; DON: deoxynivalenol; 3-aDON: 3-acetyldeoxynivalenol; 15-aDON: 15-acetyldeoxynivalenol; DAS: diacetoxyscirpenol; NEO: neosolaniol; T-2: T-2 toxin; HT-2: HT-2 toxin.
[9] FB_1: fumonisin B_1; FB_2: fumonisin B_2.
[10] ENA: enniatin A; ENA_1 enniatin A_1; ENB: enniatin B; ENB_1: enniatin B_1; BEA: beauvericin.
[11] MeOH: methanol.
[12] LC-MS/MS: liquid chromatography tandem mass spectrometry.
[13] QqQ: triple quadrupole.
[14] IT: ion trap.
[15] NRC: natural roasted coffee.
[16] TRC: torrefacto roasted coffee.
[17] DC: decaffeinated coffee.
[18] IC: instant coffee.
[19] ICS: instant coffee with sugar.
[20] ICSM: instant coffee with sugar and milk.

2.3. Coffee preparation

Ground coffee samples (NRC, TRC, and DC) were processed to reproduce home brewing using an Italian coffeepot (Italian moka). This pot consists of three compartments: the first contains water (50 ml); the second is funnel shaped, separated from the first compartment by a metallic filter, and contains ground coffee (5 g); and the third is initially empty on the top of the coffeepot. When the water in the first compartment is heated, the temperature and pressure in this compartment increase; when the internal pressure overcomes the external pressure, the water flows through the coffee in the second compartment at approximately 98.6 °C, and the final coffee beverage then rises into the third compartment. The process starting with the heating of the water until the final coffee beverage is produced is 5 to 7 min long (Gianino, 2007).

Instant coffee beverages (IC, ICS, and ICSM) were prepared according to the brand recommendations. Water was heated to 85 °C on a hotplate and was added to a glass beaker with the measured instant coffee powder.

The beverages were kept in specific glass containers and analyzed immediately.

2.4. Extraction procedures

2.4.1. Clarification

The pigments of coffee samples increase the matrix effects during mass spectrometry detection. To improve the sensibility and accuracy, different cleanup procedures were studied. Cleanup methods that have been previously used in mycotoxin analysis, such as reverse phase silica C_{18} cartridges and graphitized carbon black (Rahmani et al., 2009), along with methods used previously in the extraction of different compounds from coffee samples, such as precipitation with Carrez solutions (Perrone, Farah, & Donangelo, 2012) and casein (Niseteo, Komes, Belščak-Cvitanović, Horžič, & Budeč, 2012) were studied.

The coffee beverages (roasted or instant) were each initially clarified with 1 ml of Carrez solution in 100 ml of distilled water. The solution was shaken vigorously and filtered after 30 min. A 20-ml aliquot of this filtered solution was referenced as a purified solution for the subsequent extraction.

2.4.2. Ultra-turrax extraction (UTE)[21]

For the optimization, different analytical parameters were tested (solvent, volume, time, and cycles) varying one parameter at time. The optimum extraction solvent was chosen first because it is the most important extraction parameter (Ferrer et al., 2011). The aqueous coffee samples were preliminarily conditioned for extraction with polar solvents (MeOH and acetonitrile). The coffee beverage water was previously dried with a freeze drier. Different solvents or mixtures of them were tested: MeOH, acetonitrile, ethyl acetate, and a mixture of ethyl acetate/formic acid (95:5 v/v). In addition, the volume of solvent (10–30 ml), extraction time (1–6 min) and numbers of extractions (number of UTA times of the same aliquot with new solvent; 1–4 times) were studied to obtain the highest recoveries of the most mycotoxins. All experiments were carried out in triplicate using a standard sample spiked at concentrations of 100 LOQ for each mycotoxin. The purified solution (20 ml) was extracted with a mixture of ethyl acetate/formic acid (95:5 v/v; 20 ml × 3 cycles × 5 min) with an Ika T18 basic Ultra-Turrax (Staufen, Germany). The supernatant was evaporated to dryness with a Büchi Rotavapor R-200 (Flawil, Switzerland). The extract was dissolved with 5 ml of ethyl acetate/formic acid mixture and dried by nitrogen gas at 35 °C using a multi-sample Turbovap LV Evaporator from Zymark (Hoptikinton, USA). The extract was reconstituted with 1 ml of H_2O/MeOH (50:50 v/v) and filtered through a 0.22-μm nylon filter from Membrane Solutions (Dallas, TX, USA) prior to injection in the LC-MS/MS system.

2.5. Instrumentation and chromatographic conditions

2.5.1. LC-MS/MS-IT

An HPLC Agilent 1200 Chromatograph from Agilent Technologies (Palo Alto, CA, USA) in tandem with a 3200QTRAP mass spectrometer (Applied Bio-systems, AB Sciex, Foster City, CA, USA) equipped with a turbo electrospray ionization interface was employed. Chromatography was performed using a Gemini-NX C_{18} column (3 μm, 110 A 150 mm × 2 mm). The run time was 30 min, and the injection volume was 20 μl including phase A (H_2O in 5 mM ammonium formate and 0.1% formic acid) and phase B (MeOH in 5 mM ammonium formate and 0.1% formic acid). A pre-run time of 3 min was used for equilibration with a flow rate of 0.25 ml/min (10% eluent B). The elution gradient (with a flow rate of 0.25 ml/min) began with 70% of eluent B, which remained constant for 3 min, and then the amount of eluent B increased to 80% and 90% at minute 6 and minute 14, respectively. At minute 18, the flow increased to 0.350 ml/min and 100% B, at minute 20, the flow increased further to 0.400 ml/min with 50% B, and from minute 21 to minute 30, the flow returned to 0.250 ml/min with 10% eluent B and 90% eluent A.

The mass spectrometer parameters were as follows: ion source turbo spray; multiple reaction monitoring (MRM)[22]; positive ionization polarity (ESI+); resolution = 12.0 (unit resolution) for the first and third quadrupoles; ion energy = 0.5 V; entrance and exit energies = −3 and 1 V, respectively; multiplier = 650; collision gas = argon, 99.995% purity; pressure = 3.83×10^{-3} mbar; inter-channel delay = 0.02 s; total scan time = 1.0 s; dwell time = 0.1 ms. Analyst version 1.5.1 was used to control each component of the system and for data acquisition.

2.5.2. LC-MS/MS-QqQ

A Quattro LC triple quadrupole mass spectrometer from Micromass (Manchester, UK) equipped with an LC Alliance 2690 system (Waters, Milford, MA, USA) consisting of an autosampler, a quaternary pump, and a pneumatically assisted electrospray probe, a Z-spray interface, and Mass Lynx NT software version 4.1 was used for the MS/MS analyses. Separation was achieved using a Gemini-NX C_{18} (3 μm, 110A size: 150 mm × 2 mm) analytical column supplied by Phenomenex (Madrid, Spain). The mobile phase consisted of a gradient between phase A (H_2O in 5 mM ammonium formate) and phase B (MeOH). The optimized gradient parameters were as follows: a constant flow rate of 0.20 ml/min was used; the gradient began with 0% eluent B and increased to 100% over 10 min, decreased to 80% in 5 min and to 70% in 6 min more, decreased to 0% in 1 min, and finally remained constant at 0% eluent B and 100% eluent A for 4 min at a constant flow rate of 0.2 ml/min.

The analysis was performed in positive ion mode. The electrospray ionization source values were as follows: capillary voltage, 3.50 kV; extractor, 1 V; RF lens, 0.5 V; source temperature, 120 °C; desolvation temperature, 400 °C; and desolvation gas (nitrogen, 99.99% purity) flow, 200 l/h. The analyzer settings were as follows: resolution, 12.0 (unit resolution) for the first and third quadrupoles; ion energy, 0.5; entrance and exit energies, 5 and 3, respectively; multiplier, 650; collision gas: argon (99.995% purity) at a pressure of 0.279 Pa; inter-channel delay, 0.02 s; and total scan time, 1.0 s. The dwell time was 0.2 s for all compounds. The mass spectrometer was operated in scan, product ion scan, and MRM modes. The MRM transitions (precursor–product ion transitions) and ratios between quantification and confirmation ion, cone voltages, and collision energies were optimized for each mycotoxin during the

[21] UTE: ultra-turrax extraction.

[22] MRM: multiple reaction monitoring.

Table 2
Optimized parameters: LC-MS/MS (IT) and LC-MS/MS (QqQ) for each mycotoxin.

