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CONTROL DE MICOTOXINAS EN CEREALES Y PRODUCTOS DERIVADOS POR CROMATOGRAFIA LÍQUIDA ESPECTROMETRIA DE MASAS. EVALUACIÓN DE LAS INGESTAS.

DETERMINATION OF MYCOTOXINS IN CEREALS AND DERIVATES BY LIQUID CHROMATOGRAPHY MASS SPECTROMETRY. ESTIMATION OF INTAKES

CONTROL DE MICOTOXINES EN CEREALS I PRODUCTES DERIVATS PER CROMATOGRAFIA LÍQUIDA ESPECTROMETRIA DE MASSES. AVALUACIÓ DE LES INGESTES

TESI DOCTORAL INTERNACIONAL

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Certifiquen que:

El llicenciat en Ciència i Tecnologia dels Aliments **D. Josep Vicent Rubert Bassedas** ha realitzat baix la seva direcció el treball que porta per títol **Control de micotoxines en cereals i productes derivats per cromatografia líquida espectrometria de masses. Avaluació de les ingestes**. Aquest treball ha sigut plasmat en 12 articles, publicats o que es publicaran en les següents revistes:

1. *Analysis of mycotoxins in baby food: an unresolved matter. TrAC Trends in Analytical Chemistry. (Under review) Impact factor: 6.60*
2. *Optimization of Matrix Solid-Phase Dispersion method for simultaneous extraction of aflatoxins and OTA in cereals and its application to commercial sample. Talanta 82 (2010) 567. Impact factor: 3.72*
3. *One-year monitoring of aflatoxins and ochratoxin A in tiger-nuts and their beverages. Food Chemistry 127 (2011) 822. Impact factor: 3.45*
4. *Evaluation of Matrix Solid-Phase Dispersion (MSPD) extraction for multi-mycotoxin determination in different flours using LC-MS/MS. Talanta 85 (2011) 206. Impact factor: 3.72*
5. *Occurrence of fourteen mycotoxins in Tiger-nut. Food Control 25 (2012) 374. Impact factor: 2.81*
6. *Application of an HPLC-MS/MS method for mycotoxins analysis in commercial baby food. Food Chemistry 133 (2012) 176. Impact factor: 3.45*
7. *Study of mycotoxin calibration approaches on the example of trichothecenes analysis from flour. Food and Chemical Toxicology (Under review). Impact factor: 2.60*
8. *Analysis of mycotoxins in barley using ultra high liquid chromatography high resolution mass spectrometry: Comparison of efficiency and efficacy of different extraction procedures. Talanta (Under review). Impact factor: 3.7*
9. *Application of hybrid linear ion trap-high resolution mass spectrometry to the analysis of mycotoxins in beer. Food Additive and Contaminants 28 (2011) 1438 Impact factor: 2.23*
10. *Survey and distribution of mycotoxins in European beers using different LC-MS analysers. Food control (Under review). Impact factor: 2.81*
11. *Applicability of hybrid linear ion trap-high resolution mass spectrometry and quadrupole-linear ion trap-mass spectrometry for mycotoxins analysis in baby food. Journal Chromatography A 1223 (2012) 84. Impact factor: 4.19*
12. *Rapid mycotoxins analysis in human urine. A pilot study. Food and Chemical Toxicology 49 (2011) 2299. Impact factor: 2.60*

Per la qual cosa, autoritzen la seva presentació per optar al Grau de Doctor en el programa de doctorat de Ciències de l'Alimentació.

Burjassot, Març 2012.

Dr. Jordi Mañes Dra. Carla Soler

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

- Anàlisis de micotoxines de *Fusarium* en matèries primes i aliments processats. Avaluació de les ingesta diàries (*CTQ2007/63186*).
- Avaluació del risc de micotoxines emergents de *Fusarium* (*AGL2010-17024/ALI*).

El doctorant agreix a la Universitat de València la beca d'investigació BI08-03 (2008-2012) i al Ministeri de Educació Espanyol l'ajuda de mobilitat (2010-2011) per la menció internacional del títol de Doctor.

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. Università degli Studi di Perugia.

0. Índex

ÍNDEX

1. Introducció general

1. INTRODUCCIÓ GENERAL

1.1 MICOTOXINES

El mot micotoxina és una paraula composta que prové de la unió de 2 paraules; del grec antic "*mykes*" que significa fong i del llatí "*toxicum"* verí.

Les micotoxines són productes naturals tòxics de baix pes molecular (Pm< 800) que provenen del metabolisme secundari dels fongs filamentosos, o floridures, que s'acumulen en diferents aliments abans, durant i després de la recol·lecció. Aquests metabòlits secundaris de fongs provenen normalment dels gèneres *Aspergillus*, *Penicillium* i *Fusarium*; però altres gèneres com *Claviceps* o *Alternaria* també poden produir aquestes toxines [1-6]. Aquests metabòlits secundaris dels fongs no són essencials per al fong i se produeixen normalment en condicions d'estrès entre el final de la fase de creixement i el principi de la fase estacionària del fong.

Al llarg de la història, *Claviceps* ha sigut el responsable de les micotoxicosis amb major impacte; l'ergotisme. Aquest és el cas de sègol contaminat per ergotamines, descrit en l'antiguitat com una banya negra i nociva per al consum. De fet, en les tabletes dels Assiris es va reflectir la presència de l'espiga de sègol nociva, al igual que els escrits del Perses en l'any 400 a. C. on detallaven que el seu consum per embarassades originava l'avortament o la mort tant de la mare com del fill. Pareix ser que els Romans van descriure la simptomatologia de l'ergotisme; gangrena, convulsions, pertorbacions mentals o embruixament eren alguns dels símptomes descrits per aquest poble [1-3].

Possiblement, el foc de Sant Antoni en l'edat mitja és un dels brots d'ergotisme més coneguts al llarg de la història, el qual va assolar l'Europa Occidental durant varis segles. Els motius d'aquest brot van ser fonamentalment

dos: la inclusió del sègol en Europa i la panificació amb qualsevol cereal panificable. El "pa maleït" o "pa negre" era una mescla de cereals amb sègol contaminat que va disminuir la qualitat de vida de centenars de miler de europeus [1-3].

Les micotoxines també són responsables del cas de les bruixes de Salem: en 1692 en Salem, actualment Danvers (Estats Units d'Amèrica), el comportament anòmal d'unes dones va activar el puritanisme dels seus ciutadans. Aquestes dones eren suposadament atacades per essers invisibles, paralitzant-se i destrossant-se els membres; tot açò feia suposar a la població que estaven posseïdes i embruixades. Les hipòtesis d'aquests fets són varies, encara que sembla que les micotoxines, contretament les ergotamines, van ser les responsables. Atacs de ràbia, sordesa, sensació de cremar-se internament o visions són alguns dels símptomes de l'ergotisme que van patir aquelles dones pel consum de sègol en mal estat [1, 2]. No hem d'oblidar la semblança estructural entre les ergotamines i els dietilamida d'àcid d-lisèrgic (LSD).

També en Rússia, on les condicions climatològiques afavoreixen el desenvolupament de *Claviceps*, van sofrir varies micotoxicosis causades per les ergotamines. El primer brot va ser descrit en 1785, però no va ser fins el 1832 quan les autoritats ho van relacionar amb la presència de micotoxines. En aquest mateix país, es va descriure la primera micotoxicosis per tricotecens, anomenada en anglès *Alimentary Toxic Aleukia* (ATA), i degut a la toxina T-2 produïda per *Fusarium sporptrichoide*s, que va arribar a una taxa de moralitat del 60% [1, 2].

No obstant això, no va ser fins la meitat del segle XX quan va aparèixer el interès per les micotoxines, més concretament, se van descobrir les aflatoxines. Des del seu descobriment se coneix el seu risc per la salut dels humans i dels animals;

de fet, l'aflatoxina B_1 esta classificada com un dels cancerígens més potents de la naturalesa [7].

Tots aquests antecedents històrics ens porten al que són avui en dia les micotoxines: un problema de seguretat alimentaria arreu del món [7-9]. A més la FAO/OMS considera que el 25% de les collites mundials estan contaminades de micotoxines [10].

A part del risc per als humans i els animals com prèviament hem comentat, hem de sumar-li les repercussions negatives en l'economia produïdes per les pèrdues en les collites. Per aquests motius les autoritats sanitàries recomanen intensificar els controls de micotoxines al llarg de la cadena alimentaria [9, 11].

1.2 LEGISLACIÓ

A principis del segle XXI, la Unió Europea, per protegir al consumidor front aquests contaminats, va implantar nous reglaments on s'establien nivells màxims per certes micotoxines. En l'actualitat el Reglament (EU) 1881/2006, de 19 desembre 2006, fixa el contingut màxim de determinats contaminants en productes alimentaris [12], encara que aquest reglament esta contínuament sofrint modificacions. En aquest reglament es va fixar el contingut màxim d'aflatoxines B_1, B_2, G_1, G_2 i M_1 en fruit secs, especies, cereals i derivats com per exemple els aliments infantils, a més de llet i preparats per lactants. També se va legislar el contingut màxim per l'ocratoxina A (OTA) en cereals i derivats, així com per vi, suc de raïm, panses i especies.

Altres toxines de *Fusarium* com són desoxinivalenol (DON), zearalenona (ZEN) i fumonisines B_1 i B_2, tenen també estipulats els continguts màxims per cereals i derivats principalment. Els continguts màxim de patulina (PAT) estan

establerts per sucs de fruites, productes derivats de les pomes, begudes fermentades i aliments infantils.

El Reglament (CE) 1881/2006 ha servit de base per les modificacions successives i any darrera any s'han introduït nous aliments o nous nivells màxims de micotoxines actualitzant-se al nous estudis que es porten a terme. Aquest és el cas de les aflatoxines, el límit de les quals va ser modificat en el Reglament (EU) 165/2010 [13], així com de l'OTA en el Reglament (EU) 105/2010 [14]. Les toxines de *Fusarium* no han sigut una excepció, perquè l'any 2007 va aparèixer el Reglament (CE) 1126/2007 per productes de dacsa i els seus derivats [15]. Per tant, tots els reglaments prèviament esmentats son esforços per garantir la seguretat alimentaria dels productes elaborats i consumits en la UE.

No obstant, la liberalització econòmica o globalització del mercat dels aliments, les polítiques agrícoles i econòmiques de la Unió Europea, a més de la dependència cada cop més gran dels aliments produïts en països del tercer món feia imprescindible una legislació Europea per controlar les importacions. Per solucionar aquesta mancança legislativa va aparèixer el Reglament (CE) 669/2009 [16], el qual va ser modificat en el 2010 per el Reglament (EU) 212/2010 [17]. Vist que la reglamentació prèviament descrita era molt general i poc específica per al camp de les micotoxines, la Unió Europea va decidir l'any 2009, amb el Reglament (CE) 1152/2009, establir condicions específiques per importar determinats productes d'alguns països amb risc de contaminació d'aflatoxines [18]. Així la EU va començar a controlar les importacions centrant-se amb les micotoxines de major risc toxicològic.

Una vegada s'han establert els límits màxims per un nombre determinat de micotoxines, s'ha d'unificar com calcular i quantificar aquests límits. Amb la finalitat de garantir els resultats i l'anàlisi de micotoxines la UE, mitjançant el

Reglament (CE) 401/2006, estableix el mostreig i l'anàlisi [19]. D'aquesta forma els resultats obtinguts per al control oficial de micotoxines en els productes alimentaris estava garantit. Aquest reglament se va reforçar amb el Reglament (UE) 178/2010 per alguns aliments específics com són els cacauets, llavors oleaginoses, fruits de crosta arboris, els pinyols del préssec, la regali i l'oli vegetal [20].

Normalitzats els límits màxims de micotoxines en aliments i regulats els procediments per obtenir aquests nivells, amb els resultats obtinguts es podrien estudiar les causes de la presència de micotoxines, i els punts més crítics per l'aparició de micotoxines. Així, la UE va publicar unes recomanacions preventives per evitar i reduir la presència de toxines de *Fusarium* en cereals i derivats (Recomanació 2006/583/CE) [21], així com per evitar i reduir la presència de PAT en suc de poma i en begudes a base de suc de poma (Recomanació 2003/598/CE) [22].

1.3 TOXICITAT

Les micotoxines posseeixen una gran variabilitat d'estructures químiques [5]. Una mostra d'aquestes diferències estructurals se representen en la ***figura 1***, on s'observen les estructures de zearalenone (ZEN), deoxinivalenol (DON) i beauvericin (BEA), totes elles produïdes per el mateix gènere de fong: *Fusarium*.

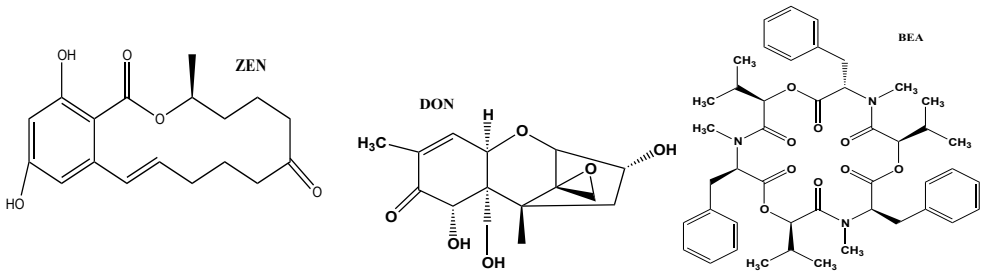

Figura 1. Estructures de les micotoxines: ZEN, DON i BEA.

Degut a la diversitat estructural, la seua toxicitat varia significativament i açò ha quedat reflectit en la classificació de "International Agency for Research on Cancer" (IARC) [23]. Aquesta organització ha classificat les micotoxines des del grup 1 fins al grup 3, o el que és el mateix, des de cancerígens com la AFB_1 (grup 1) fins a no classificat com a cancerigen, com per exemple el DON (grup 3).

Els fongs productors de micotoxines o micotoxigènics necessiten unes condicions propícies de temperatura, activitat d'aigua, humitat, oxigen, pH i substrat per proliferar, formar colònies i produir micotoxines [5, 6]. Totes aquestes circumstàncies poden donar-se durant la producció, el transport i/o l'emmagatzemament o inclús en el processat del producte alimentari, ja que moltes micotoxines poden suportar els processos tèrmics. A més les condicions ecològiques i geogràfiques en les que s'ha de desenvolupar cada gènere toxigènic pot condicionar la producció de diferents tipus de micotoxines, bé de forma individual o bé simultània [24].

Avui en dia, les micotoxines representen una de les majors preocupacions en la UE, per dalt dels additius alimentaris, els plaguicides o els residus veterinaris, degut als seus efectes a llarg termini. Esta demostrat que a curt termini les micotoxines tenen un risc baix, en canvi a llarg termini les micotoxines tenen un elevat risc per la salut, per la seua ingesta continuada al ser contaminants naturals presents en aliments bàsics.

En Europa predominen les toxines de *Fusarium* degut a les condicions climatològiques que afavoreixen el creixement de fongs d'aquest gènere, encara que podem trobar altres [24]. Tricotecens de tipus A i B, zearalenona (ZEN), fumonisines i micotoxines emergents de *Fusarium* són les principals grups de micotoxines del gènere *Fusarium*:

- Deoxinivalenol (DON), també coneguda com vomitoxina, pertany al grup de tricotecens del tipus B. A nivell molecular, DON interromp la funció normal de la cèl·lula per la inhibició de la síntesi de proteïnes mitjançant la unió al ribosoma i mitjançant l'activació de les quinases implicades en la transducció de senyals relacionades amb la proliferació, diferenciació i apoptosi cel·lular. En relació a la toxicitat, hi ha marcades diferències en la susceptibilitat de diferents espècies animals, per exemple els porcs són més sensibles al DON. Pel que fa als efectes crònics, el DON afecta el creixement, la funció immune i la reproducció en diferents espècies [25]. Recentment s'ha observat que el DON pot conjugar-se o emmascarar-se al unir-se amb altres compostos polars, com són els aminoàcids, els sucres o el sulfats. Aquest procés de conjugació és un mecanisme de defensa o descontaminació de les plantes. Un dels compostos més comú és el DON-3-Glucòsid (D3G), d'aquest contaminat emergent s'ha estudiat principalment la seua formació en diferents processos tecnològics tèrmics i no tèrmics, encara que també s'ha estudiat sobre la matèria primera. De moment, se desconeixen el risc d'aquestes micotoxines emmascarades [26, 27].
- DON pot detectar-se simultàniament amb Nivalenol (NIV) i 3 i 15-acetil-deoxinivalenol (3-ADON, 15-ADON). Depenen de l'origen geogràfic de la soca se poden produir de forma individual o simultània. DON i NIV tenen una major repercussió a nivell toxicològic, seguits per 15-ADON i 3-ADON, respectivament.
- Fusarenon X és immunosupressor, carcinogen, citotòxic, emètic, origina diarrea i hipotèrmia. Després de ser absorbit, se transforma en NIV, i d'aquesta forma s'excreta per l'orina [28].

- Les Toxines T-2 i HT-2 pertanyen als tricotecens no macrocíclics, normalment anomenats tricotecens tipus A. Les principals especies productores d'aquestes toxines poden produir-les en un ampli rang de temperatures (7-25ºC). El principal efecte advers de les toxines T-2 i HT-2, i també d'altres tricotecens, és un fort efecte citotòxic sobre les cèl·lules en divisió, com les que recobreixen el tracte gastrointestinal, pell, cèl·lules limfoides i hematopoètiques. A més de la seva activitat citotòxica, la toxina T-2 té un efecte immunosupressor que es tradueix en la disminució de la resistència als microbis infecciosos [29]. Pot disminuir els nivells d'anticossos, immunoglobulines i alguns altres factors humorals com les citocines. Les manifestacions de la malaltia són signes de problemes hematològics, pèrdua de pes o guany escàs de pes, diarrea amb sang, necrosi cutània o de bec i lesions de la boca, hemorràgies i disminució de la producció de llet i ous.
- Diacetoxiscirpenol (DAS) és un tricotecen de tipus A. Se considera carcinogen, dermatòxic i fitotòxic. A més disminueix els paràmetres zootècnics (pes, grandària...) i provoca lesions en moltes aus [30].
- Neosolaniol (NEO) origina degeneració cel·lular i cariorrexis en cèl·lules, nòduls limfàtics, melsa, medul·la òssia, intestí i testicles [5].
- La Zearalenona (ZEN) s'uneix als receptors de l'estrogen i pot donar lloc a canvis hormonals. Aquesta toxina està freqüentment implicada en trastorns de la reproducció d'animals de granja i de tant en tant en els síndromes hiperestrogènics en éssers humans. Hi ha proves que tant ZEN com els seus metabòlits tenen activitat estrogènica en els porcs, vaques, ovelles i humans. No obstant això, la ZEN és d'una toxicitat aguda relativament baixa. Aquesta micotoxina ha demostrat ser hepatotòxica, hematotóxica, inmunotóxica i genotòxica [31].

- Fumonisines afecten principalment al blat de moro, encara que s'ha trobat algun esdeveniment en altres productes bàsics com el sorgo i l'arròs. Les fumonisines inhibeixen la ceramida sintasa, causant acumulació d'intermediaris bioactius del metabolisme d'esfingolípids (esfingosina i altres bases esfingoides i derivats), així com l'esgotament dels esfingolípids complexos, que interfereix amb la funció d'algunes proteïnes de membrana. Les fumonisines afecten els animals de diferents maneres al interferir en el metabolisme d'esfingolípids, causant leucoencefalomalacia (forat en la síndrome del cap) a equins i conills; edema pulmonar i hidrotòrax en els porcs, i efectes hepatotòxics, carcinògens i l'apoptosi en el fetge de les rates. En els éssers humans, hi ha un probable vincle amb el càncer d'esòfag, defectes del tub neural i efectes inmunotòxics [32].

Les micotoxines esmentades fins ara són les micotoxines produïes pel gènere *Fusarium* amb major incidència, no obstant això en els últims anys s'ha demostrat que aquest gènere pot produir altres micotoxines, anomenades micotoxines "emergents". Les dades sobre la toxicitat, l'aparició i els nivells de contaminació d'aquestes micotoxines són encara limitats. Per exemple:

- Beauvericin (BEA) és un hexadepsipeptide cíclic que mostra activitat antimicrobiana, insecticida, citotòxica i promotora de l'apòptosi. És l'inhibidor específic més potent de colesterol aciltransferasa. BEA augmenta la permeabilitat de ions en les membranes biològiques formant un complex amb cations essencials (Ca^{2+}, Na^{+}, K^{+}) que pot afectar l'homeòstasi iònica. El paper de BEA en el desenvolupament de micotoxicosis humana i animal és encara desconeguda [33].
- Enniatins (ENs) han estat descrits com compostos amb activitat antibiòtica i insecticida. A més, els enniatins inhibeixen l'enzim acil-CoA: colesterol

acil transferasa. La capacitat de l'enniatin per formar complexos amb ions de metalls alcalins i augmentar la permeabilitat de les membranes catiòniques també ha estat documentada en una sèrie de treballs [33].

Però com hem dit abans, en Europa i arreu del món poden créixer altres gèneres de fongs, de fet, i com hem vist en la introducció, *Claviceps* és un dels gèneres amb major repercussió històrica, degut a que els episodis d'ergotisme han afectat a varies civilitzacions. Els fongs del gènere *Claviceps* proliferen a les gramínies salvatges o domesticades produint normalment els ergo alcaloides o també anomenats els compostos de la banya negra del sègol.

- Les ergo alcaloides o ergotamines són ergo pèptids que formen part de la família dels alcaloides. La seva estructura molecular és molt semblant a l'ergolina, per tant els neurotransmissors cerebrals es veuen afectats a nivell del sistema nerviós central de l'ésser humà. A dosis baixes son vasoconstrictores, encara que a dosis prou altes presenten activitat al·lucinògena i a grans dosis resulta letal [35].

Les zones més càlides i amb més humitat són un entorn més favorable per al creixement del gènere *Aspergillus*, concretament per les especies *Aspergillus flavus*, *A. parasiticus* i menys freqüent per *A. Nònius*. Les micotoxines produïdes per aquest gènere més conegut són les aflatoxines i l'ocratoxina A que se produeixen normalment en condicions inadequades d'emmagatzemament.

- Les aflatoxines més conegudes són B_1, B_2, G_1, G_2, M_1. Aquests metabòlits secundaris produeixen efectes aguts i crònics sobre els animals i els humans. A part dels efectes tòxics aguts com poden ser vòmits, diarrea i hepatitis aguda, els efectes més preocupants són de caràcter immunosupressor, mutagènic, teratogènic i cancerigen. El principal òrgan diana dels efectes tòxics i cancerígens de les aflatoxines és el fetge. No

oblidem que AFB_1 esta considerada con un dels compostos cancerígens més potents de la natura [29, 35].

- L'esterigmatocistina (STER) es un precursor de les aflatoxines. Ha estat relacionada amb carcinomes gàstrics, hepàtics i esofàgics [1].
- L'ocratoxina A (OTA) és una micotoxina produïda de manera natural per certes espècies de fongs com *Penicillinum* i *Aspergillus* durant la fase d'emmagatzemament encara que ocasionament es pot desenvolupar durant la fase de cultiu. Els efectes toxicològics que presenta OTA són: carcinogènesis, nefrotòxicitat, teratogènesis, immunotoxicitat i probablement neurotoxicitat [36].
- A més d'*Aspergillus* i *Penicillium* també *Claviceps* pot generar Penitrem A. Aquesta neurotoxina pareix ser que inhibeix els canals de potassi i relaxa la musculatura [37].

Per altra banda, les espècies d'*Alternaria* constitueixen un dels principals patògens de les plantes. En els humans són al·lèrgens i donen la febre del fenc o hipersensibilitat que de vegades ocasiona l'asma. Se calcula que com a mínim el 20% dels danys en l'agricultura els produeixen els fongs d'*Alternaria*. A més, moltes malalties humanes també les causa aquest gènere afectant la pell, la membrana de la mucosa i fins i tot els globus oculars i el tracte respiratori. Poden produir una gran varietat de compostos tòxics, com per exemple:

- Altenuene i Alternariol, els quals són mutàgens i carcinògens, però posseeixen una dèbil toxicitat aguda [38].

En conclusió, és important assenyalar que la majoria dels estudis de toxicitat realitzats, estudien una micotoxina de forma particular. No obstant, un mateix gènere pot produir varies micotoxines simultàniament. Els efectes toxicològics derivats de l'associació de varies micotoxines són encara desconeguts.

Les dades resultants dels estudis analítics de co-contaminació podrien contribuir a canvis en la normativa vigent i a estipular nivell màxims més baixos, si es demostren sinergies.

1.4 MICOTOXINES EN ALIMENTS

Els aliments se poden classificar segons el seu origen, diferenciant entre origen animal i origen vegetal. Les micotoxines se generen de forma directa sobre els aliments d'origen vegetal com poden ser els cereals i els seus derivats, així com les fruites i verdures. Però poden arribar als aliments d'origen animal de forma indirecta, quan els animals han consumit aliments contaminats amb micotoxines poden arribar al consumidor final. La carn, els ous, la llet o qualsevol derivat d'origen animal també pot presentar micotoxines o bé els seus metabòlits [1].

Les aflatoxines, les micotoxines amb major risc toxicològic, s'han detectat en qualsevol punt de la cadena alimentaria. Els fongs aflatoxigènics proliferen en cereals o llavors en mal estat a elevada temperatura i humitat. Normalment, cereals com la dacsa, l'arròs, el blat i els seus derivats són les fonts més comuns d'aflatoxines. Una altra font d'aflatoxines són els fruits secs, principalment importats de països càlids de fora de la UE en condicions de transport poc adient.

Però també, s'ha demostrat la presència de AFB_1 en els productes d'origen animal com ous, carn i sang. No obstant això, la taxa de transferència del pinso fins al producte càrnic es molt baixa. Per contra, la presència del metabòlit de l'AFB_1, la AFM_1, s'ha trobat en llet i els seus derivats, per aquest motiu les autoritats sanitàries s'han centrat en aquest metabòlit com a marcador de l'exposició a micotoxines en productes d'origen animal.

Durant l'última dècada l'OTA és una de les micotoxines més analitzades en cereals i derivats, begudes fermentades i cafè. Aquesta micotoxina es generada pels fongs en multitud de cereals: blat, dacsa, arròs, ordi, civada i sègol.

Les toxines de *Fusarium* són les micotoxines més controlades i estudiades, per exemple se coneix que les fumonisines se localitzen fonamentalment en dacsa i derivats. Hi ha dues hipòtesis al respecte d'aquesta circumstància; la primera d'elles fa referència als fongs de *Fusarium* que són més comuns degut a les condicions climatològiques en la dacsa que en altres cereals. En canvi la segona teoria responsabilitza algun component de la dacsa, el qual afavoreix la síntesi de les fumonisines.

Per altra banda, la ZEN és una altra de les toxines de *Fusarium* que afecta a cereals i derivats com el blat, la dacsa i els seus derivats com els pinsos, a més s'ha aïllat simultàniament, junt amb altres micotoxines com els tricotecens.

Generalment, els tricotecens de tipus A i B se troben en blat i derivats del blat, però també en la dacsa, l'ordi i la civada s'ha demostrat la seva presència.

Les denominades "micotoxines emergents", BEA i ENs, s'han trobat i estudiat sobretot en cereals que serveixen de matèria primera per altres aliments com cereals de desdejuni, aperitius, galetes o aliments infantils.

Les toxines d'*Alternaria* tenen una distribució completament diferent: la tomata, les olives o fruites com la poma, a més dels cereals poden contenir aquestes micotoxines.

Les toxines de la banya del sègol se localitzen en el sègol, i en molta menor quantitat en el blat. Aquestes micotoxines no s'han trobat fins ara en productes d'origen animal.

1.5 IMPACTE DE LES MICOTOXINES

Hi ha múltiples formes d'avaluar l'impacte econòmic de les micotoxines en els éssers humans, en els animals i en l'agricultura. Però, simplement considerant els efectes en la salut humana, junt a les pèrdues de producció de collites i bestiar, els costos són sempre molt elevats.