	LC-MS/MS (IT)								LC-MS/MS (QqQ)							
	DP[a]	Precursor ion	Quantification [Q]			Confirmation [q]			CV[d]	Precursor ion	Quantification [Q]			Confirmation [q]		
			CE[b]	Product ion	CXP[c]	CE[b]	Product ion	CXP[c]			CE[b]	Product ion		CE[b]	Product ion	
OTA	55	404.3	97	102.1	6	27	239.0	6	24	404.3	30	358		30	341	
AFB_1	46	313.1	39	284.9	4	41	241.0	4	47	313.2	30	241		30	269	
AFB_2	81	315.1	39	259.0	6	33	286.9	6	50	315.2	30	243		30	259	
AFG_1	76	329.0	29	311.1	6	39	243.1	6	43	329.2	40	200		30	215	
AFG_2	61	331.1	27	313.1	6	39	245.1	4	46	331.2	45	189		25	217	
STG	106	325.0	51	281.0	18	50	310.0	3	50	325.3	30	281		30	297	
NIV	50	313.4	80	115.1	3	27	175.1	3	22	391.4	35	149		35	167	
DON	36	297.1	17	249.2	4	29	161.0	4	20	297.2	10	249		10	231	
3aDON	44	339.2	20	203.1	3	20	231.1	3	30	339.2	20	185		16	203	
15aDON	50	339.2	20	137.0	3	20	261.1	3	30	339.1	26	165		26	148	
DAS	66	384.0	15	307.2	16	63	105.0	12	15	384.0	15	307		45	105	
NEO	46	400.2	25	215.0	12	29	185.0	14	20	400.2	14	258		14	285	
T-2	21	484.3	22	185.1	4	29	215.1	4	10	484.5	15	215		45	305	
HT-2	21	442.2	19	215.4	8	19	267.8	4	10	442.2	14	215		16	263	
FB_1	101	722.2	51	334.2	20	45	352.2	26	50	722.2	30	334		30	352	
FB_2	131	706.2	50	336.3	16	50	318.3	18	50	706.4	30	336		30	318	
ENA	76	699.4	59	228.2	16	35	210.1	14	40	681.9	25	210		25	228	
ENA_1	66	685.4	59	214.2	10	37	210.2	8	40	667.9	35	210		35	228	
ENB	51	657.3	39	196.1	8	59	214.0	10	40	639.8	35	196		35	214	
ENB_1	66	671.2	61	214.1	10	57	228.1	12	40	654.9	13	196		12	214	
BEA	116	801.2	27	784.1	10	39	244.1	6	35	784.4	30	244		20	262	

[a] DP: decluster potential (volts).
[b] CE: collision energy (volts).
[c] CXP: cell exit potential (volts).
[d] CV: cone voltage (volts).
[Q] Quantification ion.
[q] Confirmation ion.

infusion of pure standard. Masslynx V4.1 software was used to control each component of the system and for data acquisition.

2.6. Method internal validation

The MS/MS analytic method was optimized according to the guidelines established by the European Commission, which establishes that a substance can be identified using LC-MS/MS in MRM mode by the specific retention time and at least two ion transitions while monitoring the ion ratio (Commission Decision, EC657/2002). The most abundant product ions were selected for quantification, and the second one was selected for confirmation. Therefore, the quantification of each mycotoxin was carried out with the primary transition (transition of quantification) and confirmed with the second transition (transition of confirmation).

Table 3
Method validation.

	LC-MS/MS (IT)						LC-(QqQ)-MS/MS					
	LOD[a]	LOQ[a]	SSE[b]	R^{2c}	Recovery[d]		LOD[a]	LOQ[a]	SSE[b]	R^{2c}	Recovery[d]	
					RSD_r n = 6	RSD_{int} n = 6					RSD_r n = 6	RSD_{int} n = 6
OTA	0.24	0.42	78	0.999	94 ± 8	92 ± 8	0.93	2.90	79	0.999	92 ± 5	90 ± 7
AFB1	0.05	0.07	64	0.998	73 ± 6	77 ± 8	0.14	0.28	66	0.998	72 ± 6	67 ± 8
AFB2	0.04	0.15	60	0.992	76 ± 9	75 ± 11	0.12	0.30	59	0.993	72 ± 3	75 ± 5
AFG1	0.04	0.14	73	0.997	75 ± 5	74 ± 6	0.13	0.35	74	0.997	73 ± 4	71 ± 6
AFG2	0.05	0.27	74	0.999	82 ± 5	78 ± 10	0.14	0.37	74	0.999	81 ± 3	78 ± 9
STG	1.00	2.04	44	0.997	74 ± 10	73 ± 12	1.05	2.94	45	0.997	73 ± 4	79 ± 7
NIV	0.02	0.06	40	0.998	94 ± 7	89 ± 9	6.07	20.48	43	0.997	92 ± 5	89 ± 10
DON	8.23	16.57	13	0.994	78 ± 6	76 ± 7	10.00	22.46	15	0.994	75 ± 1	74 ± 5
3ADON	1.00	5.05	30	0.995	95 ± 4	90 ± 9	9.02	31.78	37	0.995	94 ± 3	89 ± 7
15ADON	10.00	18.94	28	0.995	73 ± 6	79 ± 8	10.00	19.06	22	0.995	77 ± 7	73 ± 8
DAS	1.38	5.99	87	0.993	88 ± 7	84 ± 9	18.70	39.64	87	0.993	81 ± 7	74 ± 9
NEO	1.22	3.81	89	0.993	73 ± 9	74 ± 7	6.04	27.99	84	0.993	81 ± 5	77 ± 6
HT-2	5.41	8.67	71	0.998	92 ± 4	89 ± 5	5.52	9.05	34	0.998	92 ± 4	89 ± 7
T-2	0.21	0.73	44	0.997	90 ± 7	83 ± 12	1.65	5.52	49	0.997	82 ± 4	79 ± 5
FB1	2.78	3.30	23	0.997	84 ± 5	80 ± 12	7.69	16.68	31	0.997	81 ± 2	80 ± 5
FB2	0.60	3.21	45	0.997	81 ± 8	84 ± 8	4.77	8.42	46	0.997	80 ± 9	76 ± 11
ENA	0.15	0.49	58	0.999	94 ± 4	93 ± 6	0.15	0.50	60	0.999	91 ± 5	90 ± 6
ENA1	0.02	0.07	56	0.997	86 ± 9	88 ± 10	0.08	0.25	57	0.997	87 ± 8	88 ± 11
ENB	0.14	0.49	58	0.998	90 ± 4	84 ± 9	0.15	0.50	65	0.998	88 ± 7	84 ± 8
ENB1	0.15	0.49	55	0.998	97 ± 11	95 ± 15	0.02	0.05	55	0.997	96 ± 5	95 ± 10
BEA	0.03	0.10	49	0.995	94 ± 4	94 ± 5	0.10	0.39	48	0.995	93 ± 3	92 ± 5

[a] LOD and LOQ are limits of detection and quantification, respectively (μg/kg).
[b] SSE: Signal Suppression–Enhancer = (slope with matrix/slope without matrix) × 100.
[c] R^2: correlation coefficient (statistical parameter).
[d] Recoveries ± RSD: experimental data from analysis performed at concentrations of 10 LOQ (data from 100 LOQ experiments not shown due to low relevance).

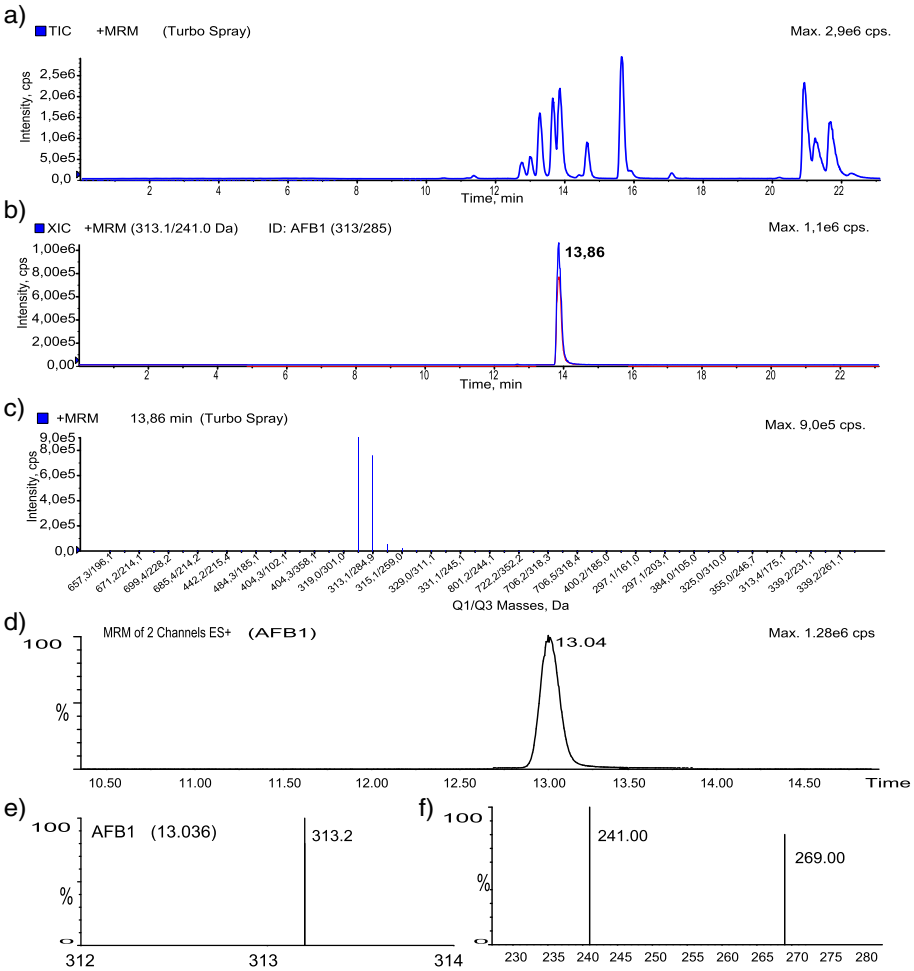


Fig. 1. AFB1 in a matrix matched multi-mycotoxins standard solution report. a) Chromatogram in TIC with all studied mycotoxins (LC-MS/MS-IT); b) chromatogram in XIC with two transitions of AFB1 (313.1/241.0 and 313.1/284.9) (LCMS/MS-IT); c) mass spectrum at 13.86 min of chromatogram "*b*"; d) chromatogram of AFB1 (LC-MS/MS-QqQ); e) mass spectrum of precursor ion at 13.04 min of chromatogram "*d*"; f) mass spectrum of products ions at 13.04 min of chromatogram "*d*".

The method internal validation included the determination of linearity, limit of detection (LOD),[23] limit of quantification (LOQ),[24] recovery, repeatability (intra-day precision; RSD_r),[25] and reproducibility (inter-day precision; RSD_{int}).[26]

To determine the linearity, calibration curves for each studied mycotoxin were constructed from the standards prepared in MeOH and from the standards prepared in extract of a mycotoxin-free sample (ICS). Standards for all mycotoxins were prepared in triplicate at six concentration levels ranging from the LOD to 100 μg/l for AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, NIV, DAS, T-2, HT-2, ENA, ENA_1, BEA, and ENB, from LOD to 500 μg/l for ENB_1, and from LOD to 750 μg/l for DON. The LODs were calculated using a signal-to-noise ratio of three. The LOQs were calculated using a signal-to-noise ratio of 10. Recovery experiments were carried out by spiking a sample ($n = 6$) on two non-consecutive days at concentrations of 10 LOQ and 100 LOQ. Intra-day precision was assessed by six determinations in the same day, while inter-day precision was assessed by six determinations on non-consecutive days. The matrix effects for each mycotoxin were evaluated using matrix-assisted calibration curves; the slopes from the calibration curves prepared with known amounts of standards and extracts of mycotoxin-free samples (with matrix) were compared with those from the calibration curves prepared with the same amounts of standards and MeOH (without matrix). A sample where none of the studied mycotoxins were detected was used as a blank sample in order to ensure representative results. The signal suppression/enhancement

[23] LOD: limit of detection.
[24] LOQ: limit of quantification.
[25] RSD_r: intraday precision.
[26] DSR_{int}: interday precision.

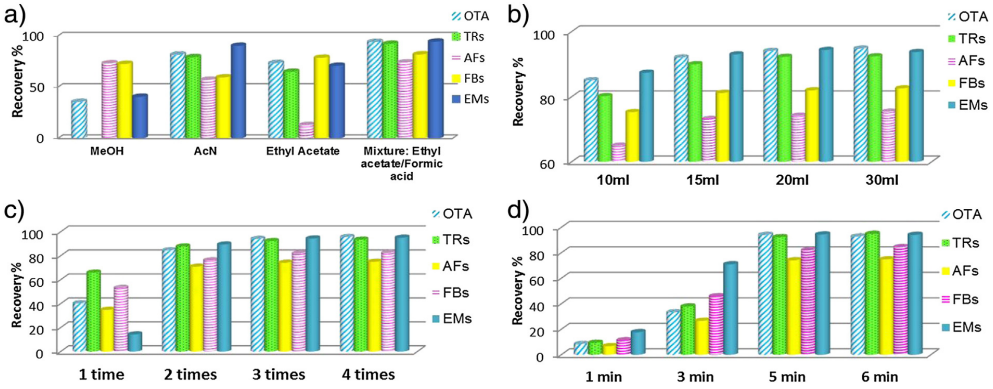


Fig. 2. UTE optimization. (a) Effect of solvents type on recovery: as it was the first step in the optimization, the other parameters were as optimum as possible (volume of solvent = 40 ml; number of cycles = 5; time of extraction = 6 min). (b) Effect of solvent volume on recovery (solvent = mixture of ethyl acetate/formic acid (95/5), number of cycles = 5, time of extraction = 6 min). (c) Effect of number of cycles on recovery (solvent = mixture of ethyl acetate/formic acid (95/5), solvent volume = 20 ml, extraction time = 6 min). (d) Effect of extraction time on recovery (solvent = mixture of ethyl acetate/formic acid (95/5), solvent volume = 20 ml, number of cycles = 3).

(SSE)[27] due to matrix effects was calculated as follows: SSE (%) = 100 (slope with matrix / slope without matrix).

3. Results and discussion

3.1. LC-MS/MS optimization

The analytical method was internally validated for coffee beverages using LC/MS-MS-QqQ and LC/MS-MS-QqQ-IT. Table 2 shows the MS/MS transitions and the instrumental parameters optimized for each compound for both instruments.

3.2. Method internal validation

The internal method validation was performed with coffee beverage samples for the simultaneous analysis of 21 mycotoxins. The analytical parameters of the method are shown in Table 3.

The results showed good correlation coefficients for both instruments ($R^2 > 0.992$). For all studied mycotoxins, the SSE values reflect high signal suppression ranging from 23 to 89%; the experimental values ranged from 72 to 97%. The relative standard deviation (RSD)[28] values ranged between 4 and 12% for RSD_r and between 5 and 15% for RSD_{int}. The final LOD and LOQ ranged from 0.02 and 0.06 to 10.00 and 18.94 μg/kg, respectively, for IT and from 0.02 and 0.05 to 18.70 and 39.64, respectively, for the QqQ instrument. For OTA, the only regulated mycotoxin in coffee, the LOD and LOQ (0.24 and 0.42 μg/kg, respectively) were lower than MLs established by the European Commission (Commission Regulation, EC1881/2006). Fig. 1 shows the chromatograms and mass spectrum of a selected mycotoxin (AFB_1) from a spiked sample of ICS (the mycotoxin-free sample). Differences between the instruments were found regarding sensibility; the IT spectrometer provides the best LOD and LOQ concentrations for most mycotoxins.

3.3. Purification and extraction procedure

3.3.1. UTE optimization

Fig. 2 shows the effects of solvent type, solvent volume, extraction time, and number of cycles on the recoveries of the different analyzed mycotoxins. The mixture of ethyl acetate/formic acid (95:5 v/v) produced recoveries ranging from 72% to 97% (Table 3). Regarding solvent volume, the best recoveries were obtained with 30 and 20 ml of solvent (Fig. 2). The extraction times affect recoveries for up to three cycles, but no significant differences were observed with larger cycle numbers. Thus, optimum recoveries without significant variations with respect to the waste of solvents were obtained using a solvent volume of 20 ml and three extraction cycles. The extraction time shows relevant differences in terms of recovery. Hence, the optimized parameters for mycotoxin extraction from coffee beverage samples using UTE with a mixture of ethyl acetate/formic acid (95:5 v/v) as the extraction solvent were a solvent volume of 20 ml, three extraction cycles, and an extraction time of 5 min. These optimized parameters are in agreement with the report of Mavungu et al. (2009), who developed a multi-mycotoxin method using a mixture of ethyl acetate/formic acid for the analysis of food supplements.

3.3.2. Cleanup optimization

Fig. 3 shows the effects of the different cleanup procedures used in each group of analyzed mycotoxins. The C_{18} cartridge and graphitized carbon blank methods were determined to be good cleanup methods in previous studies on roasted coffee. In this study, interferences (matrix substances that increase the SS or signal noise) are different with the same cleanup procedure because of the extremely acidic extraction medium and the non-polar nature of the solvent. The best recoveries for coffee samples were obtained by cleanup via clarification with Carrez solutions. This method was the best at preventing matrix effects, providing better recoveries and sensibilities.

3.4. Analysis of real samples

The developed multi-residue method was applied to six commercial coffee samples. The samples were analyzed in triplicate, and the calculated RSD values were below 10%. The obtained results are shown in Table 4. Five mycotoxins (15-aDON, DAS, AFG_2, ENA, and FB_2) were not detected in any sample. Five samples were contaminated with mycotoxins, with the co-occurrence of at least six mycotoxins. The highest co-occurrence was nine of 21 mycotoxins in the DC sample.

OTA was present in two samples: DC (1.84 μg/kg) and ICSM (4.93 μg/kg); these values are lower than the established MLs (5.00 μg/kg in roasted coffee and 10.00 μg/kg in instant coffee; EC1881/2006). These results are in agreement with those of Casal et al. (2014), who detected OTA in instant coffee at concentrations ranging from 0.15 to 11.80 μg/kg, Vanesa and Ana (2013), who detected OTA in instant coffee at concentrations ranging from 0.22 to 13.66 μg/Kg, and

[27] SSE: signal suppression/enhancement.
[28] RSD: relative standard deviation.

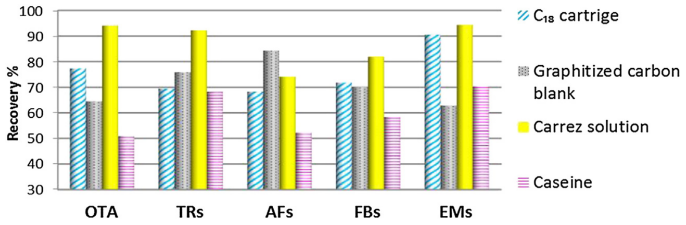


Fig. 3. Effect of cleanup on recovery (extraction conditions: solvent = mixture of ethyl acetate/formic acid (95/5), solvent volume = 20 ml, number of cycles = 3; extraction time = 5 min).

Noba et al. (2009), who found OTA in ready-to-drink samples at insignificant concentrations.

In a previous study, Bokhari (2007) found AFB_1 at concentrations between 2.10 and 219.00 μg/kg in the study of coffee beans. In our study, AFs were found in one sample with concentrations of 3.66 and 6.65 μg/kg for AFB_1, and AFG_2, respectively. However, in contrast to the study by Bokhari (2007), no samples in the current study had high AF contents. The low incidence of AFs in the present study is in agreement with Khayoon et al. (2014), who did not detect AFs in coffee samples.

Liu et al. (2014) and Alkadri et al. (2014) detected STG in wheat and sesame butter at low concentrations under 5.10 μg/kg; in the present study, STG occurred in two samples at 23.77 and 36.54 μg/kg. Yogendrarajah, Poucke, Meulenaer, and Saeger (2013) found STG at similar concentrations in white pepper.