Arreu del món la contaminació d'aliments amb micotoxines és un problema significatiu. Diversos estudis han demostrat que de forma global les micotoxines afecten a tot els països productors, augmentant les seues pèrdues econòmiques i constituint un problema de salut pública [39].

Per exemple, un article recent ha formulat el càlcul de l'impacte econòmic de les fumonisines en la dacsa mitjançant un model matemàtic [40]. En aquest estudi pilot, s'agafa com a referència la legislació americana per fumonisines aprovada per la FDA (United States FDA guidelines). Estudiant totes les dades publicades relacionades amb la contaminació de fumonisines en la dacsa durant llargs períodes de temps, la producció anual de dacsa, els percentatges de collites rebutjades i les mortalitats de cavalls, van concloure que per un any normal, un any amb poca incidència de *Fusarium* les pèrdues estarien entre 1-20 milions de dòlars. En canvi, per un any amb elevada incidència de *Fusarium* les pèrdues estarien entre 30-46 milions de dòlars. Hem de tenir present que aquest impacte econòmic teòric sols afectaria als Estats Units d'Amèrica. Per tant, al considerar la resta de països productors de dacsa arreu del món, la repercussió econòmica seria molt més elevada.

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2. Metodologia

2. METODOLOGIA

Each group or type of mycotoxin has a different chemical structure, and their toxic proprieties are related with their structure. The structural variability between mycotoxins is commonly a great problem in their extraction and detection. For this reason, multi-mycotoxins extractions are difficult and tedious, and the simultaneous detection is complicated [1]. With the aim to optimise an extraction method that allowed the simultaneous extraction of all selected mycotoxins reducing time-analysis, solvent amount and reaching the validation values required, in this thesis several extractions procedures have been checked.

The classical extraction methods were the starting point in this work since they have been widely selected and improved for multi-mycotoxin extractions [1-3]. For example, the classical liquid-liquid extraction (LLE), as well as solid-liquid extraction (SLE) have been commonly applied in different food matrices. Both procedures, LLE and SLE, are usually used as preliminary extraction. Solid-phase extraction (SPE) and immunoaffinity columns (IAC) are two clean-up procedures widely applied as clean-up methods owing to its selectivity [1-3].

On the other hand, recent and attractive alternatives, such as matrix solid-phase dispersion (MSPD) and QuEChERS have been evaluated. Both extraction methods have been commonly applied to organic contaminants as pesticides or antibiotics, but they have been scarcely used to mycotoxin extractions [4, 5]. Both techniques present some advantages, such as the extraction and clean-up are in one step, as well as the time consuming and the cost are lower than other methodologies.

The selection of extraction procedure has to be joined to a reliable detection method, which has to be able to identify target mycotoxins at ppb levels

[1-3]. The last decade, liquid chromatography (LC) coupled to mass spectrometry (MS) is the technique of choice, since LC-MS shows selectivity, sensitivity and it is a powerful tool to identify unambiguously mycotoxins from foodstuff.

During last decade, the triple quadrupole instruments have been commonly used in mycotoxins analysis. Triple quadrupole (MS/MS) permits higher selectivity, better quantification, more reliable identification, better accuracy and reproducibility than simple quadrupole or other detectors.

Nowadays, there are several important trends in mass spectrometry detectors:

- The first one, high-resolution mass detectors (HRMS); time-of-flight (TOF) or quadrupole-TOF (QTOF), as well as the last technology, Orbitrap® MS, they have been gradually applied to identify target, non-target and unknown mycotoxins from different foods. The high-resolution mass spectrometry could reach accurate mass, therefore, unambiguous identification for target mycotoxins. Furthermore, these instruments allow a retrospective data analysis, which mean that from Full-Scan, the extracted ion chromatogram of a specific analyte could be processed after the chromatogram has been acquired.
- The second one, hybrid instruments; each particular instrument provides reliable and accurate data acquired, but combining two instruments, the quality data acquired could be improved. The use of hybrid instruments could therefore overcome several drawbacks and reach the requirements and robustness data required. For this reason the applicability of hybrids instruments could complement mycotoxins analysis in foodstuff.

2.1 EXTRACTION PROCEDURES

2.1.1 SOLID-LIQUID EXTRACTION (SLE)

Mycotoxins can be present a long the food chain: since the harvest to life stocks. However, one of the most critical step in food industry is the raw material used in a process. For this reason, most of the literature is focused on the study of cereal grain and SLE procedure is the common extraction applied for mycotoxin analysis. This classical extraction needs a solvent or mixtures of them, which interact with solid matrix extracting mycotoxins from food. Matrix and solvent are continuously shaken during fixed time; in this form the interactions are improved and the final recoveries are higher.

The most common used solvents are water, methanol and acetonitrile. Moreover, in order to increase the interactions between the solvent and mycotoxins, some modifiers, such as acids or salts could be used. SLE consists in mixing an amount of sample with the extraction solvent, which is usually acetonitrile/water (80:20, v/v), and placed the mixture in the horizontal shaker during a time [6, 7]. Then an aliquot of the organic solvent is diluted and filtered, a prior to be injected.

In this form, the major of legislated mycotoxins are extracted. Little variations have been used for extraction solvent in others works, as acetonitrile/water (84:16, v/v) or acetonitrile/water (85:15, v/v), showing similar analytical parameters [8, 9]. Acid modifiers like formic and acetic acids are also used for SLE procedures [10-12]. ***Figure 1*** shows SPE workflow.

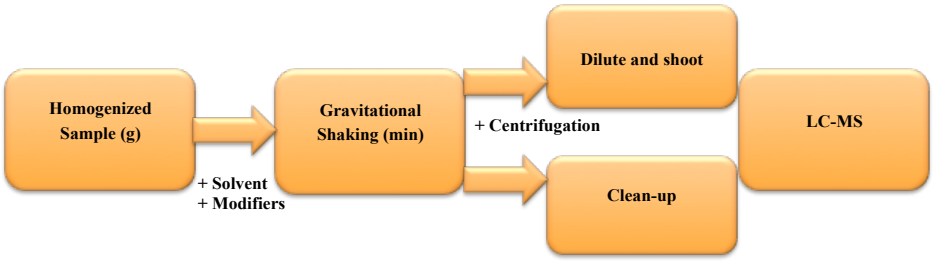


Figure 1. SLE workflow.

2.1.2 LIQUID-LIQUID EXTRACTION (LLE)

LLE is used to separate compounds based on their relative solubility in two different immiscible liquids, usually water and an organic solvent.

Most often, the matrix is mixed with a solvent by shaken during a fixed time. The final homogenized extract could be concentrated or directly injected. LLE is commonly used for analysing processed liquids products in mycotoxins field as beer, wine or animal products like milk.

The solvent election is vital, since mycotoxins must be completely recovered by solvent. As an example, mycotoxins have been extracted from beer using acetonitrile [13].

2.1.3 SOLID-PHASE EXTRACTION METHODS

Solid-phase extraction (SPE) or immunoaffinity columns (IAC) are a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties using cartridges that contain solid phases. Different steps as conditioning columns, loading of the sample, washing non-desirable compounds and elution of the analytes of interest are necessaries. ***Figure 2*** shows a clean-up workflow.

IACs contain antibodies that selectively bind the toxin of interest from the crude extract. IACs have commonly used as clean-up methods, due to obtained some advantages, as lower matrix effects and higher recoveries than other procedures. These advantages are related with the high specificity of these columns. During the clean-up step other interferences of the matrix can be washed from the column while the toxin is immobilized on the column. In the last step, the toxin is eluted from the column. Using this highly specific technology, almost all clean-up related issues can be solved. As an example, this clean up has been applied to determinate mycotoxins in infant milk and baby food demonstrating its applicability in liquid and solid matrixes [14]. Unfortunately, they have higher cost than other techniques as SPE, as well as these columns are limited to the number of compounds extracted.

Regarding SPE columns, they are able to separate target compounds from other compounds in the mixture according to their physical and chemical properties using different solid materials [15]. The extraction procedure is similar to that explained above and the main advantages are decreasing matrix effects and lower cost. SPE columns have been applied to different food, such as the analysis of mycotoxins in beer [16, 17] or cereal-based food [18].

On the other hand, MycoSep® columns allow in one-step clean-up. The interferences are adhered to the chemical packing in the column and the purified extract, containing the analytes of interest, passes through a membrane to the surface of the column [19]. The clean-up is faster than SPE or IAC, but the number of analytes are limited and depends to the column.

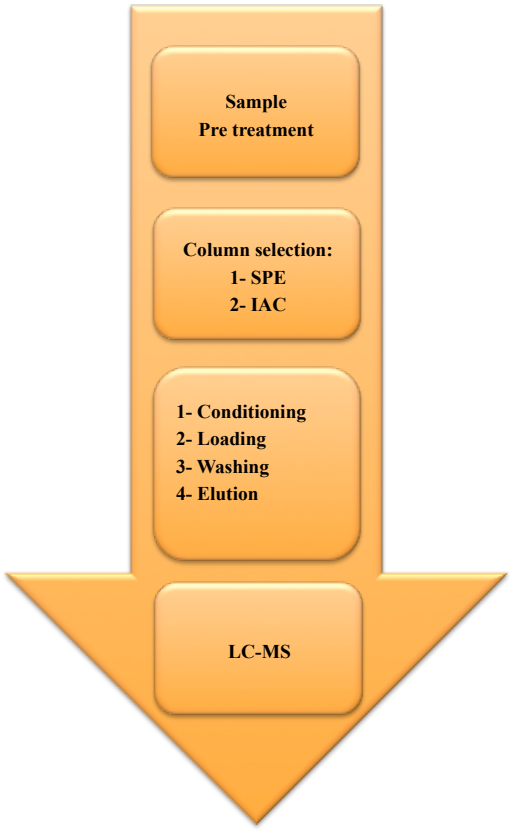


Figure 2. Clean-up workflow.

2.1.4 QUECHERS

Michelangelo Anastassiades developed the QuEChERS-method in the years 2001 and 2002. This methodology was developed for the analysis of veterinary drugs (anthelmintics and thyreostats) in animal tissues, and after the method was applied for the analysis of pesticide residues in plant material that was presented in June 2002 at the EPRW 2002 in Rome. The detailed method was published in 2003 [19].

The QuEChERS method is a two-step process: extraction followed by clean-up. The extraction step products are (i) MgSO4, to reduce water in the sample, along with either (ii) NaCl or anhydrous sodium acetate. The extraction step products are supplied in a 50mL polypropylene centrifuge tube for convenient extractions. The clean-up product used is PSA (primary/secondary amine) for the removal of organic acids and polar pigments, among other compounds: some products couple the PSA with endcapped C_{18} for the removal of lipids and sterols. ***Figure 3*** summarizes QuEChERS procedure.

QuEChERS offers the advantages of high recoveries, accurate results and high sample throughput, low solvent and glassware usage, as well as less labour and bench space and lower reagent costs.

Nowadays, QuEChERS is applied to different organic contaminants, such as pesticides, abuse drugs, pharmaceutical compounds, plant toxins and mycotoxins. In any contaminant, the selection of a suitable extraction solvent is the first challenge in this method development. Focus on mycotoxin analysis, it was firtly used for trichothecenes in wheat flour [20]. Mixture of several solvents, such as dichloromethane, ethyl acetate, acetonitrile, methanol, methanol/acetonitrile, methanol/acetonitrile/water and methanol/water, were tested for the extraction of selected mycotoxins. Recoveries ranged from 86 to 108% for five trichothecenes, were obtained with the mixture methanol/acetonitrile (85/15, v/v).

Step by step the number of mycotoxins has been increased since to determine 11 major *Fusarium* toxins in cereals and cereal-based products [5, 21]. The use of modified QuEChERS has allowed reaching acceptable recoveries.

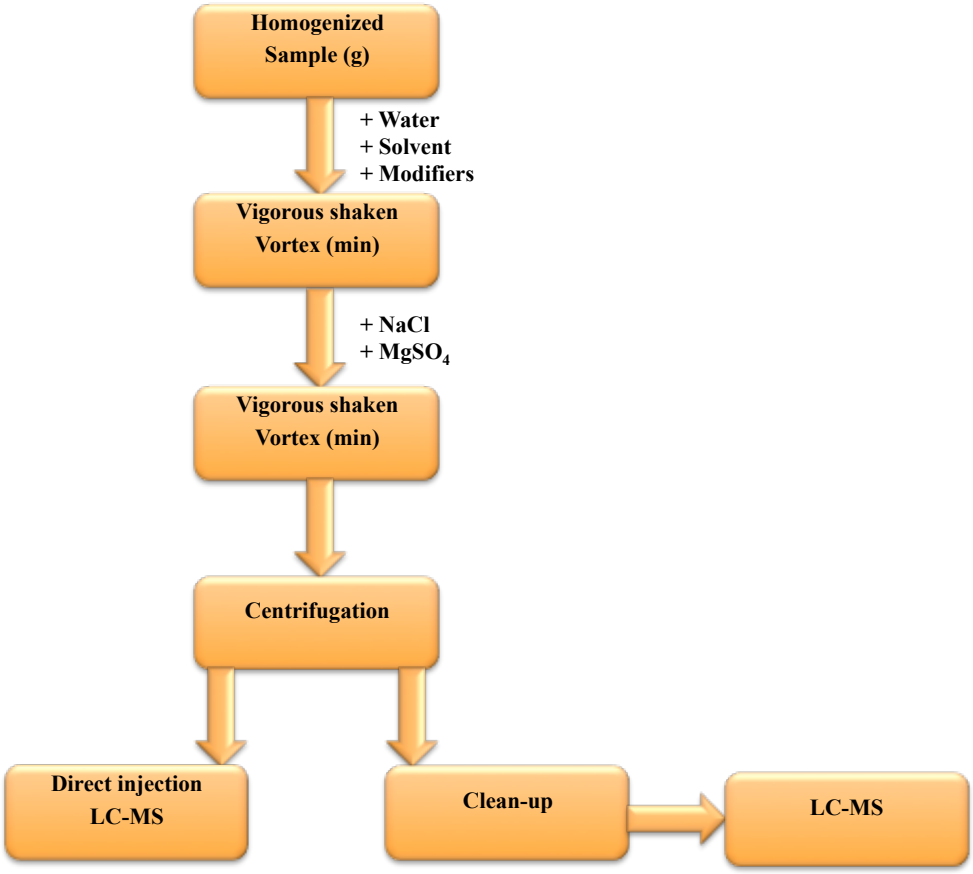


Figure 3. Modified QuEChERS workflow.

2.1.5 MATRIX SOLID-PHASE DISPERSION (MSPD)

MSPD unify extraction and clean-up in one step. It is due to MSPD is a distinct extraction for sample disruption and dispersion of semi-solid and solid samples over a bonded-phase solid support that may subsequently be used as column packing. Besides this, MSPD possesses many of the characteristics of other methods. Therefore, it possesses a unique characteristic able to provide a dimension of sample fractionation making MSPD unique.

Efficiency of MSPD extraction depends on type and quantity of dispersing phase, amount of sample and nature and volume of eluting solvents [4]. For validation studies of the extraction, the most suitable elution solvents and the polarity of solid-phase have to be assessed. ***Figure 4*** describes MSPD extraction procedure.

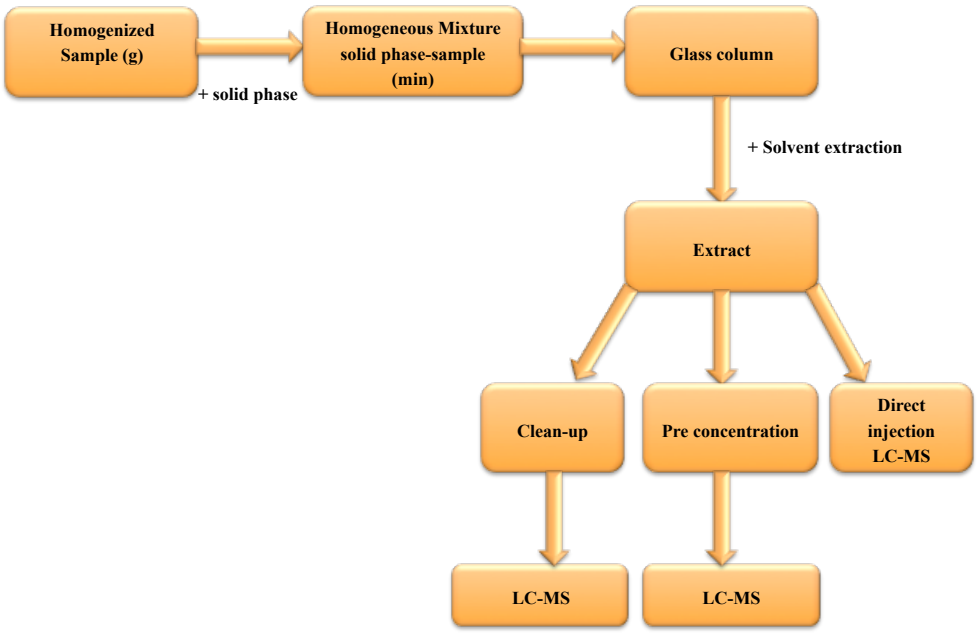


Figure 4. MSPD workflow.

2.2 SEPARATION AND DETECTION METHODS

2.2.1 SCREENING METHODS

Screening methods are very important for the early detection of mycotoxins in food and feed [1]. Usually, they are qualitative tests that only evidence the presence or absence of target analyte, but there are some tests, which can be semi-quantitative. The advantages of screening tests include the speed of detection and simplicity of the sample preparation. Even though there are some

drawbacks to such procedures as very often false-positive results could be reported [1-3].

These tests have not been applied in our research owing to its low possibilities for multi-mycotoxin analysis: all the screening studies carried out on mycotoxins analysis are focused in one mycotoxin.

2.2.2 GAS CHROMATOGRAPHY (GC)

GC has been scarcely used for mycotoxins analysis in foodstuff [22, 23]. Most often, GC has been linked to MS detector [1-3]. The problem is that normally mycotoxins are not volatile and have to be derivatised for analysis by GC [24]. For example, Cunha et al., developed a method to detect PAT in foodstuff including puree baby food [25]. However, Lombaert et al., optimized several methods to detect trichothecenes, OTA, ZEN, fumonisins and ergot alkaloids in infant cereal food [26]. The authors showed that only trichothecenes could be derivatizated and analyzed using GC-MS. The rest of mycotoxins; such OTA, fumonisins, ZEN and ergot alkaloids could not be directly determined by GC, therefore, they were efficiently analysed by liquid chromatography.

2.2.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (LC)

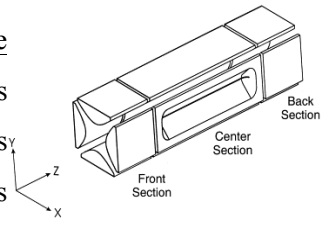
During the last decade, LC has commonly been used for mycotoxin analysis [1-3]. Step by step, HPLC have demonstrated to be cheaper and faster than other separation techniques, as well as, the best option to develop multi-mycotoxin methods linked to different detectors [1-3].

HPLC methods were initially developed using detectors as fluorescence (FLD), ultraviolet (UV) or diodearray (DAD). However, most of these procedures required derivatization before the analysis increasing the time and cost of the

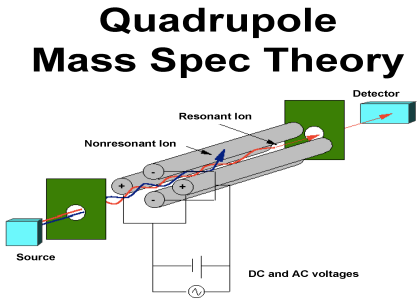
analysis, as well as providing an unreliable identification comparing with mass detectors.

Liquid chromatography mass spectrometry (LC-MS) has demonstrated to be the most reliable tool for mycotoxin analysis in foods because of it can quantify and qualify mycotoxins unambiguously [1-3]. The most common mass spectrometers detectors are simple quadrupole (Q), linear ion trap (LIT), triple quadrupole (QqQ) and high-resolution mass spectrometry (HRMS). As it has been commented before, hybrids instruments combine the advantages of two instruments in only one, improving the identification and quantification of the compounds [27].

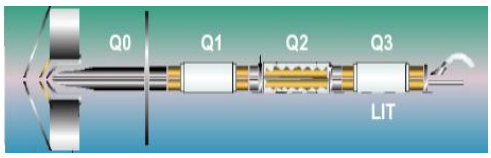
- Linear ion traps are rapidly finding new applications in many areas of mass spectrometry. In a linear ion trap, ions are confined radially by a two-dimensional (2D) radio frequency (RF) field, and axially by stopping potentials applied to end electrodes. Their use is not limited to simply storing ions. They can be combined with other mass analyzers in hybrid instruments and used to isolate ions of selected mass to charge ratios, to perform tandem mass spectrometry experiments, and to study ion-molecule chemistry.
- Quadrupoles are mass analyzers, which consist of four rods with DC and RF voltages applied. An ion of a specific mass-to-charge ratio (m/z) will be stable and can pass through the quadrupole only when a specific DC/RF voltage combination is applied. Quadrupoles are therefore called mass filters. Single quadrupole systems contain only one mass filtering quadrupole (Q), while triple quadrupole systems consist of three quadrupoles (QqQ). Q1 and Q3 are working as mass filters while Q2 is acting as collision cell. Quadrupoles



can be used in scanning or filtering mode. During a mass scan, DC and RF voltages are ramped resulting in the acquisition of full scan mass spectra. Such spectra are typically used for qualitative data analysis. However, scanning a quadrupole suffers from low sensitivity and slow scan speed. Thus, quantitative studies are performed with quadrupoles working in filtering mode.

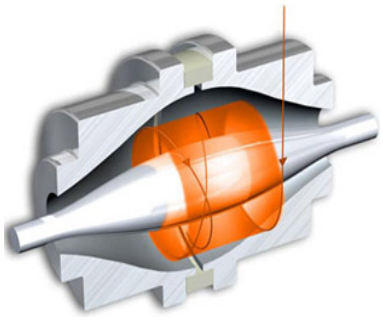


- The system triple quadrupole linear ion trap (QTRAP®) is based on a triple quadrupole platform where Q_3 can be operated either in the normal RF/DC mode or in the LIT mode. In the LIT mode, the trapped ions are ejected axially in a mass-selective fashion using fringe field effects and detected by the standard detector of the system [28]. Summarizing EPI scan, QTRAP® product ion mass spectra are generated using Q_1 as a resolving RF/DC transmission quadrupole to select the precursor ion of interest. This precursor ion is then accelerated into the pressurized collision cell inducing fragmentation and the resulting fragment and residual precursor ions are transmitted into the Q_3 linear ion trap (LIT)



where they are mass selectively scanned out toward the detector while the Q_3 LIT is performing the mass scan ions can be accumulated in Q_0 further enhancing instrument duty cycle.

- The Orbitrap® technology has been recently developed using the MS-only instrument named Exactive, which was initially developed for screening applications, however this mass spectrometer proves usefulness of high resolution and high mass accuracy also for quantitation. On the other hand, the latest implementation of the Orbitrap analyser is linear ion trap-high resolution mass spectrometry (LTQ-Orbitrap®). Hybrid linear ion trap-high resolution mass spectrometry or LTQ-ORBITRAP® combines Orbitrap analyzer with an external accumulation device such as a linear ion trap, making possible multiple levels of fragmentation (MS^n) for the elucidation of analyte structure. The use of the LTQ Orbitrap allows high-quality accurate mass and acquisition of MS^n spectra [29].



2.2.3.1 LIQUID CHROMATOGRAPHY TRIPLE QUADRUPOLE (LC-MS/MS)

HPLC-MS/MS is designed for the most demanding quantitative applications. This technique, working in Selected Reaction Monitoring (SRM) or Multiple Reaction Monitoring (MRM), consists of a series of short experiments in which one precursor ion and one characteristic fragment ion are selected by MS and MS^2. SRM or MRM are used for the detection of a specific analyte with known fragmentation proprieties in complex samples. It is accurate, robust and

reproducible tool for qualifying and measuring. During last decade, HPLC-QqQ is the wide-used detector for mycotoxin analysis [1-3].

There are different ionization sources, but Electrospray (ESI) is commonly the most important ionization techniques for the coupling of liquid chromatography mass spectrometers. The ESI works transforming the liquid (mobile phase), which contains the analytes of interest, into a fine aerosol. Because the ion formation involves extensive solvent evaporation, the typical solvents for electrospray ionization are prepared by mixing water with volatile organic compounds, such as methanol and acetonitrile. The aerosol is sampled into the first vacuum stage of mass spectrometer through a capillary, which can be heated to aid further solvent evaporation from the charged droplets.

On the other hand, atmospheric-pressure chemical ionization (APCI) is an ionization method used also in mass spectrometry. APCI allows to use directly high flow rates, often without diverting the larger fraction of volume to waste. Typically the mobile phase containing eluting analytes is heated to relatively high temperatures (350-400º C), sprayed with high flow rates of nitrogen and the entire aerosol cloud is subjected to a corona discharge that creates ions. Mycotoxins can be ionised by electrospray (ESI) and atmospheric pressure chemical ionization (APCI), usually, triple quadrupole analysers are able to ionise in both modes; therefore, the election depends of target compounds or instrumental sensitivity. The analytical methods have been optimised in both modes, but ESI is commonly more used than APCI [1-3].

This detector has been used for the validation of different extraction procedure, as well as it has been used to control the presence of mycotoxins in raw materials as tiger-nuts and processed foods as tiger-nuts beverage and beer. Owing

to its sensitivity and repeatability, it was used to evaluate different calibration approaches for effective quantification of trichothecenes.

2.2.3.2 LIQUID CHROMATOGRAPHY TRIPLE QUADRUPOLE LINEAR ION TRAP (LC-QTRAP®)

Triple quadrupole-linear ion trap-mass spectrometer or QTRAP® was born in the last decade; this hybrid instrument is a triple quadrupole (QqQ) in which the last quadrupole is replaced by a linear ion trap (LIT). This instrument combines both detectors, thereby, the LIT is capable of 3 levels of fragmentation (MS^3) with high sensitivity scan. The instrument is able to operate like a triple quadrupole or hybrid running, such as information dependent acquisition (IDA) method. Combining a triple quadrupole scanning functionality with sensitive linear ion trap scan, the analysis time can be reduced and better information from every experiment can be obtained.

However, QTRAP® instrument has been usually used as triple quadrupole for mycotoxins analysis [20, 21]. The analytical developed methods had basically confirmatory purposes, fulfilling Commission Decision 2002/657/EC. The methods

had several advantages as they were rapid, accurate and selective working in triple quadrupole mode only, but the possibilities of hybrid mode has not been normally explored.

In our research, QTRAP® has been applied for routine analysis in tiger-nuts. On the other hand, QTRAP® has been evaluated and applied for baby food and urine; in these cases, hybrid acquisition has been evaluated. Furthermore, the applicability of this hybrid instrument has been compared with other hybrid instrument.

2.2.3.4 ULTRA HIGH-PREASSURE LIQUID CHROMATOGRAPHY ORBITRAP® MS TECHNOLOGY (UHPLC-ORBITRAP®)

During The last decade, the routine analysis of mycotoxins has focused on target compounds. However, the analysis of target mycotoxins is not enough, since non-target compounds, such as metabolites or masked mycotoxins could be present.

Nowadays, the analytical methods have to be fast and effective monitoring of target, non-target and unknown compounds. In this way, the detection capability of Exactive® (Orbitrap® MS) is an attractive alternative. This high-resolution mass instrument allows the mass resolving power of up to 100000 FWHM and maintains the excellent mass accuracy lower than 5 ppm [29]. The potential of this Orbitrap® MS for the routine quantitative analysis was tested comparing different extraction methods.

2.2.3.5 LIQUID CHROMATOGRAPHY LINEAR ION-TRAP HIGH RESOLUTION MASS (LC-LTQ-ORBITRAP®)

As it has been explained hybrid linear ion trap-high resolution mass spectrometry or LTQ-ORBITRAP® has recently appeared combining Orbitrap® analyzer with an external accumulation device such as a LIT, making possible multiple levels of fragmentation (MS^n) for the elucidation of analyte structure. The use of the LTQ-Orbitrap® allows high-quality accurate mass and acquisition of MS^n spectra [29]. Focus on mycotoxin analysis by LTQ-Orbitrap® technology, it has not been commonly used for routine analysis. This hybrid instrument has been applied for beer analysis and it was compared with other hybrid instrument demonstrating its applicability for mycotoxins analysis.

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3. Objectius

3. OBJECTIUS

General objectives:

- Development of robust analytical methods in order to analyse simultaneously legislated and non-legislated mycotoxins in cereals and derivate products. The optimised analytical methods will be validated according to EU directives.
- Application of these validated methods for monitoring in different commercial samples.
- Evaluation of daily intake of mycotoxins from data obtained through the applicability of validated analytical methods.

Specific objectives:

- To introduce MSPD as a multi-mycotoxin extraction method in mycotoxin analysis field.
- To evaluate the efficiency an efficacy of wide-used extraction methods for mycotoxins analysis.
- To study the applicability of LC-MS to analyse simultaneously a wide range of mycotoxins in foodstuff.
- To estimate the daily intake for target mycotoxins of Spanish population.
- To estimate the human mycotoxin exposure determining the presence of mycotoxins in human urine.

4. Resultats

4.1 Analysis of mycotoxins in baby food: an unresolved matter.

Analysis of mycotoxins in baby food: an unresolved matter

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Abstract

Two years ago, one case had received considerable media coverage: an important company had issued a recall of some of its baby food in Canada on fears it may be contaminated with elevated levels of mycotoxins. The contamination of food is always perceived as an outrage; however, this problem is greatly exacerbated when food production intended for infants and children are incriminated. Hence the importance to review the analytical methods developed to analyse these mycotoxins in baby foods. We present a brief review of mycotoxins analysis step by step, from baby food sampling to the detection of mycotoxins, covering conventional methods and novel technologies used. Predominantly the methods specifically focused on satisfying baby food safety requirements are summarized. Additionally other methods, which would be not directly intended for baby food, but they would reach good performance characteristics at low concentration levels sufficient also for baby food analysis are also revised.

1. Introduction

In recent years, issues related to food safety have received growing attention as the stakes, rather economical than health-related, became increasingly important. During the past 20 years, several successive crises have alarmed consumers and hence led the appropriate bodies of administration to establish new control action plans to ensure the quality and safety of food products.

Contaminants in food are unavoidable. Experts in pollutant-risk assessment consider mycotoxins to be the most important contaminants with chronic dietary risk factor, above synthetic contaminants, plant toxins, and food additives or pesticides residues [1]. In fact, and despite of the efforts to control fungal contamination, fungi are ubiquitous in nature and occur regularly in worldwide food supplies agricultural products, such as cereal grains, nuts and fruits [2].

Focused on cereals, they suppose a worldwide basic raw material for production of many products, as such flours of rice, barley, wheat, rye, oat and maize which are commonly used to produce weaning cereal foods for infants. If contaminated cereals are processed, thus cereal-based products are a very considerable source of mycotoxins in infant diet, since cereal-based baby foods are considered as first solid food for infants and children constituting an important or often sole source of food for infants during their first year of life [3, 4].

The contamination of baby food with mycotoxins is often perceived, regardless of the levels found, as an outrage and it is viewed as unacceptable fact by the general public [5, 6]. The problem is that preventive measures cannot reliably exclude mycotoxins presence in these products owing to most mycotoxins are chemically stable and they tend to survive storage and processing, even when cooked at quite high temperatures.

Considering the multitude risk associated with mycotoxin intake by infants, maximum tolerance levels have been established for several mycotoxins including aflatoxins, OTA, DON, PAT, fumonisins and ZEN in foods intended for infants and young children in different guidelines [7-9] (*Table 1*). As it can be seen in

Table 1, a small number of mycotoxins have been established and the tolerance for other mycotoxins is still under discussion.

Previous studies, which were focused on determination of mycotoxins in this special group of food, clearly documented the fact that most of cereal-based baby foods frequently contain a wide range of mycotoxins at relatively low concentration levels [10]. The presence of mycotoxins in baby foods has urged the scientific community to acquire and apply the best analytical tools to protect consumers ensuring the absence of mycotoxins in these foodstuffs. During this same period, a parallel development was observed between the toxicity knowledge concerning humans and the evolution of the legislation at the food safety level [11]. Predominantly, the methods developed in the literature to detect mycotoxins in food are not directly intended to baby food, but they reached good performance characteristics at low concentration levels sufficient also for baby food analysis [12-17]. Few papers are specifically focused on satisfying baby food safety requirements [10, 18-23].

Table 1

Maximum levels (ML) for regulated mycotoxins in baby food according to EC 1881/2006, EC 1126/2007 and EC 165/2010 Commission regulations.

Compound	Maximum levels (μg/kg)
Aflatoxin B1	0.1
Aflatoxin M1	0.025
Patulin	10
Ochratoxin A	0.5
Deoxynivalenol	200
Zearalenone	20
Sum fumonisins (B_1 and B_2)	200

The ambition of this review is the representative selection of methods for mycotoxin analysis to follow the main trends and the most important approaches. Predominantly the methods specifically focused on satisfying baby food safety requirements are summarized. The attention is devoted to the brief description of the methods performance characteristics relevant for method validation.

On the other hand, this review aims to emphasis the necessity for a proactive approach to the identification of emerging mycotoxins which are detected widely in the raw materials of baby foods as rice, wheat or oats. Unfortunately, it is not yet known the extent to these mycotoxins as environmental agents can adversely affects the health of infants and children as a consequence of chronic exposure.

2. Analysis of mycotoxins in baby food: Sampling plan.

A mycotoxin sampling plan was defined by Johansson et al. [24] as a mycotoxin test procedure combined with a sample acceptance limit. The test procedure consists of sampling from the target population, sample preparation (sub-sample and homogenization) and analytical steps. The *figure 1* shows a sampling plan workflow, where sampling plan is summarized step by step.

Although literature of mycotoxin analysis is usually focused on detection and quantitative determination, mycotoxin analysis in food is generally a multistep process where each step can compromise the total variance of the testing scheme [25, 26].

2.1. Sampling

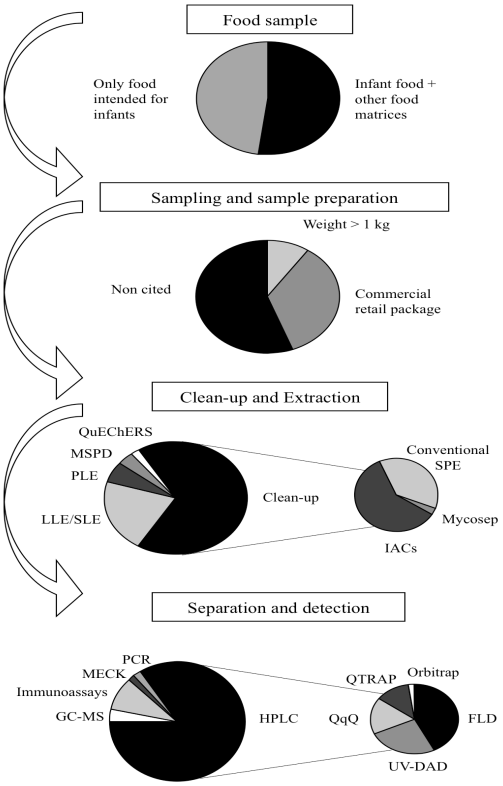
The distribution of mycotoxins in foodstuffs is an important criterion to consider (homogenization of samples); in fact EC regulations setting maximum permissible limits for mycotoxins are normally accompanied by associated Directives on sampling and analysis [27].

A number of papers have identified the particular problems associated with the sampling of commodities for mycotoxin analysis and the authors have reviewed the sampling schemes being used by various organizations. These studies have been

focused on the analysis of mycotoxins in grain or nuts. In fact, existing sample plans are mostly derived from mycotoxin distribution data for aflatoxins in peanuts and they have been extrapolated to other toxins and other commodities [28].

Regarding the analysis of mycotoxins from baby-foods, the analyses are usually carried out at the final product reporting the use of a representative retail sample to carry out a survey on the occurrence of mycotoxins in this foodstuff. Normally the baby foods are purchased in wide-range of retailers and supermarkets to ensure that survey is representative, however number or characteristics of commercial packages is rarely mentioned.

Figure 1. Sampling plan workflow.



In this sense, further studies including the acquisition of data concerning infant foods lot size and consumer packages will allow a complete overview on mycotoxin contamination in baby food [20].

2.2. Sample preparation

The second step of a sampling plan is the sample preparation, which is directly related with sampling procedure. This phase requires a careful mix of incremental samples in order to obtain a homogeneous "aggregate sample". Then a representative reduced part of the aggregate sample is obtained as "laboratory sample". Normally, the size of this laboratory sample is determined by the analytical method and the efficiency of sample homogenization. There is a trend in food analysis, to minimize amount of sample size to improve efficiency, reduce final costs and minimization of toxic solvents [29, 30].

According to EC 401/2006 [31] the aggregate samples of infant milk and follow-on milk and processed cereal-based foods for infants and young children shall be at least 1 kg or 1 L. This is limiting factor because powdered baby foods are usually marketed in a cardboard box of 300 or 600 g, which contains powdered infant food packaged in a foil pouch [20, 32].

Marin et al. [33] solved this problem homogenising thoroughly 10 individual packages in the case of apple juices intended for infants and 5 individual units in the case of solid baby food, ensuring that all came from the same batch. "Aggregate samples" were obtained on the same day of the analysis. In the sameway, Beretta et al. [21] and Biffi et al. [14] determined OTA in cereals-based baby foods working with batches composed of two samples.

However, Alvito et al. [20] reported the use of a representative commercial sample of 600g, but the authors pointed out that when the product was sold in a pack at low level, a number of retail packs were purchased ensuring that all came from the same batch, and they were mixed thoroughly.

Brera et al. [34] validated the method on bulk samples. The bulks were prepared and homogenized by mixing different packs of different brands and lots; each bulk

was about 3 kg. In the same way, Solfrizzo et al. carried out an inter-laboratory validation to determine fumonisins in baby foods; in this case, the aggregate samples were prepared mixing different commercial samples since to obtain 1.5 kg of total sample [35].

It is important to highlight that in the literature the size of "aggregate sample" is usually missing, and it is implied that retail level is used. On contrary, the size of "laboratory sample" is always indicated, ranging from 50 g necessaries to extracted aflatoxins according to AOAC International Official Method 2000.16 [19] to 1 g used to analyze DON, ZEN and its metabolites in baby foods [36]. Same amount (1 g) was recently used to detect 21 mycotoxins in baby food [37].

Once the sample is purchased, it is transported to the laboratory and processed. Since almost all the literature is focused on powdered baby food, the samples is grounded (when it is necessary) to fine powder and to homogenize it grinding the sample in a mill. Then, the powder is stored in a plastic bag, and before the analysis, a subsample from the aggregate sample is taken [38].

Little work is published in other baby food varieties as puree or liquid; most of these works are focused on the determination of PAT in juice or puree [33, 39], and in a recent work the differences in a sampling plan between three forms was evaluated for a multi-mycotoxin analysis [37].

2.3. Extraction and clean up procedures

In addition to the problem of heterogeneous distribution of mycotoxins, the complexity of baby foods can lead to serious interference during analysis of mycotoxins; a part of sugars, proteins and lipids, baby foods usually suffer addition of vitamins and minerals.

Only exceptional methods such as infrared spectroscopic techniques are able to draw data from the ground and homogenized sample without pre-treatment of the sample [40]. However, this method has been not yet applied to analyse mycotoxins from baby food. For this reason, mycotoxins need to be extracted from baby food applying extraction procedures.

In the extraction step, mycotoxin is liberated from the sample matrix; however, the presence of co-extractives can cause problems as the masking of the analytical signal for the target analyte. Therefore, clean-up procedures are used to remove these unwanted matrix components in the sample extract [25, 41].

2.3.1. Conventional extraction techniques

The methods described in literature for the extraction of mycotoxins from baby food used conventional techniques such as liquid-liquid extraction (LLE) or solid-liquid extraction (SLE), depending on the baby food variety: for liquid samples, two immiscible liquids are used, since for solid samples, a solvent can dissolve mycotoxins and extract them from powdered sample.

In the case of liquid samples, Murillo-Arbizu et al. [39] and Marin et al. [33] carried out a LLE to extract PAT from apple juice intended for infants using ethylacetate as extraction solvent in both studies. Then, a portion of organic sample was evaporated, redissolved in polar solvent, filtered and injected. In order to increase interaction between the solvent and mycotoxins, some modifiers such acid or salts could be used; in two above-cited works, salts as sodium carbonate or sodium sulphate were used. With this procedure, recoveries were ranged from 78% to 80% and from 72% to 95 % respectively.

On the other hand, for solid samples, Vendl et al. [39] determined DON, ZEN and its metabolites in baby food by SLE, using as extraction solvent a mixture of acetonitrile/water/acetic acid. For semi-solid samples, Meucci et al. [42] used SLE to extract ZEN and its major metabolites mixing the sample with chloroform by shaking and after to centrifuge, the organic phases were mixing with TRIS solution, and then, the obtained organic phase was evaporated and redissolved before the analysis. Both methodologies gave acceptable recoveries (> 70%).

Increasing the number of analyzed mycotoxins, Beltran et al. [16] used SLE procedure shaking the samples with acetonitrile/water/formic acid mixture to

extract eleven mycotoxins belonging to different chemical groups from multicereal baby-food, and Kostelanská et al. [43], using the same mixture solvent, analysed forty-five mycotoxins from cereal-based infant foods. In the first study the authors diluted the organic phase before analysis, while in the second work the authors took an aliquot of organic sample for direct analysis. The recoveries obtained were satisfactory in the both studies, with most of recoveries higher than 70%.

Ideally, selected extraction solvent should be able to extract only mycotoxins of interest from the sample matrix, while dragging as little as possible any interfering compound. However, the absence of such a completely specific extraction solvent makes necessary further purification and analyte enrichment; clean-up procedures have been employed to remove unwanted matrix components in the sample extract [44, 45].

2.3.2. Solid phase extraction: clean up procedures.

Three different applications have been reported for the SPE process: sample clean-up, sample concentration and analyte extraction with matrix removal. In the methods described in literature for the extraction of mycotoxin from baby foods, SPE has often been used for clean-up purposes. In this clean-up mode, the SPE column retains the mycotoxin and allows the impurities to pass trough the column [45].

A comprehensive compilation of different clean-up approaches for various mycotoxins has been published. During last years, there had been greater emphasis on the use of type of materials, which enable very selective binding of target molecules. The most popular is immunoaffinity column (IAC); in this column mycotoxin is bound selectively to the antibodies on the column after pre-conditioning step. However, more complex samples, as baby foods, require combination of different techniques as SLE or LLE to extract previously mycotoxins from the matrix [45, 46].

There are many experiments on SLE-IAC for mycotoxins analysis. In fact, most of studies focused on determination of aflatoxins [19, 47-49], OTA [14, 21, 32, 50,

51] or both simultaneously [20, 34, 52], were carried out using first SLE to dissolve mycotoxins and making a clean-up of the obtained extract by commercially available IAC. For trichothecenes, Dombrink-Kurtzman et al. [53] used SLE followed by IAC to extract DON from infant cereals and Romagnoli et al. [54] extracted simultaneously, DON, ZEN, T-2 and HT-2 using the same procedure. There are also commercially available columns for fumonisin; De Girolamo et al. [55] used SLE-IAC to analysed fumonisins (FB1 and FB2) in 19 maize-based baby-foods and the same research group carried out an interlaboratory validation using the same technique [35].

In these studies reached recoveries were into the limits established by the EU [31]. However, and despite the high sensitivity, immunoaffinity materials are expensive and distinctly less feasible for multi-mycotoxin analysis they are highly specific for only one (or group) target mycotoxin.

Another clean-up procedure employs multifunctional MycoSep® columns. These columns contain a mixture of charcoal, ion-exchange resins and other materials and are commercially available for aflatoxins, trichothecenes, ochratoxins, ZEN, MON and PAT. Regarding baby food analysis there is only one work in where these columns were used. Gottschalk et al. [17] extracted simultaneously trichothecenes A, B and D from 18 infant foods (5 wheat-based and 13 oat-based) using the MycoSep 226 columns, reaching good performance values. SLE previous methodology was carried out to extract mycotoxins from food matrix.

There is another experiment on comparison of Mycosep column to SPE for determining trichothecenes (DON, NIV, T-2 and HT-2) in infant semolina and infant biscuits. In this study, Lattanzio et al. [25] compared a clean-up procedure with MycoSep column (Mycosep 227) and by SPE cartridge containing C_{18} (Oasis HLB). The authors concluded that SPE columns provided good sample clean-up and higher recoveries than those obtained with MycoSep.

In fact, conventional SPE material is also used for clean-up purposes in mycotoxins analysis from baby food. For example, for clean-up PAT from different apple-

based infant products, silica-gel SPE columns have been used [15, 56, 57], while using a SPE cartridge that contained strong anion exchangers (SAX), fumonisins have been extracted from corn-based baby foods reaching good performance values [58-60].

2.3.3. Other extraction techniques

Around ninety per cent of works about mycotoxins analysis in baby food have been applied extraction techniques mentioned above; however, more recent techniques as pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE), or matrix solid phase dispersion (MSPD) are being slowly introduced in the analysis of mycotoxins in baby foods.

ASE is a SPE technique in which solvents are used at relatively high pressure and temperatures at or above the boiling point. This procedure helps to speed-up and automates the extraction process, offering a robust and time-saving alternative to classical solvent extraction techniques. So far, the high cost of an ASE apparatus has, however, limited the application of this technique in the field of mycotoxins analysis to a few laboratories [46].

As an example, Zinedine et al. [18] used ASE with acetonitrile-water for the extraction of OTA from 20 infant cereals; the mean recovery of OTA was 82 % at fortification level. In the same way, D'Arco et al. [22] validated this technique with methanol to extract fumonisins from corn-based baby food obtaining recovery values between 68% for FB_3 to 83% for FB_1. Once optimized the method, the same authors applied this technique to compare the presence of fumonisins in conventional samples and organic samples [61].

MSPD is another attractive extraction alternative where the sample and sorbent material are mixed homogenously; this mixture is then packed in cartridge and afterwards elution is performed [62]. Despite of its demonstrated advantages, MSPD is not still a mainstream sample-preparation technique in the mycotoxins field, and it use is limited to few studies in mycotoxin analysis field. In fact, Rubert et al. applied MSPD the first time for the simultaneous extraction of 21 mycotoxins

belonged to different groups from baby foods [37]. The method was optimized and then it was validated for three different baby food varieties, obtaining recovery values from 68% to 101%, demonstrating the applicability of MSPD for the simultaneous analysis of a wide range of mycotoxins in baby food.

Other modern extraction technique, QuEChERS procedure, has been only applied to analysis PAT in three baby food products including one apple-pear juice, one apple-pear puree, and one infant cereal food [63] and the recoveries were within 94–104%, demonstrating the applicability of this technique on the analysis of PAT in baby-food.

2.4. Detection and quantification techniques.

Finally, and after appropriate extraction method, mycotoxins into the extract have to be quantified. A method of analysis for mycotoxins in food should be simple, rapid, robust, accurate and selective to enable simultaneous determination [44]. It is important to keep in mind that low tolerance levels allowed in baby food require sensitive methods.

The currently used quantitative methods for the determination of mycotoxins in baby food mainly use chromatographic techniques for separation, in combination of a variety of detectors. The most important chromatographic techniques could be summarized in gas chromatography (GC) and liquid chromatography (LC).

2.4.1. Gas chromatography.

GC has been scarcely used for mycotoxins analysis in baby food [10, 64]. The problem is that most mycotoxins are not volatile, and therefore they have to be derivatised for analysis by GC. In an attempt to use this technique, several procedures have been developed for the derivatisation of mycotoxins.

Cunha et al. [64] developed a method to detect PAT in baby food using GC; in this case, silylation reaction was employed in order to obtain a volatile material able to be analyzed by GC-MS. This approach gave LOD of 0.4 μg kg^{-1} and LOQ of 1.2 μg kg^{-1}, which were below to ML established for PAT in baby food, and lower than levels obtained by other methods.

Lombaert et al. [10] carried out a survey on the occurrence of trichothecenes, OTA, ZEN, fumonisins and ergot alkaloids in infant cereal food. With this aim, different analytical techniques were optimized and, finally, only trichothecenes were derivatisated with heptafluorobutyrate and analyzed using GC-MS. The rest of mycotoxins as OTA, fumonisins, ZEN and ergot alkaloids could not be directly determined by GC, and were analyzed by liquid chromatography. LOQs reached by this methodology ranged between 20 μg kg^{-1} for DON to 40 μg kg^{-1} for NIV and HT-2. These results are according to the results obtained by other procedures.

Moreover, besides the necessity of derivatisation, several problems have been identified when GC is used as non-linear calibration curves, memory effects, matrix effects, etc [44]. All these drawbacks suggest that the use of GC detection will not increase in future years due to existence of cheaper and faster alternatives such HPLC which is able to determine all mycotoxins.

2.4.2. Liquid Chromatography

HPLC with different detectors is frequently used both for routine analyses and as confirmatory method or screening techniques. In essence, most of the protocols used for mycotoxins detection by HPLC are very similar. The most commonly found detector methods are UV or fluorescence detectors, which rely on the presence of a chromophore in the molecules [65].

Numerous protocols using fluorescence detector (FLD) exist for aflatoxins [19, 47-49], OTA [14, 21, 32, 50, 51] and aflatoxins and OTA simultaneous analysis [20, 34] from baby food which rely on sample clean-up using IAC, HPLC separation and detection by FLD. These procedures reached good sensitivity owing to the high selectivity of the detector, allowing LODs at 0.003 μg kg^{-1} concentration level for AFB_1 and LODs for OTA at 0.009 μg kg^{-1} [20].

The presence of aflatoxins and OTA has been also determined using UHPLC separation followed by mass spectrometry detector [52]. By this method, LODs reached were between 0.003 μg kg^{-1} to 0.008 μg kg^{-1} for aflatoxins and between 0.002 μg kg^{-1} to 0.009 μg kg^{-1} for OTA. These results are very similar to than

obtained by FLD. FLD has been used to determine the presence of other *Fusarium* mycotoxins as ZEN or fumonisins. For example, Meucci et al. [42] determined ZEN and its principal metabolites in meat-based infant food by HPLC-FLD and Arranz et al. [66] carried out an interlaboratory study for the quantification of ZEN in baby food by HPLC-FLD. The analytical performance showed to be enough acceptable for it application in monitoring studies.

HPLC-FLD methods have been also used to determine fumonisins in baby food. In fact, Machinski et al. [60] and de Castro et al. [67] used HPLC-FLD to carry out two different surveys of the presence of fumonisins (FB_1 and FB_2) in Brazilian corn-based products, while De Girolamo et al. [55] applied the same procedure for a survey of fumonisins, but in this case, in Italian corn-based products. In this last work, good LODs were reached; 2.8 μg kg^{-1} for FB_1 and 2.2 μg kg^{-1} for FB_2, both far below ML established for fumonisins in maize-based baby food.

Within the last 15 years, HPLC coupled to mass spectrometry (MS) or tandem mass spectrometry (MS/MS) has become the universal approach for mycotoxin analysis. In contrast to GC based methods, polar compounds are quickly reachable without the need of derivatization. Further advantages include low detection limits, the ability to generate structural information of the analytes, the minimal requirement of sample treatment, and the possibility to cover a wide range of analytes differing in their polarities. Finally, mass spectometres are rather general detectors that are not so dependent on chemical characteristics like UV absorbance or fluorescence [45].

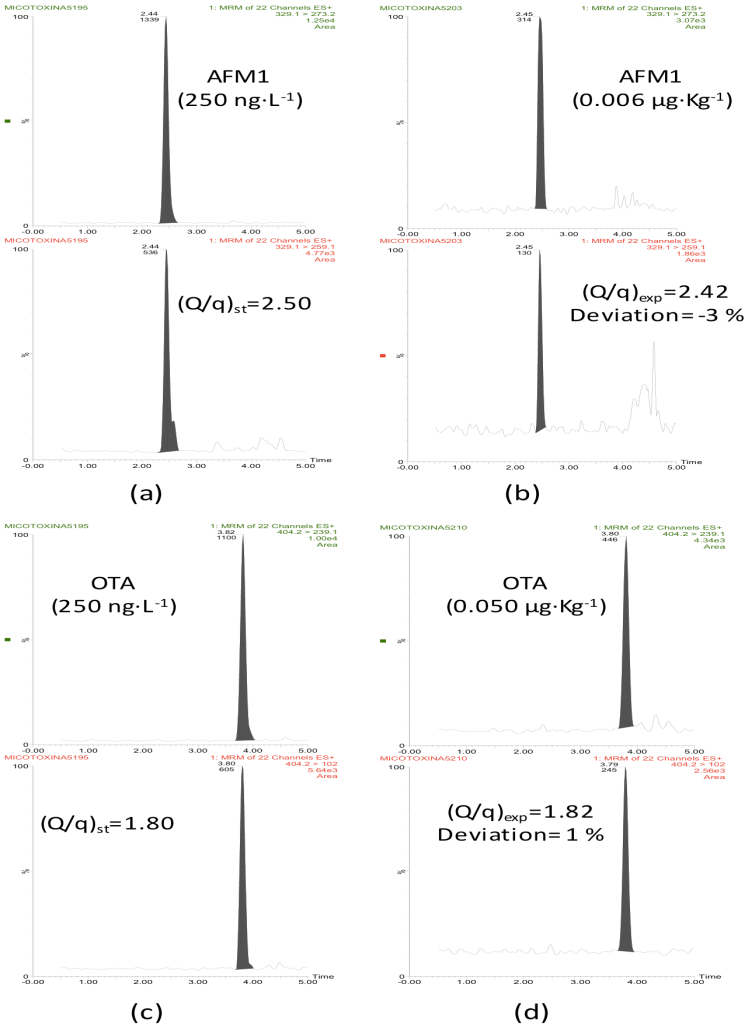
There are many types of mass analyzers such as quadrupole, time-of-flight or ion trap. The most wide-used one in mycotoxins analysis is triple quadrupole, which consist of 3 quadrupoles with 3 important functions; the first quadrupole acts as ion filter. Then, the mass separated ions pass into the collision cell and change to fragments. Finally, the selected fragment ions pass into the third quadrupole that is for detection.

Beltran et al. [52] used this instrument for the simultaneous detection of aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1) and OTA in four different infant formulae. As it has been mentioned above, LODs and LOQs obtained in this study were in high accordance with those obtained by HPLC-FLD. However, the confirmation using triple quadrupole allowed obtaining more identification points, as the *figure 2* shows the confirmation of positive samples by the accomplishment of Q/q ratios and retention times. The authors presented an UHPLC–MS/MS chromatograms corresponding to: (a) AFM1 reference standard, (b) sample of powdered milk for babies, containing 0.006 μg kg−1 of AFM1, (c) OTA reference standard, (d) sample of cereals infant formula, containing 0.050 μg kg^{-1} of OTA. The quantification transition (Q) confirmation transition (q) permitted to calculate two values. The first one was Q/qst, it is the intensity ratio for the reference standard which was used to compare with Q/qexp. This value is the experimental intensity ratio in sample. In this study, the positive samples were unambiguously confirmed by the accomplishment of both Q/q ratio and retention time obtaining deviations lower than 3%, as it can see in the *figure 2*. Fortunately, positives samples were lower than maximum permitted levels.

Gottschalk et al. [17] validated an HPLC-QqQ-MS method to estimate simultaneously the presence of type A, B and D trichothecenes in baby food classified depend on its principal cereal. LODs obtained in this study were lower than those obtained by Romagnoli et al. [54] who tested QqQ to determine DON, T-2, HT-2 and ZEN in baby foods marketed in Italy. In any case, both studies fulfilled ML established for EU.

Fumonisins have been also determined using QqQ. In fact D'Arco et al. [22, 23] carried out two different works. In the first one [22], the authors optimized the method obtaining LODs between 0.7 μg kg^{-1} for FB_1 and FB_2 to 1.2 μg kg^{-1} for FB_3; once validated, in the second work [54] the authors carried out a survey of FB_1, FB_2 and FB_3 in conventional and organic retail corn products in Spain and Italy.