The concentration of TRs ranged from 0.61 to 30.24 μg/kg, and their concentrations were low in the most cases. These contamination levels are lower than those found by other authors in different food products such as wheat, maize, and oat semola (Alkadri et al., 2014; Han et al., 2014; and Kirincic et al., 2015).

FB1 occurred in one sample with a concentration 5.18 μg/kg. Only FB_2 has been studied by other authors in coffee beans, and low concentrations between 1.30 and 9.70 μg/kg were found, in agreement with this study (Noonim et al., 2009).

EMs were found in most samples at concentrations ranging from 0.29 to 36.14 μg/kg. No data on EMs in coffee are available, although studies on Ems in peanuts and pasta indicate higher concentrations (Serrano, Font, Mañes, & Ferrer, 2013; Tolosa, Font, Mañes, & Ferrer, 2013).

Table 4
Concentration of each mycotoxin in real samples expressed in μg/kg.

	Ground coffee			Instant coffee		
	NRC[a]	TRC[b]	DC[c]	IC[d]	ICS[e]	ICSM[f]
OTA	–	–	1.84	–	–	4.93
AFB1	–	–	–	3.66	–	–
AFB2	–	–	–	5.64	–	–
AFG1	–	–	–	6.65	–	–
AFG2	–	–	–	–	–	–
STG	36.54	–	23.77	–	–	–
NIV	–	2.38	2.92	24.46	–	0.61
DON	–	–	–	–	–	18.34
3ADON	–	–	5.17	–	–	–
15ADON	–	–	–	–	–	–
DAS	–	–	–	–	–	–
NEO	–	3.82	30.24	–	–	12.37
T2	0.89	2.14	7.84	3.57	–	–
HT2	–	–	–	14.39	–	–
FB1	5.18	–	–	–	–	–
FB2	–	–	–	–	–	–
ENA	–	–	–	–	–	–
ENA1	1.64	1.18	4.82	–	–	0.64
ENB	8.44	5.51	20.66	36.14	–	0.96
ENB1	2.87	1.68	5.33	–	–	0.52
BEA	–	–	–	0.37	–	–
Co-presence	6/21	6/21	9/21	8/21	0/21	7/21

[a] NRC: natural roasted coffee.
[b] TRC: torrefacto roasted coffee.
[c] DC: decaffeinated coffee.
[d] IC: instant coffee.
[e] ICS: instant coffee sugar added.
[f] ICSM: instant coffee sugar and milk added.

4. Conclusions

A new method for the simultaneous analysis of 21 mycotoxins in coffee beverages based on liquid/liquid extraction followed by detection and quantification with LC-MS/MS (QqQ and QqQ-IT) has been optimized. The method was applied to the analysis of commercial samples.

The present study provides a multi-residue method suitable for the simultaneous detection and quantification of 21 mycotoxins in coffee beverages with LODs below the MLs established for other food commodities.

The application of this method to real samples shows the occurrence and co-occurrence of mycotoxins. No conclusion about the incidence or amount of mycotoxins in real samples can be established due to the low sample number. Further studies with more samples must be carried out. This method along with the subsequent study of commercial samples will facilitate the assessment of the safety of commercial products.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2015.02.030.

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Analysis of mycotoxins in coffee and risk assessment in Spanish adolescents and adults

A. García-Moraleja*, G. Font, J. Mañes, E. Ferrer

Laboratory of Toxicology and Food Chemistry, Faculty of Pharmacy, University of Valencia, Av. Vicent Andrés Estellés s/n, 46100 Burjassot, Spain

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ABSTRACT

Mycotoxins are toxic compounds produced by fungal secondary metabolism that cause toxicological effects. Coffee is a highly popular beverage that is susceptible to contamination by mycotoxigenic fungi. The aim of the present study was to determine the presence of the following 21 mycotoxins in coffee using liquid chromatography tandem mass spectrometry (LC-MS/MS-IT): aflatoxin B_1, B_2, G_1 and G_2; ochratoxin A; nivalenol; deoxynivalenol; 3-acetyldeoxynivalenol; 15-acetyldeoxynivalenol; diacetoxyscirpenol; neosolaniol; T-2 and HT-2 toxin; sterigmatocystin; enniatin A, A_1, B, and B_1; beauvericin; and fumonisin B_1 and B_2. We aimed to determine differences by coffee process (coffee maker, electrical machine, soluble and traditional Turkish process) and to calculate the estimated daily intake (EDI) and risk assessment of mycotoxins from coffee consumption using deterministic approach at various scenarios of food consumption in Spanish adolescents and adults. The results demonstrate that all studied mycotoxins were detected in samples with mean concentrations ranging from 0.69 μg/kg to 282.89 μg/kg. Eleven percent of samples did not show contamination with legislated mycotoxins. Only 15-acetyldeoxynivalenol, deoxynivalenol, neosolaniol, fumonisin B_1, and ochratoxin A exhibited significant differences between methods of coffee brewing. The results show that coffee intake does not represent a potential risk for consumers with respect to individual mycotoxin contamination.

1. Introduction

Coffee is a drink obtained from ground roasted beans of the coffee plant (*Coffea spp*). The genus *Coffea* includes many species, though only the following three are cultivated for commercial purposes: *Coffea arabica, Coffea robusta* and *Coffea liberica*. Coffee is the second largest trade commodity in the world. Countries with high coffee consumption include the United States of America and Brazil (I.C.O, 2013). Coffee has become an irreplaceable product in the diet, and recent studies shows that while economic crises affect the quality of purchased coffee, they do not affect the level of coffee intake by consumers (Mussatto et al., 2011).

Mycotoxins are toxic compounds that result from fungal secondary metabolism produced in different substrates under certain weather conditions (Rocha et al., 2014). Coffee is susceptible to contamination by mycotoxigenic fungi (Gamboa-Gaitán, 2012; Serani et al., 2007). These mycotoxins cause different toxicological effects in humans (Afsah-Hejri et al., 2013). The most commonly studied mycotoxin in coffee is ochratoxin A (OTA) (Paterson et al., 2014), and this is the only mycotoxin subject to current legislation (European Commission (EC) 1881/2006); the maximum levels (ML) are 5 μg/kg in roasted coffee beans and ground roasted coffee and 10 μg/kg in soluble coffee (instant coffee). Nevertheless, available studies of green and roasted coffee

Abbreviations: 15-aDON, 15-acetyldeoxynivalenol; 3-aDON, 3-acetyldeoxynivalenol; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; AFs, aflatoxins; BEA, beauvericin; DAS, diacetoxyscirpenol; DON, deoxynivalenol; EDI, Estimated Daily Intake; EFSA, European Food Safety Authority; ENA, enniatin A; ENA_1, enniatin A_1; ENB, enniatin B; ENB_1, enniatin B_1; FB_1, fumonisin B_1; FB_2, fumonisin B_2; FBs, fumonisins B; JECFA, Joint FAO/WHO Expert Committee on Food Additives; LC-MS/MS-IT, Liquid Chromatography tandem Mass Spectrometry with Triple Quadrupole and Ion Trap; LOD, Limit of Detection; LOQ, Limit of Quantification; ML, maximum levels; NEO, neosolaniol; NIV, nivalenol; OTA, ochratoxin A; PTWI, Probably Tolerable Weekly Intake; RSD, Relative Standard Deviation; SS, Signal Suppression; STG, sterigmatocystin; TDI, Tolerable Daily Intake; TRC, trichothecenes.

* Corresponding author.
E-mail address: ana.garcia-moraleja@uv.es (A. García-Moraleja).

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beans demonstrate the presence of different mycotoxins. Nielsen et al. (2015) studied OTA and fumonisins B (FBs) in green coffee, roasted coffee and soluble coffee and detected OTA in roasted coffee and FBs in green coffee. Bokhari and Ali (2009) detected OTA, aflatoxins (AFs), patulin and sterigmatocystin (STG) in coffee beans.

Coffee is a very popular beverage, and its chemical composition is affected by the brewing process (Caporaso et al., 2014). It is likely that each brewing process affects the mycotoxin content of coffee to a different degree. Studies have examined OTA and AFs as single mycotoxins in coffee, but studies of other mycotoxins such as trichothecenes (TRC) and enniatins have not been reported (Tozlovanu and Pfohl-Leszkowicz, 2010). In addition, several authors have studied the effect of different industrial and culinary processes on mycotoxin content; La Pera et al. (2008) reported a reduction in OTA (from 100% to 64%), and Napolitano et al. (2007) observed a reduction in OTA concentrations during different preparations of typical espresso. However, no data regarding the occurrence of multiple mycotoxins in a large sample of coffee beverages has been reported.

In this context, the aim of the present study was to determine the presence of 21 mycotoxins [aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), OTA, nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-aDON), 15-acetyldeoxynivalenol (15-aDON), diacetoxyscirpenol (DAS), neosolaniol (NEO), T-2 and HT-2 Toxin, STG, enniatin A (ENA), enniatin A_1 (ENA_1), enniatin B (ENB), enniatin B_1 (ENB_1), beauvericin (BEA), fumonisin B_1 (FB_1), and fumonisin B_2 (FB_2)] using liquid chromatography tandem mass spectrometry with triple quadrupole and ion trap (LC-MS/MS-IT) in different coffee beverages (caffeinated, decaffeinated, conventional, pre-portioned, soluble and Turkish coffee) to identify and assess the presence and possible co-existence of mycotoxins and to assess differences between commercial coffee types. We also calculated the EDI and performed a risk assessment for coffee consumption.