Figure 2. UHPLC–MS/MS chromatograms corresponding to: (a) AFM1 reference standard, (b) sample of powdered milk for babies, containing 0.006 μg kg^{-1} of AFM_1, (c) OTA reference standard, (d) sample of cereals infant formula, containing 0.050 μg kg^{-1} of OTA.



All the studies mentioned since now, have been focused on a group of mycotoxins because, mycotoxins constitute a structurally very inhomogeneous group what is a problem when simultaneous determination of different groups of mycotoxins is attempted. However, using HPLC-MS/MS the simultaneous determination of mycotoxins belonging to different chemical families can be performed, enabling

the efficient quantitative screening for the most important mycotoxins in food commodities for infants.

In this way, Beltran et al. [16] determined 11 mycotoxins belonging to different groups (including such as aflatoxins, OTA, fumonisins and trichothecenes) in baby food by LC-MS/MS using a triple quadrupole detector (QqQ). The proposed methodology allowed the simultaneous determination of mycotoxins with different physicochemical properties. The method was validated obtaining satisfactory LODs (concentration levels from 0.1 μg kg^{-1} for AFG_2, AFB_1 and OTA to 25 μg kg^{-1} for DON) and LOQs (concentration levels from 0.3 μg kg^{-1} for AFG_2, AFB_1 and OTA to 80 μg kg^{-1} for DON) taking into account MLs fixed by the EU.

Although the sensitivity, selectivity and efficiency of QqQ are excellent, the qualitative information needed to support the structural elucidation of compounds is lost. This liability could be overcome by using the hybrid mass spectrometer QTRAP® which is appropriate for both quantification and confirmation. This hybrid detector provides accurate results above traditional MRM owing to it capacity to carry out the enhanced product ion scan (EPI) in Information Data Acquisition (IDA) which takes structural information increasing the confidence of confirmation [68, 69].

Desmarchelier et al. [63] used this detector to determine PAT in apple-based baby food, while Lattanzio et al. [23] used it for the simultaneous determination of four trichothecenes in infant semolina and infant biscuit. An extended multi-mycotoxin method for 45 mycotoxins and metabolites in baby food [43] has been carried out in two chromatographic runs (positive and negative ESI mode) using QTRAP® detector.

In these studies, QTRAP® has been mainly used in it QqQ mode, fulfilling in all cases analytical requirements demanded for the EU legislation. However, in another study, Rubert et al. [37] analysed 21 mycotoxins from different varieties of baby foods using IDA as an extra confirmation tool for samples that contain the selected mycotoxins. Although the LOD and LOQ for AFB_1 did not reach the ML

established by the EU, all the other mycotoxins could be confirmed unambiguously in a positive sample.

Other promising detector is high resolution mass analyzer; however it has been only applied once to baby food samples for mycotoxin analysis [70]. In this study, this technology was compared with QTRAP® detector concluding that while QTRAP® instrument was more suitable for quantitative purposes, LTQ-Orbitrap® had other advantage as such unambiguous identification of non-target and unknown mycotoxins owing to ultra-high resolution mass being two complementary techniques.

Compared to the mass spectrometric and fluorescence detection, all other detections available in HPLC are seldom used in mycotoxin analysis. The reasons might be highest limits of detection unsuitable for trace amounts of the determined substances, and lack of specificity for some detectors [46].

HPLC coupled to DAD or UV detectors have been used for the analysis of mycotoxins from baby food. As an example, PAT is a mycotoxin perfectly amenable to HPLC with DAD or UV detection because of its strong UV detection λ_{max} 276 nm [71], in fact most of methods published for the analysis of this mycotoxin in baby food or juices for infant consumption have been developed using these detectors [13, 15, 33, 39, 56, 57, 72-74].

HPLC using DAD or UV detection for other mycotoxins in baby food are not frequently. This technique was employed for Stroka et al. [75] to carry out an inter laboratory validation to determine DON in baby food: developed methods showed acceptable performance for within-laboratory and between-laboratory precision as required by European legislation. Dombrink-Kurtzman et al. [53] developed also an HPLC-UV method to determine DON in infant cereals. The level of quantification for DON was 10 μg kg^{-1} for infant cereals and the level of detection was 5 μg kg^{-1}.

Enniatins and beauvericin, produced by *Fusarium*, have been also determined by HPLC-DAD in 20 infant cereals from Morocco [76]. In this case, the problem is to

establish acceptable limits, since MLs of these “emergents” mycotoxin are still under-discussion.

2.4.3. Capillary electrophoresis

In spite of the great separation power and versatility of capillary electrophoretic techniques, they have never gained such popularity as HPLC, although the same analyte can be determined with CE and the same detectors can be used. In comparison with HPLC methods, micellar electronikinetic chromatography (MEKC) and capillary electrochromatography (CEC) methods have some advantages such as being able to use a smaller volume of organic solvents, high efficient separation and producing less waste volumes [39].

However, there is scarce literature using these techniques for the detection of mycotoxins in baby food. In fact, only Murillo-Arbizu et al. [39] compared both techniques (HPLC and MECK) for determining PAT in apple juice intended for infants. The authors concluded that both methods were useful for the accurate quantification of PAT in this type of sample matrix, however, LOQ reached by HPLC was lower than the obtained by MECK; the same occurs with the time of the analysis.

2.4.4. Other methodologies: Screening techniques

Applications of analytical methods other than separation are sometimes encountered. For example, rapid methods based on immunochemical techniques often have the advantages of not requiring any clean-up or analyte enrichment steps. The microplate immunoassay (ELISA format) is one of the most frequently used rapid tests for mycotoxins. ELISA kits are commercially available for many mycotoxins as aflatoxins. As an example, Razzali-Fazeli et al. [49] compared an ELISA kit with an HPLC-FLD method to determine aflatoxins in different products (12 baby foods are included in the study). In this study, HPLC appeared to be the method of choice for the measurement of high concentration ranges of aflatoxins. Besides ELISA procedures, there is an increasing demand for immunoassay techniques for field use offering protocols for quick and reliable

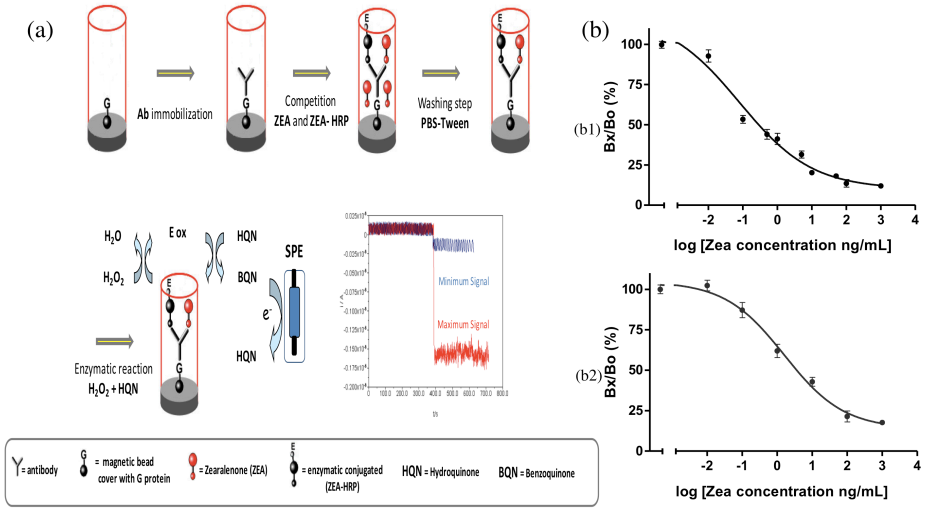
results: a promising technology for rapid mycotoxin detection is the surface plasmon resonance biosensor (SPR). The principle of SPR is based on the detection of a change of the refractive index of the medium when an analyte binds to an immobilised partner molecule (antibody) [25].

The technique has recently been adopted for determining DON in maize-based baby food using a mouse monoclonal antibody raised against DON, a DON sensor chip surface and the phenomenon of SPR; LOD of the method was 6 μg kg^{-1}, which is far below of the permitted ML [77]. The same methodology was applied for the simultaneous determination of T-2 and HT-2 in maize-based baby foods [78]. The accuracy of these assays was evaluated by comparing results of naturally contaminated samples analyzed by both SRP method and well-established LC-MS/MS method validated previously. There was extremely good agreement between the methods and in particular no false positive were observed.

ELISAs typically use a colorimetric or fluorimetric endpoint. However, they can also use substrates with products that can be measured electrochemically. The advantages of electrochemical assays may include the low cost of production of the electrodes, amenability to miniaturization, and amenability to multiplexing [79].

The development of electrochemical immunoassays has recently been applied for ZEN analysis: in first work [80], electrochemical immunoassay was evaluated trough the determination of ZEN in selected baby food containing cereals. In second work [81], the study was followed-up exploring the use of disposable immunosensing surfaces on screen-printed electrodes using magnetic beads coupled to ELISA method for the determination of ZEN in baby food samples. The *figure 3* summarizes the scheme of this procedure, as well as calibration curves for ZEN using this technique. In these two studies, electrochemical immunoassay demonstrated to be a very valuable tool for the ultrasensitive detection of ZEN in baby food.

Figure 3. Figure (a) detailed a schematic representation of immunochemical immunoassay. Calibration curves for zearalenone (b) obtained using the competitive electro-chemical immunoassay (b1) and undiluted spiked cereal milkshake samples (b2). In both cases the points correspond to the *Bx*/*Bo* percentage ± SD, calculated for n = 4 repetitions.



One of the recently developed methods for mycotoxin detection is polymerase chain reaction (PCR) method: small pieces of DNA can be amplified and detected routinely. Only one work reports the use of this technique to detect T-2 and HT-2 in baby foods. Bowens et al. [82] propose a PCR-based assay based on the ability of these trichothecenes to induce massive and fast changes of transcription in cultured human epithelial cells. The developed technique was able to measure the additive biological activity of both mycotoxins and it may further improve consumer safety by detecting the presence of new or emerging type A trichothecenes that display similar health hazards as the regulated T-2 and HT-2 toxins.

3. Conclusions and Future trends

In conclusion, based on this review, the data presented in different studies indicate that cereal-based baby food regularly contain low levels of mycotoxins. An

accurate prediction of the possible infant health impact of individual mycotoxins in baby foods is difficult; possible additive or synergistic effects of multiple mycotoxins make the task far more complex.

To control the amount that is present in infant diets, a broad range of detection techniques used for practical analysis and detection of a wide spectrum of mycotoxins in baby food are available, however, these procedures usually encompass the analysis of one mycotoxin or mycotoxins belonging to the same chemical group.

The methods able to determine and quantify simultaneously mycotoxins belonging to different groups are still rare. Efforts in this direction are being made for not only traditional methodologies but also and mainly rapid screening and multi-mycotoxin methods. We can highlight the rapid progress that represents a major step forward and lays the foundation to develop quantitative methods for the analysis of wide range of mycotoxins at trace concentration levels in baby foods thanks to the use of specific and sensitive detectors as QqQ or QTRAP.

For this reason, conventional analytical techniques as HPLC-MS with quadrupole detectors in routine baby food safety and monitoring will not be completely replaced by other techniques as ELISA or PCR, since they are essential for confirmation purposes. However, high resolution mass analyzers could become common screening techniques in the near future.

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4.2 Optimization of Matrix Solid-Phase Dispersion method for simultaneous extraction of aflatoxins and OTA in cereals and its application to commercial samples.

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Optimization of Matrix Solid-Phase Dispersion method for simultaneous extraction of aflatoxins and OTA in cereals and its application to commercial samples

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ABSTRACT

A method based on Matrix Solid-Phase Dispersion (MSPD) has been developed for the determination of 5 mycotoxins (ochratoxin A and aflatoxins B and G) in different cereals. Several dispersants, eluents and ratios were tested during the optimization of the process in order to obtain the best results. Finally, samples were blended with C_{18} and the mycotoxins were extracted with acetonitrile. Regarding to matrix effects, the results clearly demonstrated the necessity to use a matrix-matched calibration to validate the method. Analyses were performed by liquid chromatography–triple quadrupole-tandem mass spectrometry (LC–QqQ-MS/MS). The recoveries of the extraction process ranged from 64% to 91% with relative standard deviation lower than 19% in all cases, when samples were fortified at two different concentrations levels. Limits of detection ranged from 0.3 ng g^{-1} for aflatoxins to 0.8 ng g^{-1} for OTA and the limits of quantification ranged from 1 ng g^{-1} for aflatoxins to 2 ng g^{-1} for OTA, which were below the limits of mycotoxins set by European Union in the matrices evaluated. Application of the method to the analysis of several samples purchased in local supermarkets revealed aflatoxins and OTA levels.

1. Introduction

Mycotoxins are toxic chemical products formed as secondary metabolites by few fungal species that readily colonize crops and contaminate them with toxins in the field or after harvest [1]. Surveillance studies showed that mycotoxin contamination is a world-wide problem [2,3], since it is estimated that 25% of the world's crop production and 20% of crop production within the European Union may be contaminated with these contaminants [4]. Economic losses deriving from that are tremendous, including reduction of livestock production and agricultural production, health care, veterinary and regulatory costs [5].

Because of these effects on humans and animals, measures have been set up by authorities in many countries to monitor and control mycotoxins levels. In this way, aflatoxins and ochratoxin A are subject to European Union legislation for a number of years setting maximum levels for these mycotoxins in different commodities [6].

The requirement to apply these regulatory limits has prompted the development of a vast number of analytical methods for the identification and quantification of these mycotoxins in various complex samples, such as food, feed, and another biological samples, especially for the highly toxic and carcinogenic aflatoxins that maximum tolerable levels have been established at ppb level in some matrices as cereals.

Specifically, the International Agency for Research on Cancer (IARC) has classified aflatoxins; aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2) as carcinogenic to humans while ochratoxin A (OTA) has been classified as possibly carcinogenic. Moreover, they are the mycotoxins of major significance and hence there has been significant research on broad range of analytical and detection techniques that could be useful and practical.

Aflatoxins and OTA are to be found in agricultural products that are susceptible to contamination include malt, wheat, coffee, green coffee, barley, oat, chicory, maize, cacao, wine, grape juice, dried fruits, peanuts, cotton seed, corn and rice [7–10]. OTA is principally a storage mycotoxin, but it can be produced during the malting process also [11].

In the past years, a trend towards the use of liquid chromatography–tandem mass spectrometry (LC–MS/MS) in mycotoxins analysis to reach the low limits established by the legislation has been observed, since by this technique, in contrast to most screening methods, unambiguous analyte confirmation can be obtained [5,12–20]. This idea, has led some researchers to the misconception that the use of LC–MS/MS effectively eliminates matrix effects. In reality, unpredictable increase/decrease in analytical signal intensities may occur due to the co-elution of matrix

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components that disturb the ionization of the analyte [12], so it can be concluded that one of the most crucial and critical step in the analysis of mycotoxins, with independence of the determination technique, is the sample preparation and clean-up [13].

The mycotoxins are mainly determined by single compound analytical methods based on immunoaffinity column clean-up. These columns are also available for the simultaneous determination of aflatoxins and ochatoxin A [14]. Despite minor problems in cross-reactivities, this method was generally considered to be so specific that confirmation was supposed unnecessary. However, increasing quality demands altered this attitude and methods were developed in which mass spectrometry was applied for confirmation purpose [6], apart from its high cost and its matrix dependence [15,16].

For this reason, other alternative techniques, such solid-phase extraction (SPE) or Matrix Solid-Phase Dispersion (MSPD) methods, help simplify protocols, improve selective and performance characteristic and nowadays are applied to the analysis of several residues [17,18].

The possibility of achieving simultaneous extraction and clean-up has been investigated by various groups using MSPD for analysis of aflatoxins in peanuts and chilli powder, green bean and black sesame [19–21].

However, MSPD has been still scarcely used for analysis of mycotoxins from foods. In this paper an extraction procedure is presented for aflatoxins and OTA in coffee, malt and an instant cereal-breakfast beverage based on MSPD, with C_{18} as a dispersing mean and acetonitrile as an eluant after a carefully optimization of different parameters of the extraction process in order to find method that produces least matrix effect and gives high recoveries for the five mycotoxins. The identification and quantification of the analytes were carried out using LC coupled with triple quadrupole mass spectrometry detector.

These mycotoxins were selected for this study owing to the hazard they pose to human health and their high incidence in different crops. The selection of the studied matrices were according to the 2002 report on the assessment of dietary intake of OTA by the population of the EU member states [22] in which the contributions of various food commodities were estimated (just to mention the most significant) as 50% for cereals and 10% for coffee. Then cereals and coffee consumption could contribute significantly to human intake of these mycotoxins. Coffee substitutes (in general made with cereals as barley, malt, rye and chicory) are natural products that do not contain caffeine and are therefore suitable for everyone, adults and children.

The present work includes the application of the developed method in three further matrices as well as an investigation of the variability of the matrix effects between individual samples. Based on these additional data for matrix effects, the applicability of the concept of matrix-matched calibration for the developed method is evaluated. Finally, the optimized method was applied to the control of 22 commercial samples.

2. Materials and methods

2.1. Chemical and reagents

Acetonitrile, methanol, hexane, ethyl acetate and dicloromethane were supplied by Merk (Darmstadt, Germany). Solid-phase used for MSPD were silica, amino, phenile, octysilica (C_8) (50 μm), octadecysilica (C_{18}) (50 μm) bonded silica from Analisis Vinicos (Tomelloso, Spain). Florisil® (60–100 mesh) was obtained from Aldrich (Steinheim, Germany).

The standards of aflatoxins (B_1, B_2, G_1, G_2), ochratoxin A (OTA) were supplied by Sigma–Aldrich (St. Louis, MO, USA).

The individual stock solutions of aflatoxins and ochratoxin with concentration 500 mg ml^{-1} were prepared in acetonitrile, kept in security conditions at −20°. All other working standard solutions were prepared immediately before use by diluting the stock solution with acetonitrile.

Ammonium acetate (MS grade) is bought from Sigma–Aldrich (St. Louis, MO, USA). Water for LC mobile phase was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

2.2. Samples

A total of 22 samples were purchased in commercially available size during July 2009 from supermarkets located in the city of Valencia (Spain). The samples were transported to the laboratory under ambient conditions. Samples were milled using a blender Moulinex and a 200 g subsample was analysed [23].

The milled samples were analysed as quickly as possible after the purchase and they were stored at −20 °C.

2.3. Matrix Solid-Phase Dispersion

Samples (200 g) were prepared using a food processor and mixed thoroughly. Portions of 1 g were weighed and placed into a glass mortar (50 ml) and were gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain homogeneous mixture. For the preparation of fortified samples, 1 ml of the standard working solution was added to 1 g of sample. Then, they were allowed to stand at room temperature for 3 h. The homogeneous mixture after solvent evapored was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 10 ml of acetonitrile by applying a slight vacuum. Then, the extract was transferred to a 25 ml conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 ml with acetonitrile and filtered through a 13-mm/0.45-μm nylon filter purchased from Análisis Vínicos (Tomelloso, Spain) before their injection into the LC–MS/MS system.

2.4. Liquid chromatography–mass spectrometry

The triple quadrupole mass spectrometry detector (QqQ) was equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) that included an autosampler and a quaternary pump. Separation was attained on a Phenomenex (Madrid, Spain) Gemini C_{18} (250 mm × 4.6 mm i.d., 5 μm particle size) analytical column, preceded by a security guard cartridge C_{18} (4 mm × 2 mm i.d.), using a gradient that started at 35% of 5 mM ammonium acetate in water (A) and 65% of 5 mM ammonium acetate in acetonitrile (B) during 3 min. After, it was increased linearly to 95% of B in 4 min, and held constantly for 3 min. Then, the gradient backs to the initial conditions during 10 min. The flow rate was 0.25 ml min^{-1}, and 20 μl of standard solutions or extract were injected.

A QqQ mass spectrometer Quattro LC from Micromass (Manchester, UK); equipped with pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software Ver. 4.1 was used for MS/MS analyses. Parameters were optimized in positive and negative mode by continuous infusion of a standard solution (10 μg ml^{-1}) via syringe pump at a flow rate 20 μl min^{-1}. Analysis was performed in positive ion mode. The ESI source values were capillary voltage, 3.50 kV; extractor, 1 V; RF lens 0.5 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 l h^{-1}. Cone voltages and collision energies were optimized for each analyte during infusion of the pure standard and the most abundant fragment ion chosen for the selected reaction monitoring. The analyzer setting were: res-

olution 12.0 (unit resolution) for the first and third quadrupole; ion energies, 0.5; entrance and exit energies, 1 and 3; multiplier, 650; collision gas (argon, 99.99% purity) pressure 3.74×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.2 ms. The mass spectrometer was operated in scan, product ion scan, and multiple reaction monitoring (MRM) modes. All the measurements were carried out in triplicate.

2.5. Method validation

Method accuracy and precision were evaluated by performing recovery studies using "blank" samples. Recovery experiments were conducted at two levels—between 1 and 2 $\mu g\,kg^{-1}$ (quantification limits, LOQs) and between 10 and 20 $\mu g\,kg^{-1}$ ($10 \times$ LOQs). "Blank" samples (1 g) were spiked with 1 ml of a working mixture of the compounds at the appropriate concentration. Then, "blank" samples were left to stand 3 levels before the extraction. Five replicates were prepared for each spiking level after solvent evaporation.

For the estimation of the linearity and matrix effects, raw extracts of samples spots without visible fungal infections were fortified using a multi-mycotoxin standard on a range of studied concentration level, diluted and analyzed and the corresponding peak areas were compared to a standard prepared and diluted in neat solvent.

To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated and the signal suppression/enhancement (SSE) due to matrix effects was determined.

The limit of detection (LOD) was estimated from extracted samples, spiked with decreasing concentrations of the analytes, where the response of the qualifier ion was equal to 3 times the response of the blank extract. Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LOQ was defined in this study as the lowest calibrator with an acceptable relative uncertainty (coefficient of variation $\leq$19% and an accuracy $\geq 70 \pm 19\%$). The LOQ was preliminarily estimated, in the same way as the LOD, using also the criterion of S/N $\geq$ 10 for the qualifier ion.

3. Results and discussion

3.1. Optimization of the LC–MS/MS

3.1.1. Optimization of the triple-quad detection method

First, the experiments to select the optimum multiple reaction monitoring parameters (MRM transitions, interface parameters and MS/MS parameters) were performed by direct injection of individual standards at 10 mg ml^{-1}. ESI in both positive and negative ion mode were evaluated, observing that all mycotoxins exhibited higher precursor ion signal intensities or better fragmentation patterns in positive ion mode. Only OTA was efficiently ionized in the negative mode, but lower signal than positive mode, so ESI in positive mode was selected for all of them. In general, all aflatoxins exhibit good ESI ionisation efficiency in the positive ion mode with abundant protonated molecules $[M+H]^+$ and sodium adduct ions $[M+Na]^+$. To validate the identity of the parent, these ions were fragmented into daughter ions with argon gas in the collision cell of the triple quadrupole, but since the sodium adduct did not exhibit specific fragmentation during the collision induced dissociation process for any compound, the protonated molecule was chosen as the precursor ion for each studied mycotoxin in the product ion scan mode. In this context, and as it has been related in the literature [24], the formation of sodium adduct ions can easily be suppressed by the addition of modifiers (ammonium ions) to the mobile phase leading to a better MS sensitivity. The product ion spectra of the protonated aflatoxins species contains a number of abundant product ions reflecting bond cleavages and rearrangement reactions of the polycyclic ring system along with loss of water, carbon monoxide and carbon dioxide.

The pathway fragmentation of OTA has been widely studied. In positive mode ionization, the abundant ion is the protonated molecule. Applying soft energy collision energy, the fragments obtained correspond to the loss of a carboxylic group and radical cleavage [25,26].

This method has been specifically developed for confirmation analysis purposes in compliance with the European Union laws in force [27]. On this basis, a substance can be identified using LC–MS/MS, in MRM mode, by at least two transitions. For this purpose all possible fragments were studied and the two transitions with highest chromatographic signal-to-noise (S/N) ratios and with minimum interference from matrix components were chosen.

Quantification was carried out on the primary transition. Each mycotoxin was confirmed by the second transition and the ratio between primary and secondary daughter ion calculated. These ions were selected according to the highest sensitivity and to optimal selectivity for the target compounds. These product ions with the highest intensity provided by fragmentation of the precursor ion and the optimum collision energy are shown in Table 1.

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

Mycotoxin	Retention time (min)	Precursor ion	Product ion	Cone	Collision energy
AFB_1	13.04	313.2	241^Q 269^q	47	30 30
AFB_2	12.83	315.2	243^Q 259^q	50	30 30
AFG_1	12.42	329.2	200^Q 215^q	43	40 30
AFG_2	12.23	331.2	189^Q 217^q	46	45 25
OTA	11.12	404.2	239^Q 358^q	20	20 15

Q, Quantification transition.
q, Confirmation transition.

3.1.2. Optimization of the chromatographic method

The direct combination of LC with MS reduces the stress on chromatographic separations because of the mass selectivity and distinctive fragmentation patterns. However, chromatographic separation can be crucial in some cases. In order to reduce analysis time, increase sensitivity and provide good peak shape, main variables with influence on the chromatographic separation were optimized.

Modification of the mobile phase with volatile acids and salts was also carried out because the mobile phase composition has a significant effect on peak shapes and the retention behaviour of the analyte in the LC column, as well as on the MS response.

Different mixtures of water and acetonitrile modified with ammonium acetate were evaluated. Addition of salts influences markedly the mass spectrometric response since it avoid the sodium adduct formation what improve the detection of molecular precursor ion and the consequent fragmentation. Moreover, the addition of ammonium acetate improves the peak shape and the reproducibility of the retention time for OTA.

Therefore, chromatographic separation of the target analytes was finally performed with a mixture of water–acetonitrile with ammonium acetate (5 mM), using an elution time of 20 min.

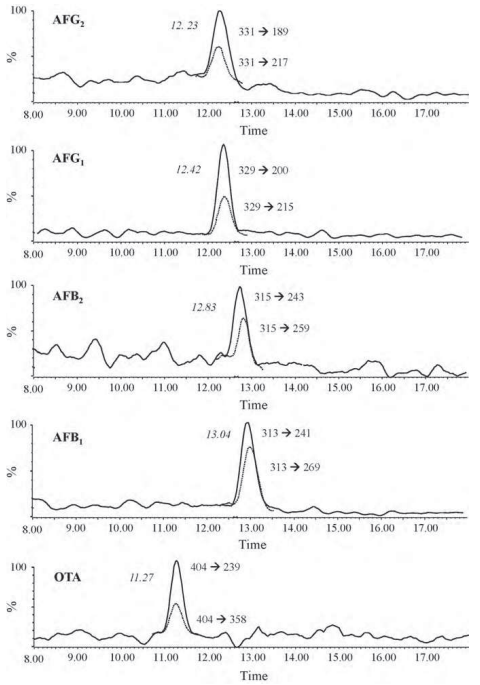

Fig. 1. LC/MS/MS chromatograms of a standard of mycotoxins in acetonitrile at LOD levels with the quantifier and qualifier ions for each compound.

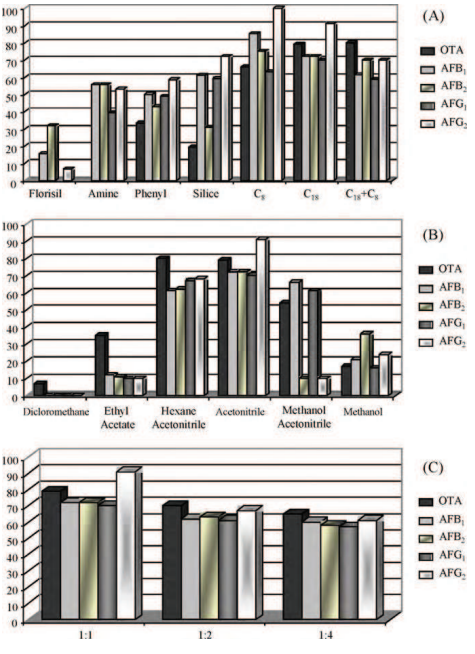

Fig. 2. Studied parameters of extraction optimization method. (A) Recoveries (%) in different solid dispersants, (B) recoveries (%) with different solvents and (C) recoveries (%) employing different ratios of sample.

Fig. 1 shows the chromatogram obtained with a standard solution under the optimum chromatographic conditions commented before at LODs levels. High repeatability and reproducibility of the injections were observed; therefore, the use of an internal standard was unnecessary.

3.2. Optimization of the extraction method

In any multi-mycotoxin method, the critical step is the extraction and clean-up procedure, specially, when the concentration of the analytes is around ppb levels. For this reason, extraction conditions had to be carefully selected to achieve the highest recovery for the mycotoxins contained in the cereals while eliminating most of the interfering matrix components.

Efficiency of MSPD extractions depends on type and quantity of dispersing phase, the amount of sample, and nature and volume of the eluting solvents. For the validation studies of the extraction, the most suitable elution solvents and the polarity of solid-phase were assessed. Malt was selected as model matrix due to its high possibility to present all the studied mycotoxins. The method was applied to the other matrix after its optimization.

3.2.1. Selection of the solid phase

Classic applications of the MSPD technique employ reversed-phase sorbent as dispersants. Octadecyl-silica (C_{18}) and octyl-silica (C_8) are by far the most often used. Theoretically, silica particles disrupt the gross architecture of biological samples whereas the bonded alkyl chains contribute to dissolving their components, providing relatively clean extracts from complex matrices when the polar solvents (acetonitrile, methanol and combinations of these) are used as extractants. In general, species of medium polarity are efficiently extracted under of these conditions.

Normal-phase, non-bonded sorbents (florisil, amino, phenyl and silica) have been proposed as dispersant in many MSPD applications. They interact with sample components solely by adsorption and, obviously, are not able to dissolve the sample matrix. The adsorption properties of these sorbents can be adjusted depending on their water content and acid or basic character.

The effect of these different sorbents on selected mycotoxins recoveries degree was studied, including the most representative as C_{18}, C_8, C_{18}-C_8, silica, florisil, phenyl, and amino. Recoveries obtained by using these solid-phases are analyzed in Fig. 2A. Here, the spiked malt samples at levels of 1 ng g^{-1} for all aflatoxins and 2 ng g^{-1} for OTA (LOQs levels) were used and, since most extraction methods reported for the extraction of mycotoxins in the literature acetonitrile was employed as a solvent, in a preliminary study.

The differences between the mean recoveries obtained with C_{18} and those obtained with phenyl, alumina, silica and florisil were statistical significance, but not those obtained with C_8. This fact was attributed to the preferential adsorption of the four solid phases (phenyl, florisil, silica and amino) by polar components.

These differences were dramatically significant in the case of the recoveries using florisil, amino and phenyl, with which the recovery values did not exceed 58.5% for AFG_2 employing phenyl. In the case of the silica, the recovery values were slightly higher than the other polar solid phases even though only in the case of AFG_1, AFG_2 and AFB_1. The presence of polar groups in the structure of aflatoxins, can

explain the interactions between these compounds and these polar solid phase that not allow their elution at the pass of the medium polar solvent as acetonitrile.

Owing to the good recovery results obtained by the use of C_8 and C_{18}, a mixture of these solid phases (50:50 p/p) was experimented. As it is reflected in the Fig. 2A, C_{18} applied alone provided higher recoveries for all the mycotoxins, maybe for the strong hydrophobic character of the first one, and eliminating the mix of solid-phase steps.

As a conclusion, and according to previous studies [24,25], C_{18} proved to be the best solid support providing high affinity for the studied compounds.

3.2.2. Study of extraction solvent

The nature of the elution solvent is an important matter since the target analytes should be efficiently desorbed while the remaining matrix components should be retained in the column. Solvents are characterized by their polarity and elution strength for a specific sorbent. The extraction solvent is often a compromise between the solvent strength required to efficiently extract mycotoxins from food and the compatibility of solvents with the analytical system.

The optimum extraction solvent was evaluated, checking a variety of solvent with very different polarities such methanol, dicloromethane, acetonitrile, ethyl acetate, hexane and mixtures of them to determine their ability to adequately elute OTA and aflatoxins on C_{18}. The only parameter changed was the type of solvent, maintaining the volume constant (10 ml). Results are presented in Fig. 2B. In this study, aqueous mixtures were discarded owing to the interaction between water and solid phase, leading a doughy consistency that makes the pass of the analytes difficult.

The most apolar organic solvents (ethyl acetate and dicloromethane) gave low mean recoveries for OTA and aflatoxins as the most polar solvent (methanol), for what it was necessary prove solutions of medium polarity as acetonitrile and mixtures of this solvent with apolar solvent (hexane) and with most polar solvent (methanol).

Regarding the acetonitrile solutions (80% of acetonitrile in all proves), adding a hexane part (20%), OTA was determined at similar levels but the aflatoxins recoveries were lower than 100% acetonitrile extraction. However, with methanolic portion (20%), the recoveries were much lower for all compounds than those obtained by acetonitrile extraction, maybe for the high polarity that this solvent has.

Considering these results and to avoid the mix step, the use of only one solvent was selected. Acetonitrile was considered the best organic solvent because of the acceptable recoveries for all the studied mycotoxins and because it gave the cleanest extracts and chromatograms.

3.2.3. Ratio of sample-to-sorbent

In MSPD, a critical parameter is the ratio between matrix and dispersing material. This ratio depends on the sample nature, although ratios of 1:1, 1:2 and 1:4 are frequently applied. For further optimization the sample amount and sorbent mass were varied to assay optimal conditions. The initial study was conducted applying the most usual sample/solid support material ratio.

To verify whether near optimum conditions were used, different amounts of C_{18} (1, 2, and 4 g) were added to the glass mortar and blended with 1 g of sample, and then elution was performed with 10 ml of acetonitrile. Results presented in Fig. 2C, showed that there were no significant differences among the recoveries of the target analytes.

With 1 g of C_{18} and 1 g of sample (ratio 1:1), recoveries were in acceptable range of 77.3–89.7% whereas when 2 and 4 g of C_{18} were used, the recoveries obtained for OTA and aflatoxins were less than 60% so any further increase of C_{18} did not improve the recovery of the studied compounds, maybe because the high dispersion of the sample into the solid-phase dispersant.

Table 2
Evaluation of matrix effects: comparison of the calibration curves slopes and calculation of signal suppression/enhancement (SSE) for selected mycotoxins in malt.

Compound		Slope	*y*-intercept	r^2	SEE
OTA	Solvent	36.421	161.46	0.9973	83
	Matrix-matched	30.255	−9.4998	0.9913	
AFB1	Standard	83.712	265	0.9965	48
	Matrix-matched	39.964	59.943	0.9897	
AFB2	Standard	35.963	84.735	0.9984	56
	Matrix-matched	20.07	30.327	0.9978	
AFG1	Standard	34.864	73.035	0.9974	53
	Matrix-matched	18.492	31.749	0.9952	
AFG2	Standard	63.193	126.22	0.9988	51
	Matrix-matched	32.322	−7.2808	0.9913	

SSE = (slope matrix-matched calibration/slope standard calibration in solvent) × 100.

3.2.4. Study of matrix effects

One of the main problems of LC–MS/MS is that the presence of matrix components can affect the ionisation of the target compounds, reducing or enhancing the response compared with standards in solvents, and the influence of the matrix effect on the response must be studied and, obviously, this affects the quantification, unless matrix effects are removed or compensated.

In order to evaluate matrix effects, the signal suppression–enhancement (SSE) for each analyte in each matrix was calculated, defined as the percentage of the matrix-matched calibration slope divided by the slope of the standard calibration in solvent.

For this objective, triplicate experiments by spiking LOQ level analyte free samples after the extraction and then following the remaining procedure reported in experimental section, were done to obtain a matrix-matched standard calibration for each matrix. The calibration curves showed high linearity ($r^2 > 0.9897$).

The matrix-matched curves slopes were compared with that of the calibration standards in solvent. Results obtained for malt as a representative matrix, are shown in Table 2, where it can be seen that notable signal suppression occurred for aflatoxins. These compounds presented slope ratios that indicate response reduction of 48% for AFB_1 and 56% for AFB_2. Therefore, a reliable quantification of these mycotoxins from food samples using LC–QqQ-MS requires malt-matched standards.

Moreover, it was considered that for accurate quantitative results, the main limitations are different matrices, as well as within given matrix. For this reason, the same experiment was carried out for the other matrices; coffee and instant cereal-breakfast beverage. The results are presented in Table 3. These matrices showed higher suppression matrix effect than those presented for malt. More concretely, SSE due to co-eluting matrix compounds was so pronounced in coffee (reaching a reduction response of 39% for AFG_1). This fact emphasized the necessity of carrying out (sample preparation and chromatography) method validation not only at different concentration but also using different matrices.

According to our results the use of matrix-matched standards calibration as it was reflected in the experimental section was required for the correct quantification of analytes and the data presented so far indicate that the most critical compounds are aflatoxins.

In practice, it is usual to prepare calibration curves for solvent and matrix to calculate the matrix effect by comparing the results.

Table 3
Calculation of signal suppression/enhancement (SSE) for OTA and aflatoxins B and G in coffee and instant breakfast beverage.

Mycotoxin	SSE (%)	
	Instant cereal-breakfast beverage	Coffee
AFB1	43	42
AFB_2	68	57
AFG_1	57	39
AFG_2	66	47
OTA	59	74

SSE = (slope matrix-matched calibration/slope standard calibration in solvent) × 100.

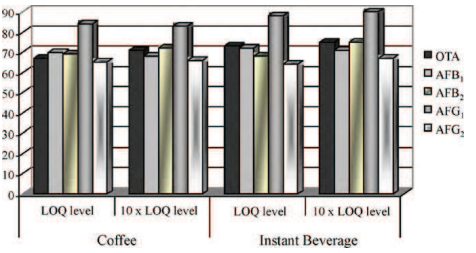

Fig. 3. Recoveries (%) obtained from spiked samples at LOQ levels and 10 times LOQ levels in coffee and instant cereal-breakfast beverage.

3.3. Validation of the method

In order to validate the developed procedure for each cereal sample, recoveries, repeatability as well as limits of detection (LODs) and limits of quantification (LOQs) were determined. The consequence of the latter matrix effects explained is the decrease of sensitivity in terms of LOD and LOQ. To compensate and improve the analytical parameters, validation is performed with a calibration curve for each analyte in real sample matrix and not in standard solution.

The LODs and LOQs were based on minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10 times the background chromatographic noise, respectively. Estimated values for all matrices of LODs were in the range of 0.8 ng g^{-1} for OTA and 0.3 ng g^{-1} for all aflatoxins, except for the aflatoxin AFG2 that was 0.4 ng g^{-1}, whereas LOQ values were in the range from 2 ng g^{-1} for OTA to 1 ng g^{-1} for the aflatoxins. The results are summarized in Table 4. The same table contains the maximum levels (MLs) for the studied mycotoxins set by the European Union (EU) considering all tested matrices.

As it can be observed LODs and LOQs were lower than the established ML (or at least similar to them), indicating that the proposed method is suitable for quantification of selected mycotoxins in the studied matrices. The main cereals contained in the composition of the instant breakfast beverage are barley, malt and chicory, for what their values are indicated in the table at individual form.

The recovery of the extraction step of each mycotoxin at two fortification levels (5 and 50 ng g^{-1} for OTA and 2 and 20 ng g^{-1} for aflatoxins) was studied, showing the obtained results in malt in Table 5. Recoveries and repeatability of the developed analytical method were carried out by injection of the same matrix-matched standard five consecutive times within the day (intra-day precision), and for five consecutive days (inter-day precision) for each analyzed compound in each selected matrix.

For all compounds mean recoveries in malt were satisfactory, ranging from 69% to 91%. The precision in the present study, estimated by the relative standard deviation (RSD) of the recovery was in the range of 7–19%. These results were very similar at those obtained in coffee and instant breakfast beverage as it can be observed in Fig. 3. From the results obtained, the developed method was found to be precise (with run-to-run instrumental RSD values between 7 and 19% and day-to-day RSD values between 8 and 17%). The method can thus be qualified as "acceptable" according to the EU criteria [27]; an average recovery ($n = 5$) between 70 and 120% and a repeatability (RSD) of 20% or less. The results of performance characteristics of the developed method are in good agreement with the performance criteria of the mentioned regulation.

The confirmation of positive samples was carried out by acquiring the full scan product ion spectra of the suspected compounds from a matrix-matched standard. The ion abundances were com-

Table 4
LODs, LOQs, and MLs for the three studied matrices.

ML (EU)						
Compound	LOD (ng g^{-1})	LOQ (ng g^{-1})	Coffee (ng g^{-1})	Malt (ng g^{-1})	Barley (ng g^{-1})	Chicory (ng g^{-1})
OTA	0.8	2	5	3	3	3
AFB_1	0.3	1	2	2	2	2
AFB_2	0.3	1				
AFG_1	0.3	1	4*	4*	4*	4*
AFG_2	0.4	1				

* Expressed as the sum of the four aflatoxins ($AFB_1 + AFB_2 + AFG_1 + AFG_2$).

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

Compound	Intra-day precision[a]				Inter-day precision[b]			
	Low level		High level		Low level		High level	
	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery
OTA	5	71 (11)	50	75 (9)	5	74 (10)	50	71 (12)
AFB_1	2	72 (13)	20	70 (10)	2	69 (14)	50	74 (12)
AFB_2	2	72 (9)	20	74 (7)	2	73 (9)	50	72 (8)
AFG_1	2	91 (14)	20	88 (12)	2	91 (12)	50	90 (13)
AFG_2	2	69 (18)	20	70 (19)	2	70 (17)	50	71 (17)

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

Table 6
LC–MS/MS ion ratios (A qualifying ion $^{(q)}$ /A quantifying ion $^{(Q)}$) for mycotoxin into matrix-matched sample and matrix sample.

Mycotoxin	Precursor ion	Product ion	Ion ratio expected[a,*] (RSD %)	Ion ratio observed[b,*]		
				Malt (RSD %)	Coffee (RSD %)	Instant breakfast beverage (RSD %)
AFB_1	313.2	$241^{(Q)}$ $269^{(q)}$	0.77	0.60 (5)	0.55 (6)	0.58 (9)
AFB_2	315.2	$243^{(Q)}$ $259^{(q)}$	0.50	0.55 (6)	0.59 (8)	0.57 (7)
AFG_1	329.2	$200^{(Q)}$ $215^{(q)}$	0.31	0.45 (11)	0.48 (10)	0.44 (10)
AFG_2	331.2	$189^{(Q)}$ $217^{(q)}$	0.37	0.35 (17)	0.40 (15)	0.39 (14)
OTA	404.2	$239^{(Q)}$ $358^{(q)}$	0.38	0.35 (7)	0.45 (6)	0.43 (9)

[a] Ratio determined in matrix-matched solution at LOQ level in acetonitrile ($n = 5$).
[b] Ratio determined in fortified sample at LOQ level in malt, coffee and instant cereal-breakfast beverage ($n = 5$).
[*] The EU guidelines [32] sets criteria for the observed ratio as follows; expected ratio >0.5, observed ratio should be within (20%, expected ratio 0.2–0.5, observed ratio should be within (25%; expected ratio 0.1–0.2, observed ratio should be within (30%; expected ratio <0.1, observed ratio should be within (50%).

pared with those calculated for fortified malt, coffee, and instant cereal-breakfast beverage samples. Table 6 lists the extracted fragment ions that were monitored to quantify and identify at the same concentration, and the calculated ratio of their abundances. Confirmatory analysis was found to be successful in all the cases. The quantification ion was the most abundant. The ratio of the two major products ions and the retention time deviation were within the interval established by the European Union Guidelines [27].

3.4. Application to differents samples

To evaluate the applicability of the method proposed, 22 samples were obtained from a local supermarket. 10 samples of malt, 7 samples of coffee and 5 samples of instant-based cereal-breakfast beverage. The results are statistically represented in Fig. 4.

Four samples of the total malt samples were positive for AFG_2 and AFG_1, and traces of AFB_1 and AFB_2 were detected. Although these levels were below the maximum level established by EU, they can indicate that more attention should be paid to storage conditions, in order to minimize the content of these analytes.

On the other hand, seven different coffee samples were collected and after the analysis, only two samples were positive for OTA, although the concentrations levels were lower than those established by the legislation.

Finally, none of the instant breakfast beverage analyzed presented traces of the mycotoxins studied.

Fig. 5 shows the chromatogram of a malt positive real sample, which maintains the good characteristics of those obtained from spiked samples.

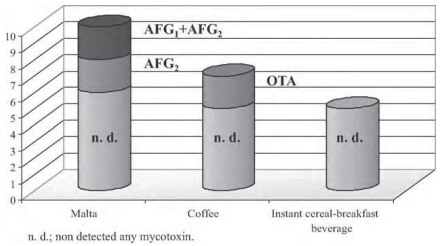

Fig. 4. Results of the analysis of 22 different real samples and the incidence of mycotoxins.

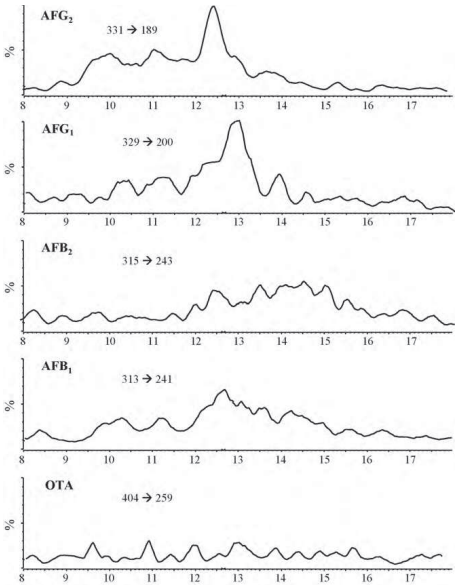

Fig. 5. Chromatogram of real malt sample that contains AFG1 and AFG2.

Although 6 samples gave evidence of contamination, this level did not exceed the ML fixed by the EU.

4. Conclusions

The MSPD method presented is a good starting point for further development of sample analysis in a single run and it can be regarded as a valuable alternative to the more classical sample preparation methods because it allows a significant reduction in both the sample size and solvent consumption needed for multi-residue analysis. Moreover, it offers a valid clean-up alternative to immunoaffinity columns, which are expensive and cannot be used to perform a multi-mycotoxin extraction, being also suitable for

routine analysis. It should be emphasize the necessity to optimize the solid-phase dispersant, the elution solvent and the ratio of the extraction method.

The results obtained in the extraction optimization confirm once again the need to carefully evaluate potential matrix effects. Only appropriate sample extraction, clean-up and good chromatographic separations allow us to considerably reduce matrix effects and to obtain the best method performances in terms of repeatability and accuracy of quantitative measurements.

This study showed that matrix effects vary from sample and from analyte, and it can considerably affect quantification accuracy. Therefore, for a full method validation, the matrix effects should be carefully evaluated on all analytes in each specific matrix under investigation.

In an application of the methodology, six out of 22 radom samples gave evidence of contamination, however these levels did not exceed the MRL fixed by the EU. These results suggest that it is important to monitor malt for the presence of aflatoxins and OTA, especially when it may be possible the store in dubious conditions. These amounts of OTA and AFs detected may be attributed to improper packaging and long storage time.

Although consumption of food with traces of mycotoxins does not in variably produce immediate or dramatic reaction, chronic exposure may have adverse effects on the consumers. For this, usually, OTA and aflatoxins analysis in these samples is required in the commercial transaction for minimizing the public health risk.

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4.3 One-year monitoring of aflatoxins and ochratoxin A in tiger-nuts and their beverages.

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Analytical Methods

One-year monitoring of aflatoxins and ochratoxin A in tiger-nuts and their beverages

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ABSTRACT

A sensitive and selective liquid chromatography–triple quadrupole–tandem mass spectrometry (LC–ESI–MS–MS) method was developed for the routine analysis of aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2) and ochratoxin A (OTA) in tiger nuts and tiger-nut beverage (horchata). A matrix solid phase dispersion was adapted to eliminate lipidic interferences. The solid support was C_{18}, while the elution solvent was acetonitrile. Mean recoveries obtained at two fortification levels were 72–83% and 71–81% for horchata and tiger nut respectively with relative standard deviations (RSDs) <13% and 15% respectively. The LC–MS–MS method allowed quantification and identification at low levels in two matrices. The method was applied for the routine analysis of tiger-nuts and horchata samples collected from different supermarkets of Valencia (Spain) during one year (March 2009–March 2010). A total of 238 samples were analysed and 32 samples were found positives for OTA, AFB_1, AFB_2 and AFG_2.

1. Introduction

In Spain tiger nut or chufa is widely used both for animal feed (chufa) and human consumption producing valencian horchata (a tiger nut milk drink). Horchata de chufa is a refreshing, non-alcoholic beverage of dairy appearance, called tiger-nut beverage, or orgeat, in English speaking country, originates from the town of Alboraya, to the north of Valencia. This typical product of Spain has a great economic importance since this country is the main producer (Sánchez-Zapata et al., 2009). The annual value of tiger nut production is close to five million euros (CRDO, 2009). In recent years, the popularity of horchata has been extended to other countries, such as the United Kingdom and France.

The processing of the tiger-nut to produce horchata is critical due to the presence of physical, chemical and biological contaminants, such as stones, pesticides, bacteria and fungi including *Fusarium spp.* and more fungi such as *Alternaria alternata*, *Aspergillus flavus*, *A. niger*, *Penicillium citrinum and Rhizopus arrhizus* (HACCP guide to elaborate tiger-nut beverage). It is demonstrated that *A. flavus* possess the ability to produce aflatoxins in tiger nuts (Bankole & Eseigbe, 1996). Furthermore, these mycotoxins could contaminate the tiger-nut beverages if no precaution is taken, arriving to the consumers (Arranz, Stroka, & Neugebauer, 2006).

Specifically, the International Agency for Research on Cancer (IARC) has classified AFB1 and natural mixtures of aflatoxins as group 1 (carcinogenic to humans); aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2). Ochratoxin A (OTA) has been classified as group 2B (possibly carcinogenic).

Nowadays, tiger nuts are cultivated in Northern Nigeria, Ghana, and Togo. These countries, and others like the Ivory Coast and India, export several tons of tubers every year to Spain. Despite of the official control, some doubts exist about the origin of tiger-nuts and its mycotoxin routine analysis in the horchata obtained with these tiger-nuts.

Thus, the aim of this study has been: (i) to develop a sensible and specific analytical method to determine aflatoxins and OTA at LOQs as low as possible; and (ii) to apply the method in a monitoring programme under strict quality assurance conditions to demonstrate the ruggedness of the total procedure. With this objective, a total of 238 real samples (both chufa and horchata) were purchased from different local markets, bars and ice-cream parlours.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile, methanol, hexane, ethyl acetate and dichloromethane were supplied by Merck (Darmstadt, Germany). Solid-phase used for the extraction was octadecylsilica (C_{18}) (50 μm) bonded silica from Analisis Vinicos (Tomelloso, Spain). The standards of aflatoxins (B_1, B_2, G_1, G_2), and ochratoxin A (OTA) were supplied by Sigma–Aldrich (Madrid, Spain). The individual stock solutions of aflatoxins and ochratoxin A with concentration 500 μg/ml were prepared in acetonitrile, kept in security conditions at −20 °C. All

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the working standard solutions were prepared immediately before use by diluting the stock solution with acetonitrile. Ammonium acetate (MS grade) was bought from Sigma–Aldrich (Madrid, Spain). Water for LC mobile phase was purified successively by reverse osmosis and a Milli-Q plus system from Millipore (Molsheim, France).

2.2. Samples and sampling

Sampling was carried out according to the EU guidance (EU, 2006a). Samples of tiger-nuts were bought from supermarkets, street vendors and little candy shops that sell nuts whereas horchata (tiger-nut beverages) were obtained from different supermarkets, street vendors, juice bars and ice-cream parlours and were stored at 4 °C until they were analysed. A total of 48 tiger-nuts and 190 horchata (including sterilized, concentrated, pasteurised and fresh serving) samples were investigated.

2.3. Extraction

Sample preparation was performed as described in a previous study of these matrices (Sebastià, Soler, Soriano, & Mañes, 2010). An aliquot of the sample (1 mL of horchata or 1 g of chufa) was placed into a mortar and 2 g of the C18 sorbent was added and they were blended using a pestle to obtain a homogeneous mixture. The mixture was introduced into a glass tube of centrifuge (Pirex) and 10 ml of hexane was added and the content was thoroughly mixed. Then the tubes were placed into a centifuge at 2490*g* for 15 min. The extract was thrown away and the solid was dried up with a stream of N_2. After that 10 ml of acetonitrile was added and centrifuged for a second time at 4500 rpm for 15 min. The extract was evaporated to dryness with gentle stream of N_2 at 45 °C. After, the extract was reconstituted in 1 ml of acetonitrile for LC–QqQ–MS.

2.4. Liquid chromatography–mass spectrometry analysis

Detection was performed as described in a previous study of these compounds (Rubert, Soler, & Mañes, 2010). The triple quadrupole mass spectrometry detector (QqQ) was equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) that included an autosampler and a quaternary pump. Separation was attained on a Phenomenex (Madrid, Spain) Gemini C_{18} (250 mm × 4.6 mm I.D., 5 μm particle size) analytical column, preceded by a security guard cartridge C_{18} (4 mm × 2 mm I.D.), using a gradient that started at 35% of 5 mM ammonium acetate in water (A) and 65% of 5 mM ammonium acetate in acetonitrile (B) during 3 min. After, it was increased linearly to 95% of B in 4 min, and held constantly for 3 min. Then, the gradient backs to the initial conditions during 10 min. The flow rate was 0.25 ml min^{-1}, and 20 μl of standard solutions or extract were injected.

A QqQ mass spectrometer Quattro LC from Micromass (Manchester, UK); equipped with pneumatically assisted electrospray probe, a Z-spray interface and a Mass Lynx NT software Ver. 4.1 was used for MS/MS analyses. Parameters were optimised in positive and negative mode by continuous infusion of a standard solution (10 μg ml^{-1}) via syringe pump at a flow rate 20 μl min^{-1}. Analysis was performed in positive ion mode. The ESI source values were capillary voltage, 3.50 kV; extractor, 1 V; RF lens 0,5 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 l h^{-1}. Cone voltages and collision energies were optimised for each analyte during infusion of the pure standard and the most abundant fragment ion chosen for the selected reaction monitoring. The analyser setting were: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energies, 0.5; entrance and exit energies, 1 and 3; multiplier, 650; collision gas (argon, 99.99% purity) pressure 3.74×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.2 ms. The mass spectrometer was operated in scan, product ion scan, and multiple reaction monitoring (MRM) modes. All the measurements were carried out in triplicate.

3. Results and discussion

3.1. Procedure performance

The present study is a combination of two published methods; on the one hand an extraction method based on a solid phase extraction (SPE)-centrifuge assisted (Sebastià et al., 2010) for determination of aflatoxins in tiger-nuts and horchata. On the second hand a detection method using LC-QqQ-MS for determining aflatoxins and OTA in coffee, barley and cereal-beverages (Rubert et al., 2010). The MS parameters optimised in this study are summarised in Table 1.

Although these methods have demonstrated its capability for analysing these mycotoxins, with the objective of to get as much information on contamination and occurrence of these mycotoxins as possible, an investigation of its combination was carried out.