2. Materials and methods

2.1. Chemical and reagents

Ethyl acetate (HPLC grade) was supplied by Alfa Aesar (Karlsurhe), and acetonitrile and methanol (HPLC grade) were supplied by Merck (Damstadt, Germany). Ammonium formiate (99%), $ZnSO_4 \cdot 7H_2O$ and $K_2Fe(CN)_6$ were supplied by Panreac Quimica SAU (Barcelona, Spain). Formic acid (reagent grade ≥95%) was provided by Sigma–Aldrich (Louis, USA), and deionized water (resistance $<18\ M\Omega\ cm^{-1}$) was purified using the Milli-Q SP purification system from the Millipore Corporation (Bedford, USA). Standards of the following mycotoxins were provided by Sigma (St. Louis, MO, USA): AFB_1, AFB_2, AFG_1, AFG_2, OTA, NIV, DON, 3-aDON, 15-aDON, DAS, NEO, T-2 and HT-2 toxin, STG, ENA, ENA_1, ENB, ENB_1, BEA, FB_1, and FB_2. Individual stock solutions with a concentration of 500 mg/l were prepared in acetonitrile. The appropriate working solutions were prepared and stored in darkness at 20 °C until LC-MS/MS-IT analysis. Carrez's Solutions were prepared within the laboratory with $ZnSO_4 7H_2O$ (1.0 M) for Carrez's solution (I) and $K_2Fe(CN)_6$ (0.3 M) for Carrez's solution (II). A mixture of ethyl acetate/formic acid (95:5 v/v) was prepared daily with 25 ml of formic acid and up to 500 ml of ethyl acetate. Prior to injection, the samples were passed through a nylon filter of 13 mm/0.22 mm (Membrane Solutions, Texas, USA). The mobile phases were filtered through a cellulose filter with a 0.45-mm diameter from Scharlau (Barcelona, Spain).

2.2. Samples

A total of 169 samples of coffee representative of general consumption habits and according market share were purchased from supermarkets in Valencia, Spain. Total samples from 28 different brands representing all qualities (name brands and own brands) and within easy reach of consumers were collected, processed and analyzed. The samples were classified according the brewing process as follows: conventional (coffee maker), soluble, pre-portioned (electric machine), and Turkish coffee. Within these types of coffee, the samples were classified as caffeinated and decaffeinated. Each coffee type was represented with as many brands as possible. All samples were analyzed before brewing according Gracía-Moraleja et al. (2015b) methodology; No significative differences in the mycotoxins contents were obtained between the different coffee type (excepting Turkish coffee).

2.3. Sample preparation and processing

To maximum the quality of the brew and minimize confounding between methods of coffee preparation, all coffee types were processed using mineral water (mycotoxin-free) of the same commercial brand. A coffee maker (Italian moka) was used to prepare ground conventional packed coffee samples similar to how coffee is prepared in the home. The coffee maker contained three compartments; the first compartment contained water, the second compartment was separated from the first by a metallic filter that holds the ground coffee and the third compartment is located on above the coffee pot and is initially empty. Water is boiled in the first compartment and flows through the coffee in the second compartment; the water is approximately 98.6 °C (Gianino, 2007). Coffee then brews in the upper reservoir. For this preparation, approximately 5 g of ground coffee and 50 ml of water are used to obtain a final cup of coffee of approximately 45 ml. Soluble coffee samples were prepared according to the manufacturer's specifications regarding the ratio of grams of coffee per milliliters of water. In this case, water was heated to 85–90 °C in a hot plate and added to a glass beaker holding the soluble coffee; the mixture was continuously stirred to dissolve the coffee. Samples of Turkish coffee were processed using the traditional Turkish method. For this, 50 ml of water was heated on a hot plate to 95–100 °C and added to a glass beaker holding 5 g of coffee; this was mixed for 30 s. The upper phase was collected after allowing 10 min for sedimentation. Samples of pre-portioned coffee of approximately 5–7 g were prepared using corresponding electrical machines. The first 45 ml produced by the coffee machines were collected.

2.4. Extraction procedure

Briefly, samples were initially clarified with Carrez's solution. Then, the clarified solution was added to a mixture of ethyl acetate/formic acid (95/5 v/v) and homogenized with Ultra-Turrax (T_{18} Basic Ika, Staufen, Germany) for 5 min; the supernatant with the organic phase was then separated. This extraction was performed in triplicate using 20 ml of mixture (ethyl acetate/formic acid) each time, and the supernatants of each extraction were collected. The supernatant was evaporated in Büchi Rotavapor R-200 (Postfach, Switzerland) under conditions of 40 °C and 140 mmHg. The extract obtained was dissolved with 10 ml of mixture (ethyl acetate/formic acid) and evaporated to dryness with a TurboVap LV Evaporator (Zymark, Hoptikinton, USA) under conditions of 50 °C and nitrogen pressure of 5 psi. The residue was reconstituted with 1 ml of methanol/water (50/50 v/v), stirred with a vortex for 1 min, and filtered through a nylon filter of 0.22 μm (Membrane Solutions, Texas, USA) to remove impurities. The extract was put in specifics

amber flaks for autosampler prior to LC-MS/MS-IT analysis (García-Moraleja et al., 2015a).

2.5. Instrumental parameters

Analyses were performed in a system equipped with an Agilent 1200 LC from Agilent Technologies (Palo Alto, CA, USA) coupled to a 3200 QTRAP mass spectrometer (Applied Biosystems, AB Sciex, Foster City, CA, USA) equipped with electrospray ionization interface Turbo IonSpray (ESI). The QTRAP analyzer combines fully functional triple quadrupole and an ion trap mass spectrometer in the same instrument. The separation of the analytes was performed with a Gemini-NX C_{18} column (3 μm, 110 A 150 mm × 2 mm) (150 mm × 4.6 mm, 5 μm particle size) preceded by a guard column C_{18}. Run time was 30 min; injection volume was 20 μl; phase A (H_2O in 5 mM Ammonium formiate and 0.1% formic acid) and phase B (MeOH in 5vmM Ammonium formiate and 0.1% formic acid) were completed; there was a pre-run time of 3 min for equilibration with flow of 0.25 ml/min (10% eluent B); the elution gradient started with flow of 0.25 ml/min with 70% of eluent B remaining constant for 3 min and increasing to 80% and 90% at minutes 6 and 14, respectively; at minute 18, the flow increased to 0.350 ml/min and 100% B; at minute 20, the flow increased to 0.400 ml/min and 50%; and from minute 21 to minute 30, the flow returned to 0.250 ml/min and 10% eluent B and 90% eluent A.

Mass spectrometer parameters were as follows: Ion source Turbo spray, type Multiple Reaction Monitoring (MRM), and positive ionization polarity (ESI+) were used; the resolution was 12.0 (unit resolution) for the first and third quadrupoles, and the ion energy was 0.5 V; the entrance and exit energies were −3 and 1 V, respectively; the multiplier was 650; the collision gas (Argon 99.995% purity) pressure was 3.83×10^{-3} mbar, the inter-channel delay was 0.02 s, the total scan time was 1.0 s, and the dwell time was 0.1 ms. Table 1 shows the specific ion transitions and energies applied for the analyses of each mycotoxin. Analyst version 1.5.1 was used to control each component of the system and for data acquisition.

2.6. Statistical analysis

An observational descriptive analysis was performed to assess differences between coffee processes (traditional, pre-portioned, soluble, and Turkish coffee) and coffee types (caffeinated and decaffeinated). The analysis included 169 samples of coffee. A one-way ANOVA test was used to assess the coffee processes, and a multi comparative post hoc study with the honest significant difference Tukey's test was used with concentration (μg/kg) as the dependent variable and coffee process as an independent factor. For the coffee type (caffeinated or decaffeinated), a Student's T-test P ($T \leq t$) was used. For both tests, P-values of <0.05 were considered statistically significant. IBM SSPS statistics 22 software for Windows 7 was used for statistical analyses.

2.7. Dietary exposure assessment

Exposure assessment is an essential element for quantifying risk and is a tool for risk managers involved in food safety (Leblanc et al., 2005). The most common methodologies for exposure assessment are deterministic or point estimates of dietary exposure and probabilistic assessments (WHO, 2009). The deterministic assessment is the simplest approach, as it is a single value of consumer exposure. The assessment is calculated as the product of food consumption and the average amount of the studied mycotoxin contained in the food. The advantage of deterministic assessments is that the simplicity of the results allows comparison between studies from different countries (Serrano et al., 2012), with tolerable daily intakes (TDI) proposed by organizations (Rodríguez-Carrasco et al., 2013) that is an estimate of the amount of a substance that can be taken in daily over a lifetime without appreciable health risk, and with total diet studies. Conversely, in the

Table 1
LC-MS/MS-QqQ-IT optimized instrumental parameters of the validated method.