3.2. Validation of the method

In order to validate the developed procedure for each matrix, recoveries, repeatability as well as limits of detection (LODs) and limits of quantification (LOQs) were determined. The LODs and LOQs were based on minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10 times the background chromatographic noise, respectively. Estimated values for horchata of LODs were in the range of 0.75 ng ml^{-1} for OTA, 0.2 ng ml^{-1} for AFB_1 and 0.3 ng ml^{-1} for AFB_2, AFG_1 and AFG_2 whereas LOQ values were in the range from 2 ng ml^{-1} for OTA to 0.75 ng ml^{-1} for AFB_1 and 1 ng ml^{-1} for AFB_2, AFG_1 and AFG_2. However, these values were different for tiger nuts: LOD ranged between 1 ng g^{-1} for OTA, 0.3 ng g^{-1} for AFB_1 and 0.4 ng g^{-1} for the rest of aflatoxins whereas LOQ values were in the range from 3 ng g^{-1} for OTA to 1 ng g^{-1} for AFB_1 and 1.2 ng g^{-1} for the rest of aflatoxins. The results are summarised in Table 2 and are coherent to the previous work (Rubert et al., 2010).

Since the goal of this work was to provide information on the occurrence of aflatoxins and OTA in tiger-nuts and horchata and a rough estimate of the related concentrations, a detailed investigation on the extraction efficiencies and matrix effects in the different matrices were deeply studied. For the matrix effects evaluation, six concentrations between LOQ and 10 times LOQ

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

Mycotoxin	Retention time (min)	Precursor ion	Product ion	Cone	Collision energy
AFB_1	12.98	313.2	241[Q]	47	30
			269[q]		30
AFB_2	12.75	315.2	243[Q]	50	30
			259[q]		30
AFG_1	12.37	329.2	200[Q]	43	40
			215[q]		30
AFG_2	11.87	331.2	189[Q]	46	45
			217[q]		25
OTA	11.23	404.2	239[Q]	20	20
			358[q]		15

[Q] Quantification transition.
[q] Confirmation transition.

Table 2
Validation parameters calculated at two concentration levels and relative standard deviations (RSD) (%) given in brackets.

Compound	Horchata						Tiger-nuts					
	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	*Intra-day*		*Inter-day*		LOD (ng g^{-1})	LOQ (ng g^{-1})	*Intra-day*		*Inter-day*	
			REC[a]	REC[b]	REC[a]	REC[b]			REC[a]	REC[b]	REC[a]	REC[b]
OTA	0.75	2	78 (11)	76 (10)	76 (11)	79 (9)	1	3	73 (8)	78 (11)	79 (10)	78 (9)
AFB_1	0.2	0.75	80 (5)	74 (7)	79 (9)	72 (5)	0.3	1	75 (6)	80 (3)	79 (7)	81 (6)
AFB_2	0.3	1	75 (8)	80 (10)	78 (13)	76 (10)	0.4	1.2	74 (15)	78 (10)	76 (9)	73 (13)
AFG_1	0.3	1	81 (11)	83 (8)	80 (13)	81 (10)	0.4	1.2	80 (10)	77 (8)	78 (10)	79 (14)
AFG_2	0.3	1	72 (9)	73 (7)	78 (8)	74 (4)	0.4	1.2	71 (14)	76 (12)	75 (9)	72 (11)

[a] Recoveries at LOQ concentration level.
[b] Recoveries at 10 × LOQ concentration level.

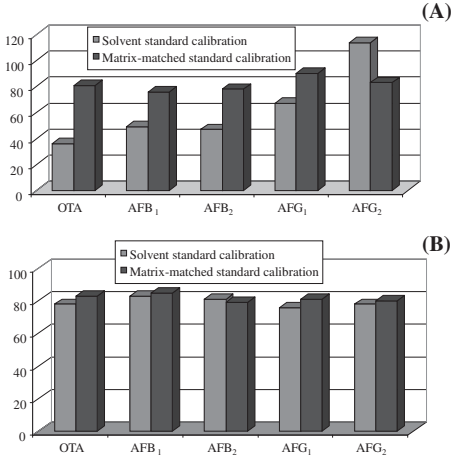

Fig. 1. Matrix effects (%) in (A) tiger nuts and (B) horchata. Solvent standard calibration: slope matrix-matched standard calibration/slope solvent standard calibration × 100. Matrix-matched standard calibration: slope spiked sample calibration/slope matrix-matched standard calibration × 100.

levels were analysed in acetronitrile and in spiked sample extracts, and the slopes of the calibration curves were compared by an analysis of covariance. Fig. 1 shows the obtained results (Fig. 1A matrix effects in tiger nuts and Fig. 1B matrix effects in horchata). In all cases, the calibration curves showed high linearity ($r^2 > 0.99$).

It can be noted that significant signal suppression occurred for OTA and aflatoxins, (except for AFG_2 that an enhancement signal occurred) when tiger-nuts were analysed. Although in the case of horchata a slight suppression of the signal it was observed, it can be considered in acceptable range in accordance with the EU guidelines (matrix effects (ME)% between 70% and 120%) (EU, 2002). This fact could be explained by the different chemical composition of the matrices: it is well-known that horchata is a beverage of tiger-nuts and obviously, its major component is water. However, tiger-nuts have about 25% of lipids, 10% of proteins and high portions of pigments which can be extracted and eluted at the same time as the analysed mycotoxins, interfering its signal.

Therefore, for reliable quantification of these mycotoxins in tiger-nuts, matrix-matched standards calibration is required to compensate and improve the analytical parameters. In the same Fig. 1 it is represented the ME calculated when matrix-matched calibration (black bar) was applied in the case of tiger nuts, confirming its capability to correct the matrix effect problems (ME% between 76% and 90%).

Bearing in mind the short extraction time and chromatographic analysis, the preparation and injection of one matrix-matched calibration does not increase the analysis time per batch considerably.

Recoveries and repeatability of the developed analytical method were carried out by injection of the spiked samples at the two concentration levels (LOQ concentration level and 10 times LOQ concentration level) five consecutive times within the day verifying the repeatability (intra-day precision), and for five consecutive days verifying the reproducibility (inter-day precision) for each analysed compound in each selected matrix.

For all compounds mean recoveries in horchata and tiger-nuts were satisfactory, ranging from 72% to 83% and from 71% to 81% respectively. The precision in the present study, estimated by the relative standard deviation (RSD) of the recovery was in the range of <13% for horchata and <15% for tiger-nuts. The results of performance characteristics of the developed extraction method are in good agreement with the performance criteria of the EU criteria (EU, 2002).

Fig. 2 shows the chromatogram of spiked tiger-nut and horchata sample at LOQs levels under the optimum chromatographic conditions commented above.

3.3. Monitoring residue in marketing tiger-nuts and horchata

The method was applied to the determination of the studied mycotoxins in horchata and tiger-nuts samples obtained from local markets over one-year. These samples comprise 48 tiger-nuts and 190 horchata, being analysed a total of 238 samples. An internal quality control was carried out for every batch of samples to check if the system is under control, and it implies a matrix-matched tiger nut calibration, a matrix blank and a spiked tiger nut blank sample at LOQ levels.

The tiger-nuts samples were purchased in different local markets, differencing between the organic and conventional harvest and different sorts of tiger nuts. Only 12 of them were identified with the Protected Origin Designation from Valencia. The other 36 samples were of unknown origin.

Regarding the horchata samples, they included different trademarks, different presentations as concentrated, sterilized and pasteurised, with or without sugar (light), freezer and with different ingredients as chocolate or lemon. This work was carried out in Valencia and obviously a great number of samples (around 110) were fresh provided by street vendors, juice bars and ice-cream parlours from the Valencian Community mainly between March and October. Also in the case of horchata samples, both packed and fresh, most of them were unknown origin since they were not identified with the Protected Origin Designation from Valencia.

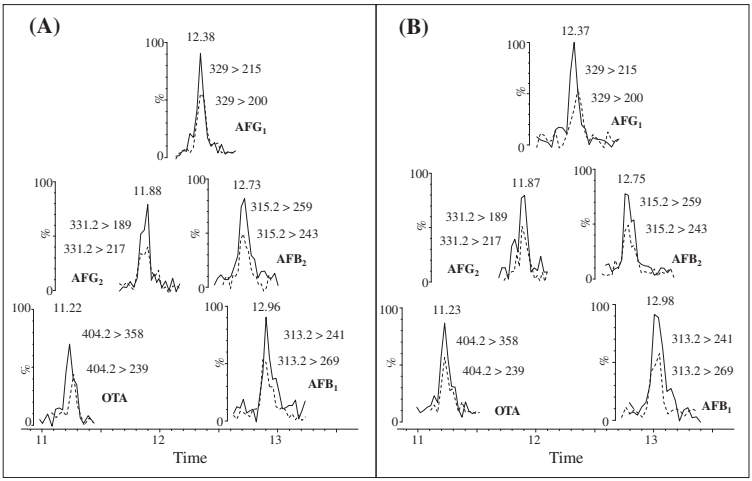

Fig. 2. LC/MS/MS chromatograms of a standard of mycotoxins in (A) tiger nuts and (B) horchata at LOQ levels with the quantifier and qualifier ions for each compound.

Table 3
Occurrence of aflatoxins and OTA in tiger-nuts (n = 48) and horchata (n = 190).

	Horchata		Tiger-nuts	
	Positives	Range concentrations (ng ml^{-1})	Positives	Range concentrations (ng g^{-1})
OTA	2	2–2.4	6	3.5–11
AFB_1	12	0.8–1.7	14	1.2–2
AFB_2	2	1.1–1.6	3	1.5–1.8
AFG_1	n.d.		n.d.	
AFG_2	1	1.2–2.3	8	1.3–3.8
Total	15		17	

Table 3 shows the results obtained in total analysed samples. Maximum levels of total aflatoxins and AFB_1 are laid down in European legislation (EC, 2006b) as 4 and 2 μg kg^{-1}, respectively, present in groundnuts and nuts and processed products thereof, intended for direct human consumption or use as an ingredient in foodstuffs. It is not specified anything about OTA, so the criteria "as low as possible" was applied.

In our study a total of 32 samples were confirmed as positive samples. The confirmation of positive samples was carried out, according to the European Commission (EU, 2006a), by acquiring the full scan product ion spectra of the suspected compounds from

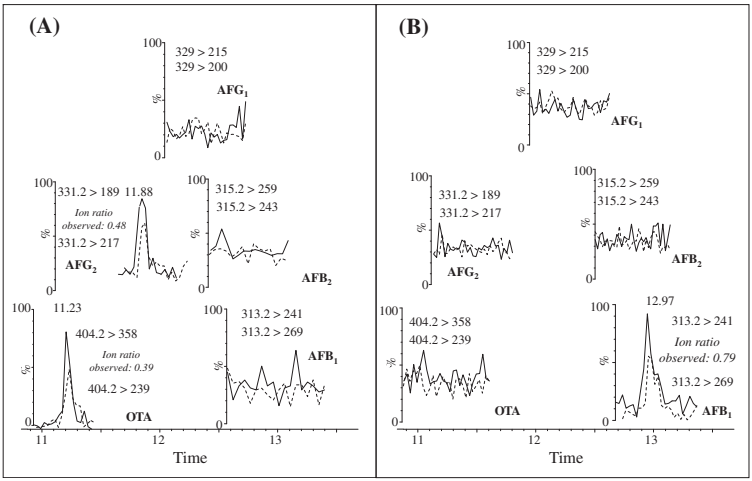

Fig. 3. Chromatogram of positive (A) tiger nut sample that contains OTA and AFG_2 and (B) horchata sample that contains AFB_1.

a matrix-matched standard. The ion abundances were compared with those calculated for fortified samples. Confirmatory analysis was found to be successful in all the cases.

These positive samples for both, horchata and tiger nuts, were of unknown origin.

In the case of the tiger nuts (Table 3), 9 of the 17 positives samples were from ecological harvest. Only two samples exceeded the ML established by EU for nuts: one sample contained AFB_1, AFB_2 and AFG_2 (1.3 ng g^{-1}, 1.7 ng g^{-1} and 1.5 ng g^{-1}, respectively) and in the second sample was confirmed the presence of AFB_2 and AFG_2 (1.8 ng g^{-1} and 3.1 ng g^{-1} respectively). No one of these samples were identified as a ecological product.

Moreover, it was demonstrated the co-occurrence of aflatoxins and OTA in four tiger nut samples, making questionable the treatment of these samples along the food chain (transport, storage, cleaning, etc.).

Of the total 15 positive samples of horchata, 9 corresponded to fresh horchata, 3 to pasteurised and 3 to refrigerated horchata. Three of them contained additional ingredients as chocolate or lemon. No contamination was observed in the case of the sterilized and concentrated horchata. The first one is a high temperature treatment that reduces the fungal growth. The second treatment increases the sugar concentration to levels that interferes the fungal growth. Obviously, two of them are strong food technologies that alter the quality of the horchata.Fig. 3 shows chromatograms of positive samples, which maintain the good characteristics of those obtained from spiked samples.

4. Conclusions

In an application of the methodology, 32 out of 238 random samples gave evidence of contamination, although only two tiger-nuts samples exceed the ML fixed by the EU for a similar products as nuts. These results reveal the importance to monitor aflatoxins and OTA in tiger-nuts, especially when the store and transport could be in dubious conditions. Amounts detected of OTA and AFs in this study may be attributed to improper packaging and long storage time. Although consumption of food with traces of mycotoxins does not in variably produce immediate or dramatic reaction, chronic exposure may have adverse effects on the consumers. It is important to keep in mind two facts: (1) the important world-wide increase in consumption of horchata in the summer period that increase the daily intake and (2) the fact that almost of the horchata consumed world-wide is exported from Spain market making evident the necessity of a control of the raw materials. For this, it is so important the analysis of OTA and aflatoxins in raw and final materials and to verify the quality control is required in the commercial transaction before to commercialise the tiger-nuts and to elaborate the horchata for minimising the public health risk.

Acknowledgement

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4.4 Evaluation of Matrix Solid-Phase Dispersion (MSPD) extraction for multi-mycotoxin determination in different flours using LC-MS/MS.

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Evaluation of matrix solid-phase dispersion (MSPD) extraction for multi-mycotoxin determination in different flours using LC–MS/MS

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ABSTRACT

An existing matrix solid-phase dispersion (MSPD) method for aflatoxins (AFs) and ochratoxin A (OTA) extraction was extended by further 14 mycotoxins. After it careful optimization, this method was applied to determine the occurrence of these mycotoxins on commercial flour samples (with different cereals composition) collected from local markets. In a total of 49 samples investigated, 9 mycotoxins were identified. Nivalenol (NIV) and Beauvericin (BEA) were the mycotoxins found most frequently. The samples that presented major contamination were wheat flours and bakery preparations. Despite of the great number of positives finding, only one wheat flour sample exceeded the maximum limits (ML) for OTA established by the European Union (EU). However, it would be interesting to calculate the total ingest of these mycotoxins along the years.

1. Introduction

The contamination of food by the intentional use of chemicals, such as pesticides or veterinary drugs, is a worldwide public health concern [1]. However, extensive contamination of food and drinks with natural toxins as mycotoxins is the main problem over the world since they can also compromise the safety of food and feed supplies and adversely affect health in humans and animals [2,3]. Owing to the toxicity and carcinogenic risk of these mycotoxins, there have been established European maximum levels (MLs) directed toward the control of these toxins in food, but only those toxins that pose a major risk (aflatoxins (AFs), ochratoxin A (OTA) and patulin (PAT)) had been regulated during years.

It was not until 2003, when the European Union started to develop new community legislations which have included, year after year, new groups of mycotoxins. Concretely, in 2006, it was published the Directive (CE) 1881/2006 which establishes MLs for 12 mycotoxins in different food commodities [4]. That directive was followed by other modifications, as the modification of 2007 based on *Fusarium* toxins in maize and maize products [5], the latest decisions for OTA in spices and liquorice [6] and the latest update for AFs in foodstuffs that it was in 2010 [7]. The need to apply these directives makes indispensable the accurate quantification of these mycotoxins in different commodities to evaluate their intake, as well as its associate risks, enabling to establish prevent measures that could protect the health of the consumers [8–15].

There exists around over 400 mycotoxins, but the well-known mycotoxins are fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), trichothecenes as deoxynivalenol (DON), nivalenol (NIV), the toxin T-2 (T-2), toxin HT-2 (HT-2) and diacetoxyscirpenol (DAS), aflatoxins B_1 (AFB_1), B_2 (AFB_2), G_1 (AFG_1), G_2 (AFG_2), ochratoxin A and zearalenone (ZEN), because they are the wide distributed. For this reason these mycotoxins can usually interfere in the safety food of the consumer [1,16].

These mycotoxins are produced for such genera as *Aspergillus*, *Fusarium* and *Penicillium*, but these genera, together to other genera as *Claviceps* and *Alternaria*, can also produce other mycotoxins less studied and less known, named "emergent" or "new mycotoxins". Focusing on these "emergent mycotoxins" it would be interesting to cite BEA, a mycotoxin generated by *Fusarium* genera and which is starting to be detected in some foods [17].

Nowadays, the trend in mycotoxins analysis is the development of multi-mycotoxin methods able to cover, in a single analysis, all the mycotoxins considered by the EU food legislation [18,19]. However, several difficulties are found to reach this objective. First, the mycotoxins exhibit a wide range of different physicochemical properties in terms of pH stability, solubility, diversity of chemical structure and molecular weight [20]. Second, very different maximum levels are admitted for mycotoxins as a function of their toxicity and type of food. Finally, the matrix food composition under study is also highly variable [21].

In this way, one of the main problems to develop a multi-mycotoxin method is the extraction and purification in a single step of the all mycotoxins from the matrix, owing to the great differences in their physicochemical properties presented for these compounds. In fact, the extraction is the most critical step since it

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should determine the recoveries for all mycotoxins under investigation in a specific food matrix [22].

An attractive alternative is the matrix solid-phase dispersion (MSPD), where the sample and sorbent material are mixed homogenously; this mixture is then packed in cartridge and afterwards elution is performed [23].

Operational steps in MSPD, and efficiency and selectivity of the extraction process, are conditioned by a number of factors, for example: the physical state of the sample, the relative concentrations and properties of analytes, the interferences of the sample, the suitable combination of sorbent, etc. To start the optimization, the attention must fall upon the careful selection of sorbent materials and elution solvents to enhance the yield of the extraction [24].

Despite of its demonstrated advantages, MSPD is not still a mainstream sample-preparation technique in the mycotoxins field, and it use is limited to few reports as determination of AFs in peanuts, chilli powder, olive oil and hazelnuts [25–28], AFs and OTA in coffee, malt and cereal beverage [29], trichothecenes in corn flour [30] and PAT in apple and apple juice [31].

In the present work, a previous MSPD–LC/MS/MS method [29] was further extended and applied for the simultaneous determination of AFs, fumonisins, trichothecenes, OTA, ZEN and BEA in flour. To reach this objective, it was evaluated in detail the performance and features of the methodology in terms of cleanliness of the extracts, efficiency of the extraction (recoveries), analytical performance, matrix effects and sensitivity (limits of detection).

2. Materials and methods

2.1. Chemical and reagents

Acetonitrile, methanol, hexane, ethyl acetate and dichloromethane were supplied by Merck (Darmstadt, Germany). Solid-phases used for MSPD were silica, amino, basic alumina, acid alumina, neutral alumina, celite, phenyl, octy-silica (C_8) (50 μm) and octadecy-silica (C_{18}) (50 μm) bonded silica from Analisis Vinicos S.L. (Tomelloso, Spain). Florisil® (60–100 mesh) was obtained from Aldrich (Steinheim, Germany).

The standards of AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEN, NIV, DON, DAS, FB_1, FB_2, BEA were purchased from Sigma–Aldrich (Madrid, Spain). T-2 and HT-2 toxin stock solution (in acetonitrile), as internal standard Deepoxy-deoxynivalenol (DOM-1) and Aflatoxin M_1 (AFM_1) stock solution (in acetonitrile) were obtained from Biopure referenzsubstanzen GmBH (Tulln, Austria).

The individual stock solutions of AFs, OTA, ZEN, NIV, DON, FB_1, FB_2, BEA at concentration of 500 μg ml^{-1} were prepared in methanol. On the other hand, stock solutions of DAS, T-2 and HT-2 at concentration of 100 μg ml^{-1} were prepared in methanol. Internal standard compounds used were AFM_1 at 0.05 μg ml^{-1} and DOM-1 at 0.150 μg ml^{-1} were prepared by dilution of individual stock solutions in methanol. All these solutions were prepared and kept in safety conditions at −20 °C.

All other working standard solutions were prepared immediately before use by diluting the stock solution with methanol.

Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade reagent formic acid (purity > 98%), and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

Reference material was purchased from Biopure referenzsubstanzen GmBH (Tulln, Austria).

2.2. Matrix solid phase dispersion

Samples (200 g) were prepared using a food processor and mixed thoroughly. Portions of 1 g were weighed and placed into a glass mortar (50 ml) and were gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain homogeneous mixture. For the preparation of fortified samples, 0.2 ml of the standard working solution was added to 1 g of sample. Then, they were allowed to stand at room temperature for 3 h before the extraction for the evaporation of the solvent and the equilibration between the mycotoxins and the flour sample. The homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 20 ml of acetonitrile/methanol (50/50, v/v) 1 mM ammonium formate by applying a slight vacuum.

Then, extract was transferred to 25 ml conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 ml with methanol and filtered through a 13-mm/0.45-μm nylon filter purchased from Análisis Vínicos S.L. (Tomelloso, Spain) before the injection of 20 μl into the LC–MS/MS system.

2.3. Matrix effect measurements

The matrix effect (ME) was assessed by employing matrix-matched standards. MS/MS areas of known amounts of standards (A) were compared with those measured in a blank multicereal flour extract spiked after extraction with the same analyte amount (B). The ratio ($B/A \times 100$) is defined as the absolute matrix effect (ME %). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%. Tests were conducted in triplicate on blank multicereal flour samples, originating from a local supermarket, and spiked to obtain the experimental concentration of each mycotoxin for the HPLC-QqQ-MS/MS analysis. Flour samples were first examined for the presence of possible contaminants.

In order to evaluate the possible differences between flour varieties, three flour samples of different cereal (wheat, rice or corn) composition were used to calculate matrix effects.

2.4. Liquid chromatography–mass spectrometry

The triple quadrupole mass spectrometry detector (QqQ) was equipped with an LC Alliance 2695 system (Waters, Milford, MA, USA) that included an autosampler and a quaternary pump. Separation was attained on a Phenomenex (Madrid, Spain) Gemini-NX C_{18} (150 mm × 4.6 mm I.D., 5 μm particle size) analytical column, preceded by a security guard cartridge C_{18} (4 mm × 2 mm I.D.), using a gradient that started at 100% of A (5 mM ammonium formate and 0.1% of formic acid in water) and 0% of B (5 mM ammonium formate in methanol), increased linearly to 100% B in 10 min. After, it was decreased linearly to 80% of B in 5 min and it was gradually decreased to 70% B in 10 min. Afterwards, the initial conditions were maintained for 5 min. Flow rate was maintained at 0.3 ml min^{-1}.

A QqQ mass spectrometer Quattro LC from Micromass (Manchester, UK) equipped with pneumatically assisted electrospray probe, a Z-spray interface and Mass Lynx NT software Ver. 4.1 was used for MS/MS analyses. Parameters were optimized in positive (ESI+) and negative (ESI−) ionization mode by continuous infusion of a standard solution (10 μg ml^{-1}) via syringe pump at a flow rate of 20 μl min^{-1}. Finally, analysis was performed in ESI+ and ESI−. The ESI source values were capillary voltage, 3.50 kV positive ionization mode and 3 kV negative ion mode; extractor, 1 V; RF lens 0.5 V; source temperature, 120 °C; desolvation temperature, 400 °C; desolvation gas (nitrogen 99.99% purity) flow, 800 l h^{-1};

cone gas 50 l h^{-1} (nitrogen 99.99% purity). Cone voltages and collision energies were optimized for each analyte during infusion of the pure standard and the most abundant fragment ion chosen for the selected reaction monitoring. The analyzer setting were: resolution 12.0 (unit resolution) for the first and third quadrupoles; ion energies, 0.5; entrance and exit energies, 5 and 3; multiplier, 650; collision gas (argon, 99.99% purity) pressure 3.83×10^{-3} mbar; interchannel delay, 0.02 s; total scan time, 1.0 s; dwell time 0.1 ms. The mass spectrometer was operated in scan, product ion scan, and single reaction monitoring (SRM) modes. All the measurements were carried out in triplicate.

2.5. Method validation

The method was validated for linearity, accuracy, precision and sensitivity.

Linearity was evaluated using the standard calibration curves that were constructed for each mycotoxins by plotting the signal intensity versus the analyte concentration and the internal standard calibration curves were constructed from the peak area ratio of each analyte to the corresponding I.S. In the same manner, matrix-matched standards of the studied mycotoxins were prepared using MSPD sample treatment, by adding known amount of working solution to the obtained extracts in order to reach the desired concentration range and the signal intensities obtained were plotted.

Recovery experiments were conducted at two concentration levels between 0.25 and 85.24 $\mu g\,kg^{-1}$ (limits of quantification, LOQs) and between 2.5 and 852.4 $\mu g\,kg^{-1}$ (10 times LOQs) added before the corresponding extraction procedure. Intraday precision was assessed by calculating the RSD of six determinations per concentration in a single day and interday precision by one determination per concentration on 6 days. In case of calculations with internal standards, areas ratios (area analyte/area internal standard) were used.

Sensitivity was evaluated by limit of detection (LOD) and limit of quantification (LOQ) values. The LOD was estimated from blank extract, spiked with decreasing concentrations of the analytes, where the response of the qualifier ion was equal to 3 times the response of the blank extract. Once evaluated, three samples were spiked at the estimated levels and extracted according to the proposed procedure. The LOQ (coefficient of variation $\leq$19% and an accuracy $\geq 70 \pm 19\%$) was preliminarily estimated, in the same way as the LOD, but using the criterion of $S/N \geq 10$ for the qualifier ion.

2.6. Application to commercial sample

Around 50 flour samples with different cereal composition (wheat, corn, rice, soy, oats, etc.) were collected from several local markets of Valencia (Spain) and analyzed in order to investigate the presence of selected mycotoxins. Samples were stored in plastic (high density polyethylene) containers and stored in the dark at $<-18\,^{\circ}C$ until analyses.

In every sequence of analysis, multicereal flour (blank sample previously analyzed) MSPD extracts were injected by duplicate between two calibration curves. Recoveries were considered satisfactory if they were in the range 70–120% for every analyte.

Confirmation of positive findings was carried out by calculating the peak area ratios between the quantification (Q) and confirmation (q) transitions and comparing them with ion-ratios obtained from a reference standard. The sample was considered positive when the experimental ion-ratio fulfilled the tolerance range, according to EU Decision 2002/657/EC [32].

3. Results and discussion

3.1. Optimization of liquid chromatography–mass spectrometry

3.1.1. Optimization of MRM mode

As in the previous work [29], preliminary experiments were conducted to find instrumental conditions that allow unambiguous identification of the analytes in real samples at low levels. The optimization of MS and MS/MS parameters were carried out by infusing individual solutions of the analytes. All the mycotoxins showed acceptable sensitivity in ESI+ to undertake food analysis, excepting ZEN that it was detected better in ESI− than ESI+. The results are summarized in Table 1.

According to Commission Decision 2002/657/EC, two specific transitions must be acquired for each compound reaching the minimum number of identification points (IPs) required for unambiguous confirmation [32]. In the present work, two transitions for reliable confirmation were possible for all the mycotoxins analyzed (Table 1). Given the high number of transitions (28 without I.S. and 32 when the I.S.s were used) to be monitored, SRM detection was separated in eight segments, in order to perform detection with sufficient instrumental sensitivity. Each segment included 1–5 substances, based on its retention time, as it is shown in Table 1.

Regarding the chromatographic analysis, due to the different physical–chemical characteristics of the mycotoxins selected, it was necessary to find a compromise to guarantee a sharp peak shape and reproducible retention times. With this goal, several mobile phases which varied in concentration and type of buffer (ammonium formate and ammonium acetate), pH (by addition of formic or acetic acids) and organic solvent (methanol or acetonitrile) were tested. The final selected mobile phase is described in the experimental section.

For the choice of gradient elution programme, results of multiple injections indicated that a gradient that starts with a high percentage of water is required, being more sensible the detection of NIV owing to it high polarity. After this step, the percentage of methanol was increased as it is indicated in experimental section, achieving good peak shape and high sensitivity. Fig. 1 illustrates typical ion chromatograms obtained under selected time-scheduled conditions for multicereal flour spiked with the mycotoxin mixture at concentration of LOQ level, providing evidence that the LC optimized conditions fulfilled the separation requirements.

Our results were according to the literature about multi-mycotoxin methods for food published [20,24,33–36]. However, in none of them MSPD has been applied.

3.2. Optimization of the sample pre-treatment

As this work supposed an expansion of the previous work [29], the authors decided to study the same parameters in this experiment, to get as much information on MSPD as possible. With this objective, the most suitable elution solvents and the polarity of solid-phase were assessed. For the optimization and development of the extraction, all the tests were performed in triplicate. It must be kept in mind that these conditions were a compromise resulting from the chemically diverse set of mycotoxins and may be far from optimal for some compounds.

3.2.1. Solvent extraction selection

In a first series of experiments, different extraction solvents or mixtures of them, and the volume required were tested. Since the study included different compounds (from polar compounds as NIV to apolar compounds as BEA) the extraction solvent was evaluated, checking a variety of solvents with different polarities such methanol, dichloromethane, acetonitrile, ethyl acetate,

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

Mycotoxin	Precursor ion (*m/z*)	Time window (range min)	Product ion	Cone (V)	Collision energy (eV)	
NIV	313.0 $[M+H]^+$	1 (10.5–13)	125^{Q} 177^{q}	23	12	12
DON	297.2 $[M+H]^+$	2 (11–14)	249^{Q} 203^{q}	20	10	10
DOM-1 (IS)	281.1 $[M+H]^+$		108^{Q} 137^{q}	20	14	14
AFB_1	313.2 $[M+H]^+$		241^{Q} 269^{q}	47	30	30
AFB_2	315.2 $[M+H]^+$		243^{Q} 259^{q}	50	30	30
AFG_1	329.2 $[M+H]^+$	3 (13.5–17)	200^{Q} 215^{q}	43	40	30
AFG_2	331.2 $[M+H]^+$		189^{Q} 217^{q}	46	45	25
AFM_1 (IS)	329.2 $[M+H]^+$		273^{Q} 259^{q}	30	20	20
DAS	384.0 $[M+NH_4]^+$	4 (14.5–16.5)	307^{Q} 105^{q}	15	15	45
FB_1	722.0 $[M+H]^+$	5 (14–18)	334^{Q} 352^{q}	50	25	20
FB_2	706.4 $[M+H]^+$		336^{Q} 318^{q}	50	30	30
OTA	404.2 $[M+H]^+$		358^{Q} 239^{q}	24	15	15
HT-2	442.2 $[M+NH_4]^+$	6 (15.5–18)	263^{Q} 215^{Q}	10	12	13
T-2	484.5 $[M+NH_4]^+$		305^{Q} 215^{q}	10	12	15
ZEN	317.1 $[M-H]^-$	7 (16.5–18.5)	131^{Q} $175q$	25	25	25
BEA	784.4 $[M+H]^+$	8 (18.5–22)	244^{Q} 262^{q}	35	25	25

Q, Quantification transition.
q, Confirmation transition.

and mixtures of them. As sorbent, C_{18} was applied to this experiment in view of the good results obtained in the previous study.

An illustration of these tests appears in Fig. 2, representing the intensities of target analytes obtained using the different solvents. In this study, aqueous mixtures were discarded owing to the interaction between water and flour, leading a doughy consistency that makes the pass of the analytes difficult.

When dichloromethane was used, only AFB_2, OTA, FB_2 and ZEN were extracted and giving low mean recoveries for the other mycotoxins. Employing ethyl acetate, no acceptable recoveries were obtained for AFG_1, AFG_2 and FB_1 (<40%). The mixture methanol:acetonitrile provided the best results for all the compounds: while using methanol the fumonisins signal improved about 20%, using acetonitrile the recoveries of AFs (principally of AFG_1) and trichothecenes type A were improved.

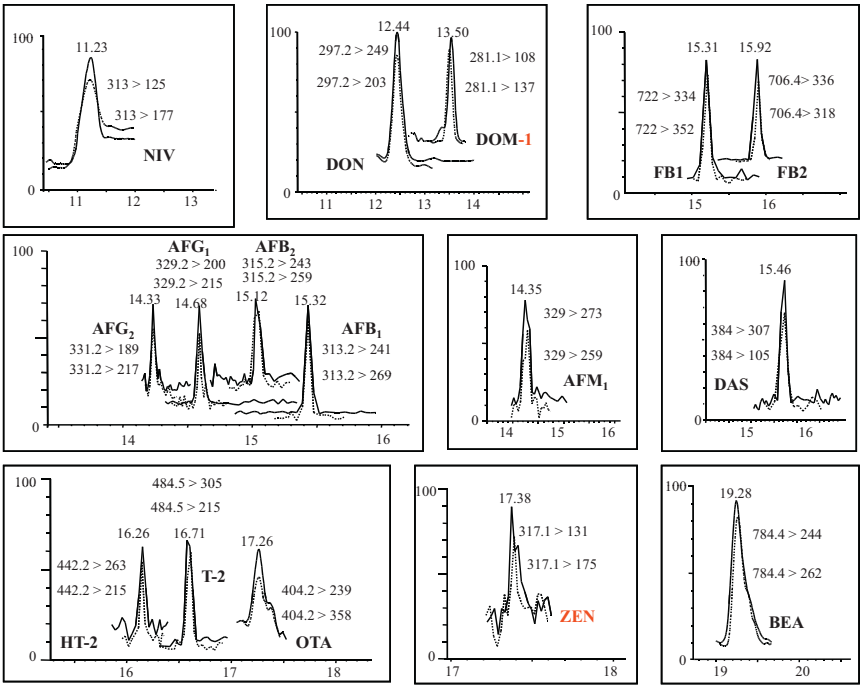

Fig. 1. Chromatogram obtained under optimized conditions of spiked multicereal flour at LOQ levels.

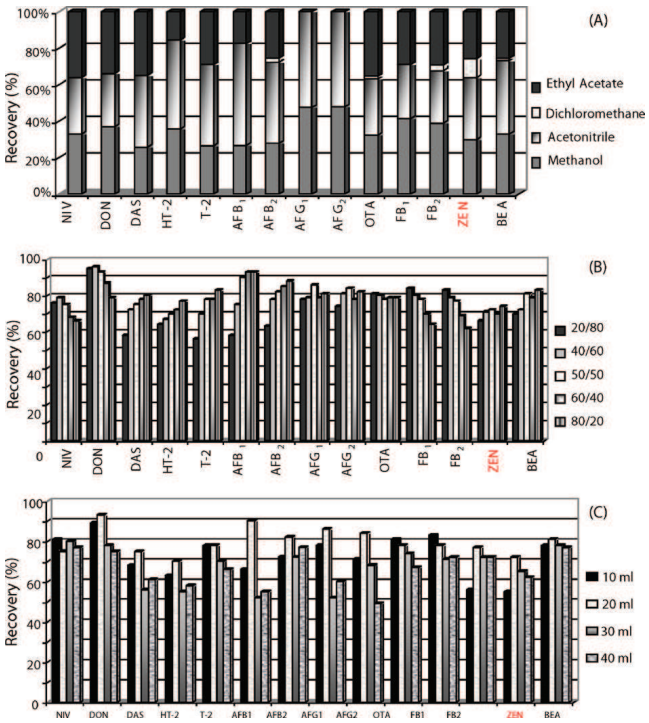

Fig. 2. Optimization of the solvents parametres. (A) Recoveries obtained using different solvents. (B) Recoveries obtained by different methanol:acetonitrile mixture rates. (C) Study of the optimum extraction volume.

For this reason, mixtures of methanol:acetonitrile at different ratios were checked (Fig. 2B). Although the results were very similar, a good compromise between all the mycotoxins was observed when the percentage 50:50 (v/v) of mixtures was used (recoveries ranged from 70% for HT-2 to 93% for DON).

On the other hand, to study the effect of pH and ionic strength, several assays were performed employing different acids (formic and acetic) and different salts (ammonium acetate and formate). For both tests, the same procedure as it is explained above was carried out; C_{18} was the sorbent and methanol:acetonitrile (50/50) was the eluting solvent.

In the case of the pH study, the addition of formic acid only improved the extraction of fumonisins, and it was dramatically detrimental to other compounds such AFs or ZEN. The addition of acetic acid not demonstrated any improvement in the extraction and for this reason pH variation was rejected.

The addition of salts in extraction solvents is known as "salting out" and it may enhance the extraction efficiency of compounds and improve sensitivity and precision of the analysis. The combination of this salting effect with MSPD is simple, fast, and results in extracts that are in an organic solvent that can be evaporated [35]. In this study, the addition of ammonium formiate improved the extraction of HT-2, DAS, and maintained constant the recoveries of the other mycotoxins. For this objective, different amounts of this salt were evaluated, obtaining the best results when 1 mM of this salt was used.

Finally, the volume of this extraction mixture was evaluated, and it was observed that when 20 ml of the mixture methanol:acetonitrile 1 mM ammonium formate were employed, reproducible results and good recoveries were obtained (Fig. 2C).

3.2.2. Optimization of the solid phase

Classic applications of the MSPD technique employ octadecyl-silica (C_{18}) and octyl-silica (C_8). However normal-phase as florisil, amine, phenyl and silica, have been also proposed as sorbent in many MSPD applications.

In this work, eight widely used solid phases were tested: (1) C_{18}, (2) C_8, (3) celite, (4) silica, (5) florisil, (6) phenyl, (7) alumina (acidic, neutral and basic) and (8) amine. Fig. 3 depicts the recoveries obtained by using these solid-phases expressed as percentage between the spiked sample after and before the extraction method.

Fumonisins were only extracted when C_{18} and C_8 were used. This circumstance limited the selection of the solid phase sorbent for this expanded experiment.

In the case of trichothecenes, they are divided into two groups according to their chemical structures: type A (such DAS, T-2 and HT-2) that are characterized by an oxygen function different from a carbonyl group at the C-8 position, and type B (NIV and DON) that posses a carbonyl function at this position. As a consequence, the polarity of these trichothecenes varies considerably (from the polar type B (NIV) to the less polar type A (T-2)). While NIV and DON reached acceptable recoveries with all the solid phases, DAS,

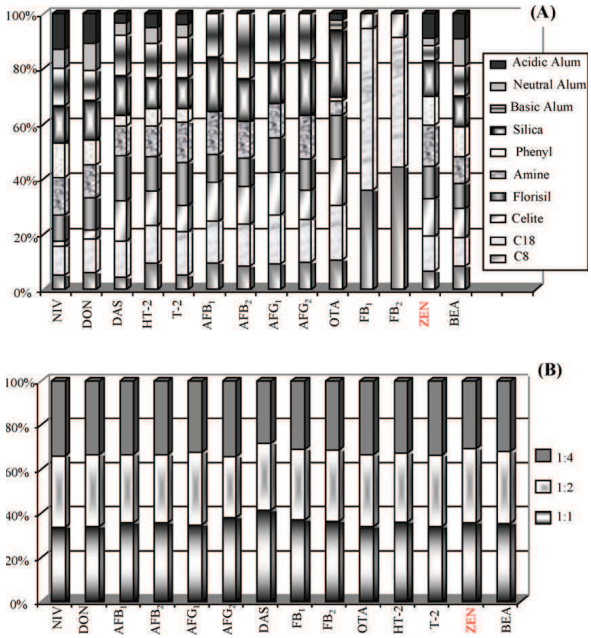

Fig. 3. Representation of the recoveries obtained by MSPD evaluating different solid sorbents (A) and different ratios (B) from a multicereal spiked sample at LOQ levels.

T-2 and HT-2 only reached recoveries > 70% when silica or acidic alumina were used. Even though using C_{18} the best results were not obtained, a compromise had to be made for the optimal extraction procedure for all mycotoxins.

As it was explained in the previous work [29], the presence of polar groups in the structure of AFs, could explain the interactions between these compounds and the polar solid phase as phenyl (Rec. 0%) or florisil (Rec. < 62%) that not allow their elution when medium polar solvent as acetonitrile or methanol were used. As a conclusion, and according to our previous studies [29], C_{18} proved to be a good solid support for the extraction of AFs.

Paying attention to the other mycotoxins (OTA, ZEN and BEA), all of them were extracted from the flour employing almost all the solid phases studied. The results obtained by C_{18} were into the acceptable range (88%, 72% and 81%, respectively).

In light of the above results, it was decided to use C_{18} alone, since acceptable recoveries were obtained for all the studied mycotoxins from multicereal-flour samples (from 72% of ZEN to 93% of DON).

3.2.3. Ratio of sample-to-sorbent

Another critical parameter in the MSPD is the ratio between matrix and sorbent material. This ratio depends on the sample nature, although the ratios often applied are 1–4. For further optimization different amounts of C_{18} (1, 2, and 4 g) were added to a glass mortar and blended with 1 g of sample, and then elution was performed with 20 ml of the mixture methanol:acetonitrile 1 mM ammonium formate. Fig. 3B shows that there were no remarkable differences between the recoveries obtained with the different ratios. In order to minimize the use of inorganic material sorbent, 1:1 ratio (1 g of multicereal-sample and 1 g of C_{18}) was selected for this study.

3.3. Matrix effects

Despite the elimination of some interfering components during the extraction, complex matrix as flour could cause analytical errors due to the presence of interferents, leading to inaccurate results. Indeed, the residual components of the matrix can promote either ion suppression or enhancement of the analyte signal in the ES interface.

In order to evaluate the influence of the flour matrix in this experiment, the obtained slopes by matrix-matched standards calibration were compared with those obtained by solvent-based standards calibration, calculating matrix/solvent slope ratios for each mycotoxin.

When different samples of a similar matrix are analyzed, some authors suggest performing a calibration plot for a standard matrix similar to that of the samples to be investigated, but free from analytes [36]. In this study, multicereal flour was used as a "standard matrix", owing to the fact that multicereal flour contains all the main cereals and therefore it could present all the selected mycotoxins.

Blank flour extracts were spiked at six concentration levels between LOQ and 10 LOQ to obtain a matrix-matched standard calibration and all the standards were prepared in triplicate. Based on empirical results presented in Table 2, the impact of matrix interferences was different for each compound. For example, significant suppression (<70%) was only observed for AFs and trichothecens type A.

Table 2
Evaluation of the matrix effects of mycotoxins in multicereal flour.

Compound	Without IS							With IS						
		Slope	r^2		Slope	r^2	ME (%)		Slope	r^2		Slope	r^2	ME (%)
NIV[1]	(a)	0.27	0.999	(b)	0.28	0.998	103.7	(a)	1×10^{-4}	0.999	(b)	1×10^{-4}	0.999	100
				(c)	0.21	0.995	75.0				(c)	1×10^{-4}	0.994	100
DON[1]	(a)	3.63	0.999	(b)	3.08	0.998	84.8	(a)	17×10^{-4}	0.999	(b)	14×10^{-4}	0.999	82.3
				(c)	2.80	0.998	90.9				(c)	13×10^{-4}	0.998	92.8
DOM-1[(IS)]	(a)	33.24	0.999	(b)	27.81	0.998	83.7							
				(c)	20.86	0.998	75.0							
AFB_1[2]	(a)	340.70	0.992	(b)	217.61	0.996	63.8	(a)	0.76	0.999	(b)	0.62	0.996	81.1
				(c)	205.10	0.994	95.9				(c)	0.59	0.996	94.9
AFB_2[2]	(a)	15.78	0.995	(b)	9.85	0.994	62.4	(a)	0.24	0.994	(b)	0.20	0.996	83.9
				(c)	8.89	0.998	90.2				(c)	0.19	0.993	92.1
AFG_1[2]	(a)	296.07	0.993	(b)	168.11	0.992	56.8	(a)	0.50	0.997	(b)	0.47	0.994	92.5
				(c)	142.21	0.994	84.6				(c)	0.44	0.996	93.7
AFG_2[2]	(a)	85.29	0.999	(b)	49.46	0.992	57.9	(a)	0.24	0.999	(b)	0.16	0.992	67.6
				(c)	43.19	0.996	87.3				(c)	0.15	0.997	93.7
AFM_1[(IS)]	(a)	84.35	0.995	(b)	57.63	0.996	68.3							
				(c)	44.89	0.993	90.8							
DAS[1]	(a)	16.35	0.998	(b)	10.91	0.999	66.7	(a)	75×10^{-4}	0.998	(b)	50×10^{-4}	0.999	66.6
				(c)	10.10	0.991	92.5				(c)	39×10^{-4}	0.990	78.0
FB_1[1]	(a)	4.07	0.990	(b)	5.05	0.990	107.4	(a)	21×10^{-4}	0.994	(b)	23×10^{-4}	0.990	109.5
				(c)	4.17	0.994	82.6				(c)	16×10^{-4}	0.998	69.6
FB_2[1]	(a)	10.96	0.996	(b)	12.85	0.990	117.2	(a)	51×10^{-4}	0.997	(b)	59×10^{-4}	0.992	115.6
				(c)	9.22	0.993	71.1				(c)	43×10^{-4}	0.995	72.9
OTA[1]	(a)	8.54	0.993	(b)	10.84	0.997	106.9	(a)	54×10^{-4}	0.997	(b)	49×10^{-4}	0.996	90.7
				(c)	10.03	0.992	92.5				(c)	49×10^{-4}	0.997	100
HT-2[1]	(a)	3.57	0.990	(b)	2.41	0.998	67.5	(a)	17×10^{-4}	0.999	(b)	11×10^{-4}	0.997	64.7
				(c)	2.26	0.994	93.7				(c)	9×10^{-4}	0.998	81.8
T-2[1]	(a)	8.98	0.994	(b)	5.29	0.991	58.9	(a)	43×10^{-4}	0.997	(b)	25×10^{-4}	0.990	58.1
				(c)	4.03	0.995	76.3				(c)	23×10^{-4}	0.994	92.0
ZEN[1]	(a)	3.01	0.991	(b)	3.07	0.994	102	(a)	14×10^{-4}	0.992	(b)	14×10^{-4}	0.996	100.1
				(c)	2.60	0.993	84.7				(c)	12×10^{-4}	0.994	85.7
BEA[1]	(a)	341.56	0.997	(b)	208.07	0.997	60.9	(a)	0.14	0.998	(b)	0.08	0.995	59.9
				(c)	156.02	0.995	74.9				(c)	0.07	0.991	93.9

(a) Standard in pure solvent (solvent calibration).
(b) Standard spiked after extraction (matrix-matched calibration).
(c) Standard spiked before extraction (fortified sample).
Internal standard used: [(1)] DOM; [(2)] AFM_1.

In these cases, and as the European guide SANCO [37] recommends, the matrix-matched calibration standard was evaluated to minimize these matrix effects; the slope of fortified samples calibration is compared with the slope obtained by matrix matched calibration. In light of the results included in the same Table 2, using the matrix matched calibration, the matrix effect was compensate for all the studied mycotoxins in multicereal flour (ME% between 71.1% and 95.5%).

Although this method showed to be efficient, in the last years there is a trend toward the use of other methods to minimize the matrix effects: for example, internal standard calibration approach [27,35,38–40]. Usually, an isotopically labelled internal standard is preferred to correct analytes signal suppression or enhancements resulting from matrix interferents. Ideally, each analyte would be corrected by own isotope-labelled molecule. However, this ideal situation is problematic in a multiresidue method due to economical restrictions to acquire a large number of these compounds.

An alternative normally applied within the mycotoxins field is the use of established internal standard, as DOM-1 and AFM_1 since they are metabolites of DON and AFB_1, which could not be present in cereal products [26,39].

To obtain more information about accurate quantification, these two compounds were checked for correction of matrix effects since, only AFs and trichothecenes presented suppression problems. NIV, DON, DAS, T-2, HT-2 were quantified using DOM-1 and AFB_1, AFB_2, AFG_1, AFG_2 using AFM_1.The dilemma was selected an IS for the emergent mycotoxin BEA. In this study, it was decided to calibrate with DOM-1, owing to the similar polarities between the trichothecenes group B and this mycotoxin.

As it can be deduced from the results in Table 2, suppression effects were also compensated by use of these internal standards. Moreover, it was interesting to prove that normalized ME using IS were close to those obtained by matrix matched standard calibration, confirming that these two methods were suitable to compensate the matrix effects presented in this study.

The linear regression coefficient of all calibration curves are also presented in this Table 2, showing that good results were achieved, with corresponding correlation coefficients (r^2) higher than 0.990.

3.3.1. Matrix effect from flour to flour

To obtain more accurate results, matrix effects were evaluated in different main cereal flours: corn, rice and wheat were selected since they are the three principal cereals world wide-used.

This part of the work was divided in two steps: in the first phase, it was evaluated ME (%) using the standard calibration in pure solvent (A). In view of the results (Table 3), statistically significant variations were presented. For example, in wheat flour analysis, a general suppression effect existed except for OTA, T-2 and HT-2 which suffer an enhancement in their signal. Moreover, in the analysis of corn flour and rice flour, the matrix effects were very variable.

Once evaluated the existence of ME, the second phase of this study was based on minimizing the matrix effects by different strategies: the matrix-matched calibration of the studied flour (B),

Table 3
Matrix effects (%) in different cereal flours (A) slope matrix matched in cereal flour/slope of standard in solvent, (B) slope spiked cereal flour/slope of matrix matched cereal flour, (C) slope spiked cereal flour/slope of matrix matched multicereal flour, (D) area ratios with IS.

Compound	Wheat flour				Corn flour				Rice flour			
	ME %(A)	ME %(B)	ME %(C)	ME %(D)	ME %(A)	ME %(B)	ME %(C)	ME %(D)	ME %(A)	ME %(B)	ME %(C)	ME %(D)
NIV[1]	42.6	77.8	80.1	90.1	62.6	79.8	82,3	97.6	66.6	73.2	97.6	99.6
DON[1]	56.3	76.6	81.0	89.2	77.7	85.4	84.9	92.3	73.5	84.6	83.2	90.3
AFB_1[2]	56.5	77.7	80.3	80.1	68.4	81.9	83.6	88.9	69.1	70.1	76.5	80.5
AFB_2[2]	64.5	76.8	81.2	81.3	66.6	82.7	88.6	89.7	69.8	72.4	75.7	83.4
$AFG1$[2]	67.1	72.3	75.4	79.8	67.2	82.1	84.2	88.2	68.1	79.9	81.0	81.5
$AFG2$[2]	63.2	74.3	81.2	77.6	69.5	80.6	87.6	91.1	70.2	78.4	78.5	80.3
DAS[1]	58.3	88.2	78.2	71.2	89.4	80.2	83.2	93.1	83.3	92.2	88.3	72.3
FB_1[1]	67.9	77.2	84.3	93.2	89.4	97.7	96.7	105.3	93.6	94.4	99.1	101.2
FB_2[1]	79.8	74.3	76.2	89.2	91.3	104.3	106.2	112.3	96.7	93.9	92.3	105.2
OTA[1]	124.4	90.2	92.3	94.4	89.4	93.2	94.1	96.7	92.4	94.5	93.5	94.8
HT-2[1]	101.4	80.2	81.5	70.2	74.6	89.2	88.6	88.9	77.8	92.2	87.4	84.3
T-2[1]	120.7	67.7	70.6	72.4	81.2	88.1	78.2	79.6	87.1	79.6	70.9	72.8
ZEN[1]	90.2	100.2	98.7	99.8	108.3	106.4	102.1	103.2	101.2	102.9	99.3	101.2
BEA[1]	48.6	69.0	71.1	74.2	82.3	78.9	78.2	70.3	77.9	80.3	78.5	77.3

$ME\%^{(A)}$: slope matrix matched selected flour/slope standard in solvent × 100.
$ME\%^{(B)}$: slope spiked selected flour/slope matrix matched selected flour × 100.
$ME\%^{(C)}$: slope spiked selected flour/slope matrix matched multicereal flour × 100.
$ME\%^{(D)}$: correction with IS × 100.
Internal standard used: (1) DOM-1; (2) AFM_1.

employing the matrix matched calibration of multicereal flour as a "standard model" (C) for all the samples and the internal standard calibration approach (D). The results are presented also in Table 3.

When, according to other authors [40,41], matrix-matched calibration of the selected flour was applied, matrix effects were compensated. However, it means that each matrix should be evaluated in each analysis, what complicates the analysis and makes it very tedious. To simplify the dependence of matrix effect on variety of samples, and according to the literature, careful study about the selection of a "standard matrix" was carried out. As it was cited before, multicereal flour was selected with this aim and the results (Table 3) demonstrated that this approach resulted in further reductions of the matrix effects and it allowed accurate quantification of the selected mycotoxin in different cereal flours. Comparable slopes, intercepts and response linearity were observed for each mycotoxin among the calibration curves in these three matrices (Table 2).

3.4. Validation of the method

The performance of the method was evaluated according to EU guidelines [32,42]. All these parameters were calculated in multicereal flour.

LODs and LOQs were calculated analysing fortified flour sample and the results obtained are shown in Table 4. In the same table are indicated the maximum levels recommended by the European Union for multicereal flour or cereal derivates [5] to compare the results obtained. It is important to emphasize those only AFs, fumonisins, OTA, ZEN and DON are regulated by the European legislation in this food commodity. In all these cases, LOQs were always lower than the MLs established by EU.

Although no limits are set for NIV, DAS T-2, HT-2 and BEA in multicereal flour, results presented in Table 4 indicate that the developed method was suitable for the detection of these mycotoxins at convenient concentration level (comparing with the MLs established by EU for other mycotoxins).

Table 4
Maximum levels and performance parameters (recovery values (%) and relative standard deviations given in brackets (%)).

Compound	ML (EU) ($\mu g\,kg^{-1}$)	LOD ($ng\,g^{-1}$)	Intra-day precision[d]				Inter-day precision[e]			
			Low level (LOQs)		High level (10 × LOQs)		Low level (LOQs)		High level (10 × LOQs)	
			Concentration	Recovery	Concentration	Recovery	Concentration	Recovery	Concentration	Recovery
NIV		75	85.24	76.6 (6)	852.4	72.6 (4)	85.24	74.5 (4)	852.4	76.3 (5)
DON	750[a]	20	31.25	89.3 (4)	312.5	85.6 (8)	31.25	79.8 (8)	312.5	84.6 (7)
DAS		3	5	77.4 (3)	50	81.9 (6)	5	78.6 (11)	50	80.0 (9)
HT-2		10	35.5	72.6 (4)	355	73.1 (4)	35.5	68.7 (5)	355	72.8 (7)
T-2		5	12.5	79.2 (9)	125	82 (12)	12.5	88.4 (14)	125	84.3 (11)
AFB_1	2	0.1	0.25	76.7 (12)	2.5	83.3 (7)	0.25	72.7 (13)	2.5	81.1 (11)
AFB_2		1	1.5	68.8 (11)	15	76.1 (9)	1.5	69.1 (13)	15	74.5 (9)
AFG_1	4[b]	0.1	0.25	80.3 (4)	2.5	75.4 (5)	0.25	71.0 (9)	2.5	73.8 (6)
AFG_2		0.5	0.75	78.7 (6)	7.5	81.7 (5)	0.75	77.1 (12)	7.5	77.9 (10)
OTA	3	1	3	71.1 (11)	30	83.5 (8)	3	76.7 (14)	30	78.2 (12)
FB_1	1000[c]	40	83.33	81.3 (6)	833.3	83.9 (5)	83.33	77.2 (11)	833.3	80.1 (12)
FB_2		60	83.75	89.6 (10)	837.5	87.5 (8)	83.75	85.2 (4)	837.5	87.6 (7)
ZEN	75[a]	6	12.5	79.4 (14)	125	77.1 (6)	12.5	78.6 (9)	125	77.4(7)
BEA		0.5	1	74.1 (11)	10	79.8 (8)	1	73.1 (10)	10	80.4 (9)

[a] EC 1126/2007 amending EC 1881/2006.
[b] Expressed as the sum of the four aflatoxins ($AFB_1 + AFB_2 + AFG_1 + AFG_2$).
[c] Expressed as the sum of fumonisins ($FB_1 + FB_2$).
[d] Number of replicates: 6.
[e] Different days: 6.

Table 5
Occurrence of mycotoxins in analyzed flours.