	DP[a]	Precursor ion	Quantification[Q]			Confirmation[q]		
			CE[b]	Product ion	CXP[c]	CE[b]	Product ion	CXP[c]
OTA	55	404.3	97	102.1	6	27	239.0	6
AFB_1	46	313.1	39	284.9	4	41	241.0	4
AFB_2	81	315.1	39	259.0	6	33	286.9	6
AFG_1	76	329.0	29	311.1	6	39	243.1	6
AFG_2	61	331.1	27	313.1	6	39	245.1	4
STG	106	325.0	51	281.0	18	50	310.0	3
NIV	50	313.4	80	115.1	3	27	175.1	3
DON	36	297.1	17	249.2	4	29	161.0	4
3aDON	44	339.2	20	203.1	3	20	231.1	3
15aDON	50	339.2	20	137.0	3	20	261.1	3
DAS	66	384.0	15	307.2	16	63	105.0	12
NEO	46	400.2	25	215.0	12	29	185.0	14
T-2	21	484.3	22	185.1	4	29	215.1	4
HT-2	21	442.2	19	215.4	8	19	267.8	4
FB_1	101	722.2	51	334.2	20	45	352.2	26
FB_2	131	706.2	50	336.3	16	50	318.3	18
ENA	76	699.4	59	228.2	16	35	210.1	14
ENA_1	66	685.4	59	214.2	10	37	210.2	8
ENB	51	657.3	39	196.1	8	59	214.0	10
ENB_1	66	671.2	61	214.1	10	57	228.1	12
BEA	116	801.2	27	784.1	10	39	244.1	6

[Q] Quantification ion.
[q] Confirmation ion.
[a] DP: Decluster potential (Volts).
[b] CE: Collision Energy (Volts).
[c] CXP: Cell Exit Potential (Volts).

probabilistic approach, consumption is represented by a distribution instead of a single value (WHO, 2009).

While coffee makes up a small part of the overall diet, on basis of its weight equivalence in percentage, many individuals drink coffee daily, and segments of the population consume large quantities of coffee. Calculating mycotoxins exposures from coffee consumption and including them in total diet studies would increases the precision of the estimates of coffee consumption in the total population.

The estimated daily intake (EDI) of each group of mycotoxin was calculated based on the recommendations of the International Program on Chemical Safety: Principles and Methods for the Risk Assessment of Chemicals in Food (WHO, 2009).

Data on mycotoxin concentrations in 169 coffee samples purchased at supermarkets were obtained following coffee preparation using traditional consumer methods. Dietary exposure assessments (deterministic) use mean consumption data from the general population at different scenarious: mean, 95th 97.5th or 99th percentiles to define the population expected to have high exposure. The assessment of mycotoxin intake from coffee, abbreviated as EDI_{Coffee}, is calculated using the equation EDI_{Coffee} (ng/kg bw day) = coffee consumption (g/kg bw day) x mycotoxin content (ng/g).

2.8. Food consumption data

The European Food Safety Authority (EFSA) has compiled food consumption data in diverse Member States (EFSA, 2015). This compilation classified food and beverages in a standardized way. In addition, consumption of a single food is specified for various segments of the population, and these segments represent the mean population of highly exposed consumers. As samples were purchased from Spain, which is included in the EFSA database, data from the Spanish Agency for Food Safety Survey for long-term exposure (chronic) food consumption statistics in grams per day per kilogram of body weight was selected. This survey from 2009 contains 1067 subjects, which include 86 adolescents and 981 adults. Children were not included. The survey was conducted in accordance with a harmonised methodology approved by EFSA.

3. Results

3.1. Method validation

The analytic method that we used to assess the 21 mycotoxins in coffee was previously validated in accordance with the European Commission (EC657/2002). For validation parameters such as linearity (R^2), the limit of detection (LOD), the limit of quantification (LOQ), recovery, signal suppression (SS) due to matrix effects, and intra- and inter-day precision were evaluated. Table 2 shows the parameters of the method validation. The method showed good correlation coefficients ($R^2 > 0.992$). For all studied mycotoxins, the SS values reflect high signal suppression ranging from 13 to 89%; the experimental recovery values ranged from 72 to 96%. The relative standard deviation (RSD) values ranged between 1 and 9% for RSD_r and between 5 and 11% for RSD_{int}. The LOD and LOQ ranged from 0.02 to 10.00 and from 0.07 to 18.94 μg/kg, respectively.

3.2. Occurrence of mycotoxins in samples

The samples were analyzed in triplicate, and the calculated RSD values were below 10%. The obtained results are shown in Table 3. The results are expressed as μg of mycotoxins per kg of coffee (commercial roasted coffee) to standardize the data. All studied mycotoxins were detected in some samples. Nineteen samples did show contamination with the legislated mycotoxins, and only 5 samples did not show contamination with the emerging mycotoxins.

Aflatoxins were present in 53% of the studied samples. As mentioned above, there are no regulations for AFs in coffee. In the present study, no samples exceeded 2 μg/kg of AFB_1, but 15% of samples had a concentration of total AFs above 5 μg/kg. Samples

Table 2
Analytical parameters of the validation method.

	LOD (μg/kg)	LOQ (μg/kg)	SSE[a]	R^{2b}	Recovery			
					10 LOQ		100 LOQ	
					Intra day (n = 6)	Inter day (n = 6)	Intra day (n = 6)	Inter day (n = 6)
OTA	0.24	0.42	78	0.999	92 ± 5	90 ± 7	98 ± 4	96 ± 7
AFB_1	0.05	0.07	64	0.998	72 ± 6	67 ± 8	84 ± 5	84 ± 4
AFB_2	0.04	0.15	60	0.992	72 ± 3	75 ± 5	86 ± 3	87 ± 5
AFG_1	0.04	0.14	73	0.997	73 ± 4	71 ± 6	85 ± 4	89 ± 4
AFG_2	0.05	0.27	74	0.999	81 ± 3	78 ± 9	85 ± 3	85 ± 9
STG	1.00	2.04	44	0.997	73 ± 4	79 ± 7	79 ± 5	88 ± 5
NIV	0.02	0.06	40	0.998	92 ± 5	89 ± 10	95 ± 3	98 ± 9
DON	8.23	16.57	13	0.994	75 ± 1	74 ± 5	80 ± 2	80 ± 4
3aDON	1.00	5.05	30	0.995	94 ± 3	89 ± 7	100 ± 4	99 ± 4
15aDON	10.00	18.94	28	0.995	77 ± 7	73 ± 8	88 ± 4	88 ± 6
DAS	1.38	5.99	87	0.993	81 ± 7	74 ± 9	92 ± 7	92 ± 7
NEO	1.22	3.81	89	0.993	81 ± 5	77 ± 6	93 ± 5	93 ± 5
T-2	5.41	8.67	71	0.998	92 ± 4	89 ± 7	89 ± 3	89 ± 3
HT-2	0.21	0.73	44	0.997	82 ± 4	79 ± 5	95 ± 6	97 ± 6
FB_1	2.78	3.30	23	0.997	81 ± 2	80 ± 5	89 ± 4	89 ± 6
FB_2	0.60	3.21	45	0.997	80 ± 9	76 ± 11	90 ± 5	91 ± 8
ENA	0.15	0.49	58	0.999	91 ± 5	90 ± 6	96 ± 4	96 ± 4
ENA_1	0.02	0.07	56	0.997	87 ± 8	88 ± 11	95 ± 9	94 ± 9
ENB	0.14	0.49	58	0.998	88 ± 7	84 ± 8	91 ± 7	91 ± 6
ENB_1	0.15	0.49	55	0.998	96 ± 5	95 ± 10	97 ± 9	95 ± 7
BEA	0.03	0.10	49	0.995	93 ± 3	92 ± 5	98 ± 3	98 ± 5

[a] SSE: Signal Suppression − Enhancer//SSE = (slope with matrix/slope without matrix) × 100.
[b] Linearity: expressed as R^2 from calibration curves matrix matched.

Table 3
Contamination levels in brewed coffee samples n = 169.

	Occurrence (%)[b]	Mean (μg/kg)		Range[c]	
		Total samples	Positive samples	Min (μg/kg)	Max (μg/kg)
OTA	36	0.67	1.87	0.67	4.70
AFB_1	22	0.20	0.91	0.75	1.14
AFB_2	10	0.07	0.69	0.51	1.57
AFG_1	15	0.46	3.05	2.54	50.80
AFG_2	25	1.01	4.03	0.99	25.87
AFS_T[a]	53	2.70	5.07	0.59	26.93
STG	16	3.62	22.63	7.65	63.19
NIV	43	0.72	1.68	0.12	37.83
DON	43	43.95	102.22	16.58	954.91
3aDON	20	2.09	10.45	6.31	24.57
15aDON	41	38.89	94.86	61.04	884.25
DAS	7	13.73	196.20	195.91	402.23
NEO	13	11.27	86.73	29.06	313.84
T-2	29	0.69	2.37	0.74	21.17
HT-2	9	1.10	12.20	8.41	37.01
FB_1	4	0.62	15.55	15.52	18.25
FB_2	4	0.90	22.46	20.02	23.99
ENA	59	166.91	282.89	5.88	2875.57
ENA_1	66	162.82	246.69	2.22	12039.12
ENB	93	163.92	176.26	4.68	4147.96
ENB_1	75	103.93	138.57	5.16	3932.47
BEA	5	0.05	0.97	0.52	3.07

[a] AFS_T: total aflatoxins ($AFB_1 + AFB_2 + AFG_1 + AFG_2$).
[b] Occurrence in total samples n = 169.
[c] Range of concentration in positive samples.

with concentrations of total AFs above 5 μg/kg are due to AFG_2 contamination; AFG_2 was present in the samples in greater concentrations but is the less toxic aflatoxin. In most cases of contamination, the samples were contaminated with one or of two AFs, and 3 of the 169 samples were contaminated with three aflatoxins; no samples were contaminated with four of the studied aflatoxins. In comparison, Khayoon et al. (2014) analyzed 21 samples of canned coffee but did not detect AFs; Bokhari (2007) detected AFB_1 in green coffee with contamination levels of 110.5 μg/kg. In a previous study, García-Moraleja et al. (2015b) detected AFB_1 and AFB_2 in roasted coffee and soluble coffee samples with a mean concentration of 1.95 μg/kg. This shows that AFs concentrations are higher in green coffee than in roasted coffee; during the brewing process, water liberates few AFs.

Thirty-six percent of the samples were contaminated with OTA, but none exceeded the limit established by ML for OTA in coffee (EC/1881/2006). The results from the present study are in accordance with Noba et al. (2009), who detected OTA in all samples of ready-to-drink coffee, which had concentrations ranging from 0.01 μg/l to 0.04 μg/l; Casal et al. (2014) detected OTA in soluble coffee samples with a mean concentration of 2.50 μg/kg. In a previous study, García-Moraleja et al. (2015b) found concentrations of 1.50–32.40 μg/kg in roasted coffee, and 35% of samples were positive. As reported by Santini et al. (2011), a reduction in OTA concentration has been observed. According to previous studies, coffee beverages have less OTA contamination than roasted coffee. Nevertheless, the brewing process does not completely remove OTA from samples, according to Pérez De Obanos et al. (2005), who studied the effects of the brewing process on the OTA content in nine positive samples; reductions in the concentrations were achieved in all cases, and only a single case had a reduction to a level below the limit of detection.

STG was present in few samples (16%). The results from the present study are in accordance with those of Bokhari and Aly (2009), who detected STG in two of 13 analyzed samples of coffee beans with concentrations of 13 and 11 μg/kg.

The trichothecenes analyzed in the present study (NIV, DON, 3-aDON, 15-aDON, DAS, NEO, HT-2 and T-2) presented variable concentrations from 1.68 μg/kg in the case of NIV to 196.20 μg/kg in the case of DAS, and the occurrence of contamination was low. García-Moraleja et al. (2015b) analyzed trichothecenes in 103 samples of roasted coffee and detected NIV, DON, DAS, HT-2, and T-2 with mean concentrations of 6605.73, 67.40, 6.50, 91.10 and 33.00 μg/kg, respectively. The results showed degradation of most mycotoxins (NIV, DAS, HT-2, and T-2). Concentration of DON was higher in beverages than in roasted coffee. The mycotoxin that showed the most degradation was NIV.

Fumonisins were analyzed in the present study, and FB_1 and FB_2 had a low occurrence and low contamination levels. García-Moraleja et al. (2015b) analyzed FBs in roasted coffee samples and detected mean concentrations of 58.62 and 85.45 μg/kg, respectively; the FBs were present in 87% and 81% of samples, respectively. Noonim et al. (2009) detected FB_2 in concentrations from 1.3 to 9.7 μg/kg in roasted coffee samples. Fumonisins that consist of carbonated chains are susceptible to heat processes, particularly during brewing. These results are in accordance with those of Nielsen et al. (2015), who detected concentrations of up to 164 μg/kg in samples of green coffee beans and demonstrated degradation of fumonisins with a thermal process (roasting). The low incidence and concentrations in the present study show that the brewing process reduces and in the most cases removes fumonisins contamination.

The following emerging mycotoxins were analyzed in the present study and were detected in samples: ENA, ENA_1, ENB, ENB_1 and BEA. Enniatins were the most concentrated mycotoxins and were present in the most samples. BEA presented low contamination levels. García-Moraleja et al. (2015b) analyzed the same mycotoxins in roasted coffee samples and detected mean concentrations of 374.30, 179.40, 577.00, 10.20 and 0.6 7 μg/kg, respectively, and these mycotoxins were present in 37%, 87%, 54%, 8% and 35% of samples, respectively. These results suggest that the brewing process does not affect the concentrations of emerging mycotoxins.

3.3. Effects of processing for mycotoxin concentration

According to Fig. 1, each brewing process shows different levels

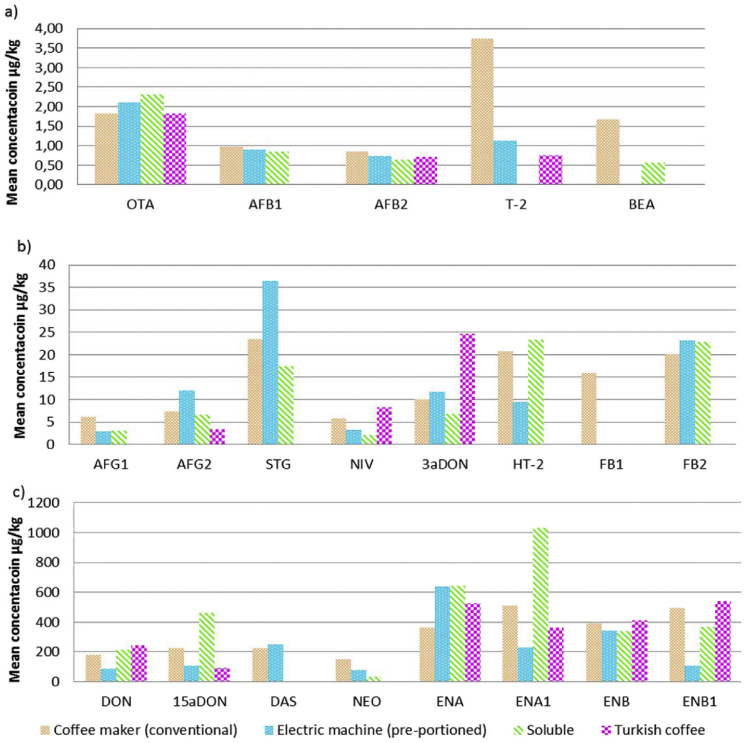

Fig. 1. Mean concentration of mycotoxins in positive samples using different brewing processes. a) Mycotoxins with low levels of mean concentration. b) Mycotoxins with medium level of mean concentration. c) Mycotoxins with high level of mean concentration.

of degradation of the mycotoxins; however, statistical analysis shows that in most cases, mycotoxins exhibited the same behavior in each process. Sixteen of the studied mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, NIV, 3a-DON, DAS, HT-2, T-2, STG, ENA, ENA_1, ENB, ENB_1, BEA and FB_2) did not differ according to the brewing process.

15a-DON, DON, NEO, and FB_1 showed extensive degradation after pre-portioned electric machine brewing, and the level of degradation was next highest in soluble coffee. The traditional process with the moka machine showed the least degradation. However, degradation of OTA was greatest with the conventional process compared to the pre-portioned and soluble processes.

Turkish coffee had a high incidence of AFG_2 contamination (80%), but it was not contaminated with other aflatoxins or STG, and contamination levels were similar to other coffee types. The Turkish coffee preparation probably affects aflatoxin contamination levels in the same way. However, Turkish coffee samples were produced in countries in North Africa, and this geographical locale or this climate could stimulate AFG_2 production over the production of other AFs.

3.4. Difference between caffeinated and decaffeinated coffee beverages

Two sub-groups of caffeinated coffee (n = 109) and decaffeinated coffee (n = 60) were compared. Mean concentrations are shown in Fig. 2. The figures show that decaffeinated coffee had higher concentrations than caffeinated coffee, though the difference was not significant ($p \geq 0.05$) for OTA, AFs, NIV, DON, DAS, or the emerging mycotoxins. There were significantly lower concentrations in caffeinated coffee for STG, 3a-DON, 15a-DON, and NEO. Differences between caffeinated and decaffeinated samples do not result from the brewing process. It is likely that caffeinated commercial roasted coffee samples have lower contamination levels of STG, 3a-DON, 15a-DON, and NEO than decaffeinated samples. Soliman (2002) detected higher production of mycotoxins in decaffeinated coffee than in caffeinated coffee, but in this study, differences between caffeinated and decaffeinated samples were detected for few mycotoxins and not for the same mycotoxin found by Soliman (2002).

3.5. Risk assessment

The dietary exposure assessment characterizes the population exposure. The results can be used for comparison with the TDI. All samples of the present study have been used for the statistical analysis. Samples with contamination levels under the LOD and LOQ were considered to be not contaminated (0 µg/kg).

The estimated daily intake (Table 4) of mycotoxins in coffee was calculated among diverse segments of the population; they were calculated in adults and adolescents and in different scenarios corresponding to population consumption habits, including the mean population and in highly exposed consumers (95th, 97.5th

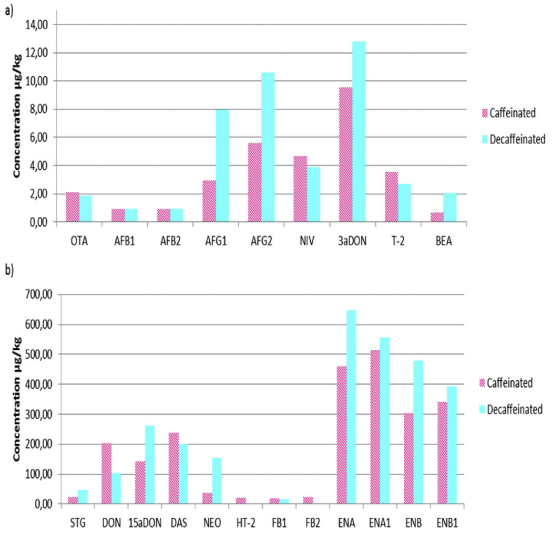

Fig. 2. Mean concentration of mycotoxin in positive caffeinated and decaffeinated samples. a) Mycotoxins with mean concentrations under 15 µg/kg; b) Mycotoxins with mean concentration above 15 µg/kg.

and 99th percentiles). The EDI in the mean population is not alarming with the exception of emerging mycotoxins, whose toxicology in humans is unknown; nevertheless, in highly exposed segments, the increased values of EDI are considerable. For example, for trichothecenes, specifically for DON and its metabolites, the mean consumption of the 99th population is not so concerning, though the effects of high doses of mycotoxins are uncertain. In a study of dietary exposure to OTA, higher levels of OTA exposure due to coffee consumption were observed in two segments of the population; the mean level was 0.05 ng/kg bw d, and the 95th percentile was 0.22 ng/kg bw d (Leblanc et al., 2005).

For the risk assessment (Table 5) the mean and less favorable scenario were chosen for the study. Adults and adolescent in the mean and 99th percentile of consumption and an average sample were assumed. The risk was characterized via comparison with the TDI or the probable tolerable weekly intake (PTWI) proposed by The Joint FAO/WHO Expert Committee on Food Additives (JECFA) and with available studies of total diet from Spain, France, Japan and Libano (Coronel et al., 2011; Raad et al.,. 2014; Sirot et al., 2013; Sugita et al., 2013). As Table 5 shows, any mycotoxins in coffee represent a risk to human health. The higher EDI was the result of emerging mycotoxins, and 15 ng of each enniatin per kg of body weight day was estimated. The EDI of DON was higher than the EDI of the other mycotoxins; however, DON toxicity is lower than that of the other mycotoxins. The risk characterization shows that DON and its metabolites (sum of DON, 3aDON, and 15aDON) represent 0.77% of the TDI; DON levels in coffee do not represent a risk to consumers. These results are in agreement with those of Rodríguez-Carrasco et al. (2013), who studied 10 mycotoxins in wheat rice and maize. Only total aflatoxins presented a considerable risk (15.68% of the TDI), and the other mycotoxins showed low EDI. It is important to consider that synergism and/or additive effects of mycotoxins by co-occurrence have been described (Wan et al., 2013) unfortunately no TDI or similar value has been stablished and comparisons are not available.

Table 6 shows the comparison of the EDI between coffee consumption among the detailed percentile of the population and that from total diet examined in recent studies. This comparison shows that coffee is a low contributor food to the overall exposure to most mycotoxins.

4. Conclusions

A study of multi-mycotoxins in ready-to-drink coffee

Table 4
Results of the dietary intake assessment of the studied mycotoxins for various scenarios expressed as (ng/g bw d).

	Adults				Adolescent			
	EDI_{mean}[b]	EDI_{P95}[b]	$EDI_{P97.5}$[b]	EDI_{P99}[b]	EDI_{mean}[b]	EDI_{P95}[b]	$EDI_{P97.5}$[b]	EDI_{P99}[b]
OTA	0.009	0.042	0.052	0.061	0.002	0.018	0.023	0.031
AFB_1	0.003	0.013	0.016	0.018	0.001	0.005	0.007	0.009
AFB_2	0.001	0.004	0.005	0.006	0.000	0.002	0.002	0.003
AFG_1	0.006	0.029	0.036	0.042	0.001	0.013	0.016	0.021
AFG_2	0.014	0.063	0.079	0.091	0.003	0.028	0.035	0.047
AFs_T[a]	0.036	0.169	0.210	0.244	0.008	0.074	0.093	0.125
STG	0.049	0.226	0.282	0.327	0.011	0.099	0.125	0.167
NIV	0.010	0.045	0.056	0.065	0.002	0.020	0.025	0.033
DON	0.593	2.747	3.418	3.973	0.138	1.204	1.516	2.028
3aDON	0.028	0.131	0.163	0.189	0.007	0.057	0.072	0.096
15aDON	0.524	2.431	3.025	3.515	0.122	1.065	1.341	1.795
DAS	0.185	0.858	1.068	1.241	0.043	0.376	0.473	0.634
NEO	0.152	0.704	0.877	1.019	0.035	0.309	0.389	0.520
T-2	0.009	0.043	0.054	0.062	0.002	0.019	0.024	0.032
HT-2	0.015	0.069	0.086	0.099	0.003	0.030	0.038	0.051
FB_1	0.008	0.039	0.048	0.056	0.002	0.017	0.021	0.029
FB_2	0.012	0.056	0.070	0.081	0.003	0.025	0.031	0.042
ENA	2.251	10.432	12.982	15.088	0.523	4.573	5.756	7.704
ENA_1	2.196	10.176	12.664	14.718	0.510	4.461	5.614	7.515
ENB	2.211	10.245	12.749	14.818	0.513	4.491	5.652	7.566
ENB_1	1.402	6.496	8.083	9.395	0.325	2.847	3.584	4.797
BEA	0.001	0.003	0.004	0.005	0.000	0.001	0.002	0.002
Intake[c]	0.013	0.063	0.078	0.090	0.003	0.027	0.034	0.046

[a] Mean concentration in µg/kg in all samples; those samples under the LOD are assigned as 0 µg/kg and LOQ as LOD.
[b] EDI Estimated Daily intake in the different exposed consumers (mean, 95th, 97.5th, 99th) expressed as ng/kg bw day.
[c] Consumption: expressed as g/kg bw day.

Table 5
Risk characterization for each mycotoxin and groups of mycotoxins, and total mycotoxins.

	TDI[a]	Adults				Adolescents			
		RA_{Mean} (%TDI)[b]	RAg_{Mean} (%TDI)[b]	RA_{P99} (%TDI)[b]	RAg_{P99} (%TDI)[b]	RA_{Mean} (%TDI)[b]	RAg_{Mean} (%TDI)[b]	RA_{P99} (%TDI)[b]	RAg_{P99} (%TDI)[b]
OTA	16	0.06		0.38		0.01		0.19	
AFB_1	1	0.27	2.35	1.81	15.68	0.06	0.54	0.92	8.03
AFB_2	1	0.09		0.62		0.02		0.32	
AFG_1	1	0.62		4.14		0.14		2.12	
AFG_2	1	1.36		9.11		0.32		4.66	
STG		0.05[c]		0.33[c]		0.01[c]		0.17[c]	
NIV	700	0.00		0.01		0.00		0.00	
DON	1000	0.06	0.11	0.40	0.77	0.01	0.03	0.20	0.39
3aDON		0.00[d]		0.02[d]		0.00[d]		0.01[d]	
15aDON		0.05[d]		0.35[d]		0.01[d]		0.18[d]	
DAS	2000	0.01		0.06		0.00		0.03	
NEO		0.15[c]		1.02[c]		0.04[c]		0.52[c]	
T-2	100	0.01	0.02	0.06	0.16	0.00	0.01	0.03	0.08
HT-2	100	0.01		0.10		0.00		0.05	
FB1	2000	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00
FB2	2000	0.00		0.00		0.00		0.00	
ENA		2.25[c]	8.06	15.09[c]	54.02	0.52[c]	1.87	7.70[c]	27.57
ENA_1		2.20[c]		14.72[c]		0.51[c]		7.51[c]	
ENB		2.21[c]		14.82[c]		0.51[c]		7.57[c]	
ENB_1		1.40[c]		9.39[c]		0.33[c]		4.80[c]	
BEA		0.00[c]		0.00[c]		0.00		0.00	
Total		14.46		72.43		3.34		49.44	

[a] TDI: tolerable daily intake.
[b] Risk assessment and risk assessment of the groups of mycotoxins. RA = EDI/TDI × 100.
[c] Risk assessment with respect to HT-2 TDI: 100 ng/kg bw d.
[d] Risk assessment with respect to DON TDI: 1000 ng/kg bw d.

Table 6
Assessment of mycotoxin exposure by coffee consumption according to studies from the literature that assessed total diet.

Studies of total diet	Mycotoxin	Intake from total diet in mean population (ng/kg bw d)	Intake from total diet high exposed population (ng/kg bw d)	Share from coffee[a] (%)	Share from coffee in high exposed population[a] (%)
Raad et al., 2014	AFB_1	0.63		0.48	
	OTA	4.28		0.21	
	DON	1560.00		0.06	
Sugita et al., 2013	OTA		1.49 (95th)		2.82
	FB		5.30 (99th)		2.58
Sirot et al., 2013	OTA		3.23 (95th)		1.30
	AFB_1		0.39 (95th)		3.33
	AFB_2		0.30 (95th)		1.33
	AFG_1		0.30 (95th)		9.67
	AFG_2		0.30 (95th)		21.00
	DON		722.00 (95th)		0.38
	3-aDON		28.50 (95th)		0.46
	15-aDON		26.90 (95th)		9.04
	NIV		66.60 (95th)		0.07
	T-2		36.50 (95th)		0.12
	HT-2		58.90 (95th)		0.12
	FB_1		65.60 (95th)		0.06
	FB_2		42.20 (95th)		0.13
Coronel et al., 2011	OTA	2.91		0.31	

[a] Share from coffee calculated as: EDI_{coffee}/Intake from total diet* 100.

demonstrated the presence and coexistence of mycotoxins in coffee beverages. The study of coffee brewing shows similar effects by different coffee processes for most mycotoxins. In a few cases, as with the pre-portioned electric machine, there was greater degradation of 15a-DON, DON, NEO, and FB_1, and with the traditional process, there was significantly greater degradation of OTA. The risk assessment has been performed for Spanish adults and adolescents; population exposure was estimated at various scenarios. The results show that coffee intake does not represent a potential risk for consumers with respect to individual mycotoxin contamination. However, contamination of coffee by mycotoxins likely affects the EDI of the total diet, especially in highly exposed segments of the population. New parameters which take into account the co-occurrence and co-intake of mycotoxins are required to ensure a suitable understanding of this health hazard.

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Transparency document

Transparency document related to this article can be found

online at Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fct.2015.10.014.

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