Sample	Wheat flour		Corn flour		Rice flour		Oats flour		Bakery preparation	
	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)	Positive sample	Range ($\mu g\,kg^{-1}$)
NIV	9	<LOQ–105	1	92	–	–	–	–	1	76
DON	5	45–367	–	–	–	–	1	153	2	32.5–180
AFB_2	1	2	–	–	–	–	1	1.60	–	–
AFG_1	2	0.53–0.72	–	–	–	–	–	–	–	–
AFG_2	1	1	–	–	–	–	–	–	2	<LOQ–1.2
FB_2	–	–	2	230–468	–	–	–	–	1	<LOQ
OTA	3	<LOQ–3.5	–	–	–	–	–	–	1	<LOQ
ZEN	1	39.3	1	70.5	–	–	–	–	–	–
BEA	6	150–720	–	–	3	327–575	2	226–325	3	115–705

<LOQ = only detected, below the LOQ level.

Recoveries, repeatability (intra-day precision) and reproducibility (inter-day precision) were determined at two spiked levels (LOQ and 10 times LOQ). Results are also summarized in Table 4.

Precision, calculated as relative standard deviation percentages (RSD) was between 3% and 14% for the intra-day test and from 4% to 14% for the inter-day test. The recovery ranges in low and high spiked levels were 68.8–89.6% and 72.6–87.5%, respectively for the intra-day test and 68.7–88% and 72.8–87.6% for the inter-day test at LOQ and 10 times LOQ, respectively.

Therefore, the method was considered "acceptable" according to the EU criteria [32]; an average recovery ($n = 5$) between approximately 70% and 120% and a repeatability (RSD) lower than 20%.

Similarly to matrix effects, recoveries and its repeatability were studied in the three varieties of cereal flour (wheat, corn and rice) by three replicates. Recoveries were satisfactory (between 70% and 120%) in all matrix tested. To overcome matrix effect problems and for accurate quantification, matrix-matched multicereal flour was used in all the experiments as it has been explained in Table 3.

To demonstrate the efficiency of the developed method, it was applied to the analysis of reference certified material: DON in wheat flour. The measured concentration was $973 \pm 12\ \mu g\,kg^{-1}$ ($n = 3$): this concentration showed satisfactory concordance with the certificated values ($1062 \pm 110\ \mu g\,kg^{-1}$).

Table 6
LC–MS/MS ion ratios (A qualifying ion$^{(q)}$/A quantifying ion$^{(Q)}$) for mycotoxin into matrix-matched sample and matrix sample.

Mycotoxin	Ion ratio expected[a,c] (RSD %)	Ion ratio observed[b,c] Multicereal flour (RSD %)
NIV	0.86 (4)	0.79 (11)
DON	0.72 (5)	0.75 (8)
AFB_1	0.45 (2)	0.48 (5)
AFB_2	0.74 (3)	0.76 (6)
AFG_1	0.21 (3)	0.25 (11)
AFG_2	0.67 (7)	0.59 (12)
DAS	0.81 (6)	0.83 (9)
FB_1	0.71 (8)	0.66 (10)
FB_2	0.78 (7)	0.82 (8)
OTA	0.39 (3)	0.36 (7)
HT-2	0.83 (9)	0.79 (12)
T-2	0.68 (7)	0.76 (5)
ZEN	0.85 (5)	0.90 (6)
BEA	0.58 (2)	0.65 (14)

[a] Ratio determined in matrix-matched solution at LOQ level ($n = 5$).
[b] Ratio determined in fortified multicereal flour sample at LOQ level ($n = 5$).
[c] The EU guidelines [32] sets criteria for the observed ratio as follows; expected ratio >0.5. Observed ratio should be within (20%). expected ratio 0.2–0.5. Observed ratio should be within (25%); expected ratio 0.1–0.2. Observed ratio should be within (30%; expected ratio <0.1. Observed ratio should be within (50%).

3.5. Application to commercial samples

The applicability of the MSPD developed method was evaluated carrying out a survey of fourteen selected mycotoxins in around 50 commercial flours produced by different companies and which were purchased in several valencian supermarkets. These samples included wheat flour (25 samples), corn flour (9 samples), rice flour (3 samples), soy flour (1 sample), oats flour (3 sample) and including different bakery preparations that consist on several mix of different cereal flours for muffins, bread, pizza or similar (8 samples). The obtained results are summarized in Table 5.

According to the European Commission [32], the ion ratio of the primary and secondary product ions were monitored. The results are summarized in Table 6. In light of these results the trueness of the method was assessed and it was in good agreement with this European Commission performance criterion for qualitative analysis.

An internal quality control was carried out for every batch of samples to check if the system was under control, and it implied a matrix-matched calibration, a matrix blank and a fortified multicereal flour blank sample at LOQ levels.

Among these 49 tested samples, NIV, DON, AFB_2, AFG_1, AFG_2, FB_2, OTA, ZEN and BEA were detected in 25 different samples. All these mycotoxins were detected in wheat flour samples. It was not surprising since most of the flour samples (50%) belong to this cereal; it is the wide-used flour in Europe and there exist different commercial samples [43–45]. To our surprise, one of these samples exceeded the maximum tolerable level established for the EU for OTA in this food commodity [46].

NIV and BEA were presented in a high percentage of samples. While the first one has been reported in the literature [47,48], the second one has been scarcely cited [43,49]. These results showed the necessity to study and analyze this mycotoxin in deep as a future trend.

Despite of the great number of positives finding, all of them were set below the tolerable levels established by the EU, however, it would be interesting to calculate the total ingest of these mycotoxins along the years. Moreover, these results indicate that more attention should be paid to storage conditions, in order to minimize the content of mycotoxins.

4. Conclusion

The MSPD method presented is a good starting point for further mycotoxins analysis and it can be regarded as a valuable future alternative in this field. However, this study confirms once again the need to carefully evaluate potential matrix effects.

These matrix effects should be solved by using appropriate calibration method. The problem is the absence of one unified way to

solve these matrix effects since all the analytical published works used different methods demonstrating the validity of all of them. In this paper, authors decided to evaluate matrix-matched calibration and internal standard addition, without to cast doubt upon the efficiency to use isotopically labelled internal standard. Moreover, this work goes deeper into the possibility to use a "model" matrix-matched calibration which was used to validate the methods obtaining reliable results. In light of the results obtained in this work, matrix-matched calibration approach allows correct quantitative analysis.

Although consumption of food with traces of mycotoxins does not produce immediate or dramatic reaction, chronic exposure may have adverse effects on the consumers. For this, usually, mycotoxin analysis in samples is required in the commercial transaction for minimizing the public health risk.

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4.5 Occurrence of fourteen mycotoxins in Tiger-nuts.

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Occurrence of fourteen mycotoxins in tiger-nuts

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ABSTRACT

A previous developed matrix solid-phase dispersion (MSPD) extraction method was applied for the routine analysis of aflatoxins (AFs), ochratoxin A (OTA), fumonisins (FB_1 and FB_2), beauvericin (BEA), nivalenol (NIV), deoxynivalenol (DON), the toxin T-2 (T-2), toxin HT-2 (HT-2), diacetoxyscirpenol (DAS) and zearalenone (ZEN) in tiger-nuts by liquid chromatography–triple-quadrupole linear ion trap (HPLC–QTRAP®). The extraction solid support used was C_{18}, while the elution solvent was acetonitrile/methanol (50/50, v/v) 1 mM ammonium formate. Using matrix-matched calibration, recoveries and repeatabilities were in the range 67–89% and 2–11% relative standard deviation (RSD), respectively. The method was applied to determine the occurrence of the fourteen selected mycotoxins in a total of 83 tiger-nut samples purchased from different local markets of Valencian Community (Spain) during (March–June 2010 and March–May 2011). DON, OTA, AFs and BEA were detected in 26 samples of the total number of samples.

1. Introduction

Tiger-nuts, or "*chufa*", are consumed as by humans as by animals. In the case of animals, tiger-nuts are transformed in flour and added to the feed. Nevertheless, the main derivate product is "*horchata*" or "*tiger nut milk*". This beverage is a typical product from Valencia (Spain) which has a great national economic importance (Sánchez-Zapata et al., 2009) that makes necessary controls of it quality (Cortés, Esteve, Frıgola, & Torregrosa, 2005). In fact, this tuber has increased its production year to year: the annual production value of tiger-nut is approximately 5 million Euros (Consejo Regulador de la Denominación de Origen Chufa de Valencia, 2009).

These quality controls are important since tiger-nuts can contain physical, chemical and biological contaminants, such as stones, pesticides, bacteria and fungi. Regarding fungi, species such as *Fusarium* spp. and *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium citrinum* and *Rhizopus arrhizus* can growth in this tuber (HACCP guide to elaborate tiger-nut beverage). Moreover, although UE does not establish a regulation of mycotoxins in tiger-nut or its derivates, the presence of AFs and OTA have been demonstrated in tiger-nuts and their beverage in previous works (Rubert, Sebastià, Soriano, Soler, & Mañes, 2011; Sebastià, Soler, Soriano, & Mañes, 2010).

Apart from Valencia area, tiger-nuts are cultivated in Africa (Northern Nigeria, Ghana, and Togo) and India. These countries export several tons of tubers every year to other countries. It is important to keep in mind that mould contamination is strongly related to geographical area and climate; mycotoxins can be developed at various stages and under various conditions. This means that tiger-nuts of different areas can be contaminated by one or several different mycotoxins (Kroes et al., 2002).

The occurrence of such mycotoxins is of great concern because their presence in feeds and foods is often associated with chronic or acute mycotoxicosis in livestock and could threaten human health (Richard, 2007). Moreover, several mycotoxins are remarkably stable during processing and can be found in final products. Concentration may even increase during this processing.

For this reason, the aim of this study was to develop a sensible and specific analytical method expanding up the previous works, to determine AFs, OTA, ZEN, fumonisins FB_1 and FB_2, BEA, type A and B trichothecenes at concentration levels as lower as possible. Validated method was applied on monitoring programme under strict quality assurance: a total of 83 commercialized tiger-nuts were purchased during two years (2010–2011) from different local markets and cooperatives from Valencian Community (Spain).

2. Materials and methods

2.1. Chemical and reagents

HPLC grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Sorbent used for MSPD was octadecy-silica (C_{18}-E) (50 μm) bonded silica from Phenomenex (Torrance, USA).

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The certified standards of AFB_1, AFB_2, AFG_1, AFG_2, OTA, ZEN, NIV, DON, DAS, FB_1 and FB_2, BEA were purchased from Sigma Aldrich (Madrid, Spain). T-2 and HT-2 toxin stock solutions (in acetonitrile) were obtained from Biopure referenzsubstanzen GmbH (Tulln, Austria). The individual stock solutions of AFs and OTA at 500 μg ml^{-1} were prepared in acetonitrile and ZEN, NIV, DON, FB_1, FB_2, BEA were prepared at the same concentration in methanol. On the other hand stock solutions of DAS, T-2 and HT-2 at 100 μg ml^{-1} were prepared in acetonitrile. The standards were kept in safety conditions at −20 °C.

All other working standard solutions were prepared immediately before use by diluting the stock solution with methanol/water (50/50, v/v).

2.2. Sampling

Sampling was carried out according to the EU guidance (EU, 2006). Samples of tiger-nuts were purchased from different local markets, supermarkets and cooperatives of Valencian Community (Spain). At the end, a total of 83 tiger-nuts samples were investigated. The samples were recollected during 2010 and 2011 seasons.

2.3. Extraction

Sample preparation was performed as described in a previous study (Rubert, Soler, & Mañes, 2011). Tiger-nut samples (200 g) were prepared using an Oster® food processor (Professional Series Blender model BPST02-B00) mixed thoroughly. Representative portions of 1 g (tiger-nut flour) were weighed and placed into a glass mortar (50 ml) and were gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain an homogeneous mixture. The homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 15 ml of acetonitrile/methanol (50/50, v/v) 1 mM ammonium formate by applying a slight vacuum. Consequently, the extract was transferred to a 25 ml conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hopkinton, USA). The residue was reconstituted to a final volume of 1 ml with methanol/water (50/50, v/v) and filtered through a 13 mm/0.22 μm nylon filter purchased from Membrane solutions (Texas, USA).

For the preparation of fortified samples, 1 g of tiger-nut "blank" sample (it was corroborated before the analysis that no analytes were present) was spiked with 0.2 ml of working mixture of the mycotoxins at the appropriate concentration. Then, spiked samples were left to stand 3 h at room temperature before the extraction to allow the evaporation of the solvent and to establish equilibration between the mycotoxins and sample. Ten replicates were prepared for each spiking level.

2.4. Liquid chromatography–mass spectrometry analysis

LC–tandem MS analyses were carried out in a system consisting of a Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTRAP® mass spectrometer (Applied Biosystems, AB Sciex, Foster City, CA, USA) equipped with a Turbo-V™ source (ESI) interface. The QTRAP® analyzer combines a fully functional triple-quadrupole and ion trap mass spectrometer within on the same instrument. An extra confirmation tool, Information Dependent Acquisition (IDA), was carried out only for samples that contain the selected mycotoxins since the inclusion of this IDA experiment provides an unequivocal identification of mycotoxins in the matrix (Rubert, Soriano, Mañes, & Soler, 2011).

Separation of analytes was performed using a Gemini C_{18} (Phenomenex, 150 mm × 2 mm, 3 μm of particle size) analytical column preceded by a guard column with the same packing material. The flow rate was set to 0.250 ml min^{-1} and the oven temperature was 35 °C, being eluent A water (mobile phase A) slightly acidified with 0.1% of formic acid with 5 mM ammonium formate, and B (mobile phase B) methanol with 5 mM ammonium formate. The elution gradient started with 10% of eluent B, increasing to 70% in 1.5 min and kept as isocratic during 1.5 min. After this step, B was increased to 80% in 5 min. The last step was to increase 100% B in 10 min. During the further 8 min the column was re-equilibrated to the initial conditions. The volume to injection was of 20 μl.

The analyses were performed using Turbo-V™ source in positive mode. The operation conditions for the analysis in positive ionization mode were the followings: Ion spray voltage 5500 V, curtain gas 15 (arbitrary units), GS1 and GS2, 50 and 60 psi, respectively, probe temperature (TEM) 500 ºC. Nitrogen served as nebulizer and collision gas. SRM experiments were carried out to obtain the maximum sensitivity for the detection of target molecules. The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) were performed by flow injection analysis for each compound; entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively for all analytes. The QTRAP® instrument was operated in SRM mode and with a resolution set to unit resolution for Q1 and Q3. For HPLC–MS/MS analysis, scheduled SRM (sSRM) was used with 60 s of SRM detection window and 1.5 s of target scan time. Analyst® version 1.5.2 software (AB Sciex) was used to control all components of the system and also for data collection and analysis. The MS/MS parameters optimized in this study are summarized in Table 1.

2.5. Validation study

The following parameters were evaluated in order to ensure the quality method: linearity, accuracy, precision, specificity, limits of quantification (LOQ), limits of detection (LOD) and q/Q ratios of the SRM transitions acquired, which were used for confirmation of

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

Mycotoxin	Retention time	Precursor ion (*m/z*)	Product ion	DP (V)	CE (eV)	CEP
NIV	5.90	313.10	175.60^{Q}	50	21	20
		$[M+H]^+$	125.10^{q}		40	
DON	6.60	297.00	175.10^{Q}	36	81	18
		$[M+H]^+$	115.10^{q}		51	
DAS	8.05	384.05	105.00^{Q}	36	53	20
		$[M+NH_4]^+$	115.00^{q}		113	
HT-2	8.90	442.10	215.00^{Q}	31	19	18
		$[M+NH_4]^+$	105.00^{q}		57	
T-2	9.60	484.10	215.00^{Q}	36	23	20
		$[M+NH_4]^+$	185.00^{q}		27	
FB1	8.50	722.30	334.30^{Q}	101	51	26
		$[M+H]^+$	352.30^{q}		45	
FB2	10.50	706.30	336.30^{Q}	131	49	18
		$[M+H]^+$	318.30^{q}		51	
ZEN	10.90	319.10	301.10^{Q}	46	13	20
		$[M+H]^+$	187.10^{q}		25	
AFB_1	8.10	313.10	241.10^{Q}	76	43	22
		$[M+H]^+$	128.00^{q}		87	
AFB_2	7.95	315.10	259.60^{Q}	60	40	32
		$[M+H]^+$	288.60^{q}		40	
AFG_1	7.70	329.08	200.10^{Q}	81	53	22
		$[M+H]^+$	243.10^{q}		35	
AFG_2	7.55	331.10	217.60^{Q}	50	43	20
		$[M+H]^+$	189.60^{q}		43	
OTA	11.35	404.10	239.10^{Q}	60	40	14
		$[M+H]^+$	102.00^{q}		100	
BEA	16.90	801.40	244.20^{Q}	96	35	32
		$[M+NH_4]^+$	262.20^{q}		35	

Q: quantifier q: qualifier.

positive findings. Quantification of each compound was performed by means of two SRM transitions (EU, 2002). To evaluate matrix effects, external matrix-matched calibration was used. At the end, the matrix-matched calibration curves were used for effective quantification in tiger-nuts. Linearity was evaluated using matrix-matched calibrations in triplicate at six concentrations levels between LOQ and 100 times LOQ. The recoveries ($n = 10$) were carried out by spiking tiger-nut at LOQ concentration level and 100 times LOQ level. Repeatability and reproducibility of the method were carried out by spiking tiger-nut at LOQ concentration level and 100 times LOQ. The precision of the method, was estimated by determining the intra- and inter-day, % RSD, by the repeated analysis ($n = 10$) of a spiked tiger-nut at LOQ and 100 times LOQ, it was obtained during the same day and on different five days.

LOQ and LOD were estimated for a signal-to-noise ratio of 10 and 3, respectively, from the chromatograms of the samples spiked at the lowest level validated.

The q/Q ratios were evaluated from reference standards in solvent and compared to those experimentally obtained from spiked samples.

3. Results and discussion

3.1. Validation of the method

In the present work, a previous developed MSPD method (Rubert, Soler, et al., 2011) was further applied for the simultaneous determination of 14 mycotoxins in tiger-nuts. However, in order to validate the developed procedure for this matrix, parameters as recoveries, repeatability and reproducibility, as well as limits of detection (LODs) and limits of quantification (LOQs), were evaluated.

The LODs and LOQs were based on minimum amount of target analyte that produced a chromatogram peak with a signal-to-noise ratio of 3 and 10 times the background chromatographic noise, respectively. Calculated values for tiger-nuts are listed in Table 2.

To guarantee quantification, matrix effects in tiger-nuts were deeply studied. For the matrix effects evaluation, six concentrations between LOQ and 100 times LOQ levels were analyzed in methanol/water (50/50, v/v) and in matrix-matched (spiked after blank) samples, and the slopes of the calibration curves were compared by the formula (slope matrix-matched tiger-nut/slope standard in solvent × 100). Table 2 shows the obtained results. In all cases, the calibration curves showed high linearity ($r > 0.9832$).

The application of the extraction method from tiger-nuts, showed suppression for AFs (<57%), in contrast of the other selected mycotoxins that were considered into acceptable range values (65–117%) in accordance with the EU guidelines (ME % between 70 and 120%) (EU, 2002). Therefore, in order to obtain reliable quantification of these mycotoxins in tiger-nuts, matrix-matched standards calibration was required to correct the matrix effect problems (ME% between 71% and 99%). Matrix-matched calibration does not increase the time of analysis since extraction procedure and chromatographic analysis were fast methods.

Recoveries and repeatability of the developed analytical method were carried out by injection of the spiked samples at the two concentration levels (LOQ concentration level and 100 times LOQ concentration level) ten consecutive times within the day (intra-day precision), and for five consecutive days (inter-day precision) for each analyzed compound.

For all compounds mean recoveries in tiger-nuts were satisfactory, ranging from 67% to 79% (LOQ level) and from 72% to 89% (100 × LOQ level). The precision in the present study, estimated by RSD of the recovery was in the range of <8% for intra-day test and <11% for inter-day test (Table 2). These results were according to the performance criteria of the EU criteria (EU, 2002).

Fig. 1 shows the chromatogram of spiked tiger-nut at 2 times LOQs levels under the optimum chromatographic conditions commented above.

3.2. Occurrence of mycotoxins in tiger-nuts

The method was applied for monitoring selected mycotoxins in 83 tiger-nuts samples obtained from local markets over two-year. An internal quality control (matrix-matched tiger-nut calibration, a matrix blank and a spiked tiger nut blank sample at LOQ levels) was strictly carried out for every batch of samples in order to check if the system is under control. Maximum levels (MLs) of mycotoxins in tiger-nuts are not legislated; only AFs and OTA could be fixed as similar products as nuts. For this reason, in this study, "positive sample" was considered when the concentration detected was upper than LOQ level.

A total of 35 samples were named protected origin designation from Valencia and the other 48 were unknown origins (Table 3).

Moreover, the confirmation of these "positive samples" was carried out, according to the European Commission (EU, 2002): the q/Q ratios were evaluated from reference standards in solvent and

Table 2
Linearity, matrix effect (%), limits of quantification (LOQs), recovery values (%) and relative standard deviations (RSD%) given in brackets calculated at two concentration levels (μg kg^{-1}).

Compound	Linear dynamic range (μg kg^{-1})	Correlation coefficient (r)	Matrix effect (%)[a]	LOD (μg kg^{-1})	LOQ (μg kg^{-1})	Intra-day[b]		Inter-day[c]	
						Low level (LOQ)	High level (100 × LOQ)	Low level (LOQ)	High level (100 × LOQ)
NIV	50–5000	0.9832	87	18	50	74 (3)	76 (3)	70 (5)	72 (4)
DON	30–3000	0.9974	77	10	30	77 (5)	76 (4)	69 (9)	78 (8)
DAS	5–500	0.9982	61	1.5	4	69 (3)	77 (2)	79 (4)	80 (3)
HT-2	10–1000	0.9999	97	3	10	71 (2)	74 (4)	73 (5)	76 (6)
T-2	6–600	0.9990	69	2	6	77 (3)	89 (4)	75 (5)	78 (7)
FB_1	45–4500	0.9976	117	15	45	68 (3)	72 (5)	72 (8)	73 (11)
FB_2	45–4500	0.9985	94	15	45	71 (3)	73 (4)	69 (7)	74 (9)
ZEN	10–1000	0.9913	65	3	10	72 (4)	76 (8)	72 (6)	77 (5)
AFB_1	0.75–75	0.9931	50	0.25	0.75	72 (3)	75 (5)	70 (8)	72 (5)
AFB_2	1–100	0.9908	45	0.3	1	72 (5)	78 (4)	75 (7)	79 (8)
AFG_1	1–100	0.9974	52	0.35	1	73 (4)	76 (6)	68 (6)	74 (8)
AFG_2	0.75–75	0.9903	57	0.25	0.75	75 (3)	79 (5)	71 (4)	75 (5)
OTA	1–100	0.9931	65	0.3	1	72 (5)	78 (6)	70 (6)	76 (7)
BEA	2–200	0.9902	110	0.7	2	70 (8)	75 (6)	67 (11)	71 (8)

[a] ME%: slope matrix-matched tiger-nut/slope standard in solvent × 100.
[b] Number of replicates:10.
[c] Different days: 5.

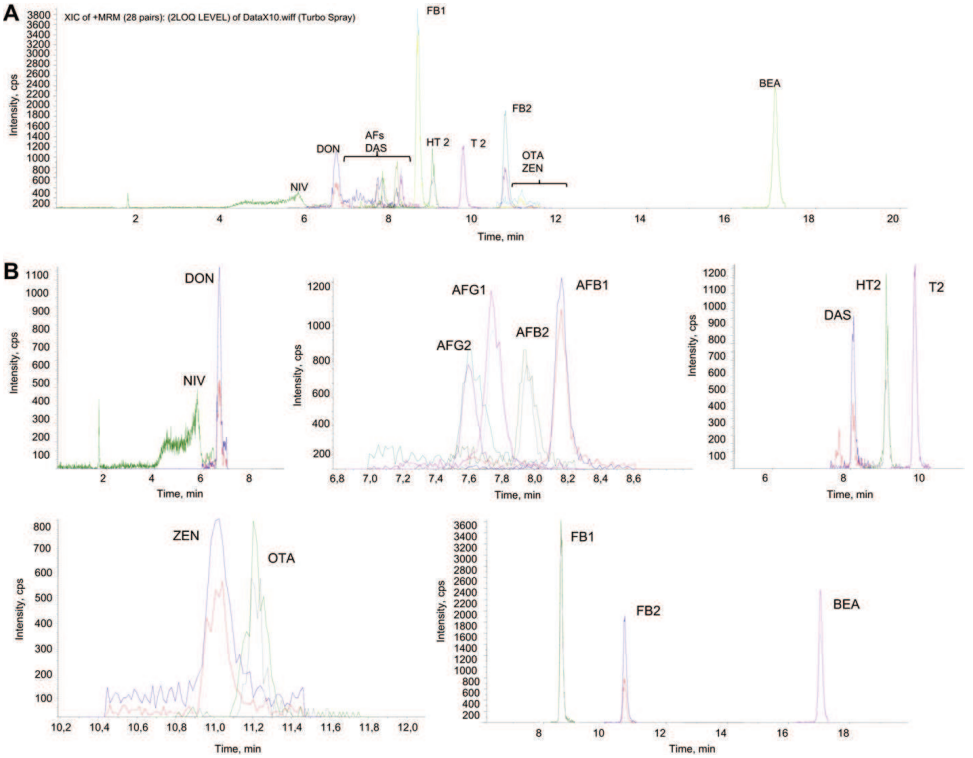

Fig. 1. (A) shows TIC of total SRM. (B) XIC from the TIC, chromatograms of spiked tiger-nut at 2 times LOQs levels under the optimum chromatographic conditions.

compared to those experimentally obtained from spiked samples. With this criterion, a total of 26 samples were confirmed as positive samples. Confirmatory analysis was found to be successful in all the cases; Fig. 2 shows an example of unknown origin sample.

BEA, DON, OTA and AFG_2 were identified and quantified in 5 samples of a total of 35 protected origin designation from Valencia samples. Only two of these "positive samples" contained more than one mycotoxin. Fig. 2 shows chromatograms of positive sample that contained BEA and DON (co-occurrence of *fusarium* toxins).

On the other hand, 21 samples of the total 48 samples of unknown origins were positives. The identified mycotoxins were: BEA, DON, OTA, AFB_1, AFB_2, AFG_1 and AFG_2. In 15 of these positive samples were detected two mycotoxins (AFs, mainly) and in 4 samples were detected the presence of three mycotoxins.

As it is known, the growth of moulds, and posterior production of mycotoxins are related to environmental conditions and conditions of storage. This may be the reason of the difference between the samples with protected origin designation from Valencia and samples with unknown origin. Owing to the economical importance of this product in Valencia area, the HAPPC system applied will minimize further elaboration of the toxin by toxigenic fungi.

These results are according to previous works that evaluated the presence of AFs and OTA in tiger-nuts-samples. Sebastià et al. (2010), detected the presence of AFs and OTA in the 8% of the total tiger-nuts samples by liquid chromatography fluorescents detector. Recently Rubert, Sebastià, et al. (2011), developed HPLC–MS/MS method to analyse OTA and AFs and the 31.3% of the samples were contaminated. To our knowledge, since this moment, only AFs an OTA have been monitored in this raw material. Arranz, Stroka, and Neugebauer (2006) analyzed aflatoxin B_1 in tiger nut-

Table 3
Occurrence of selected mycotoxins in tiger-nuts ($n = 83$).

	Valencia guaranteed origin ($n = 35$)		Unknown origin ($n = 48$)	
	Positives	Range concentrations ($\mu g\,kg^{-1}$)	Positives	Range concentrations ($\mu g\,kg^{-1}$)
DON	2	33–43	3	34–69
BEA	3	5–125	8	2.5–161
AFB_1	n.d.		14	1.2–1.9
AFB_2	n.d.		2	1.4–1.8
AFG_1	n.d.		2	1.3–1.5
AFG_2	1	1.3	9	1.8–3.1
OTA	1	1.6	7	1.2–37
Total	5		21	

n.d. Not detected.

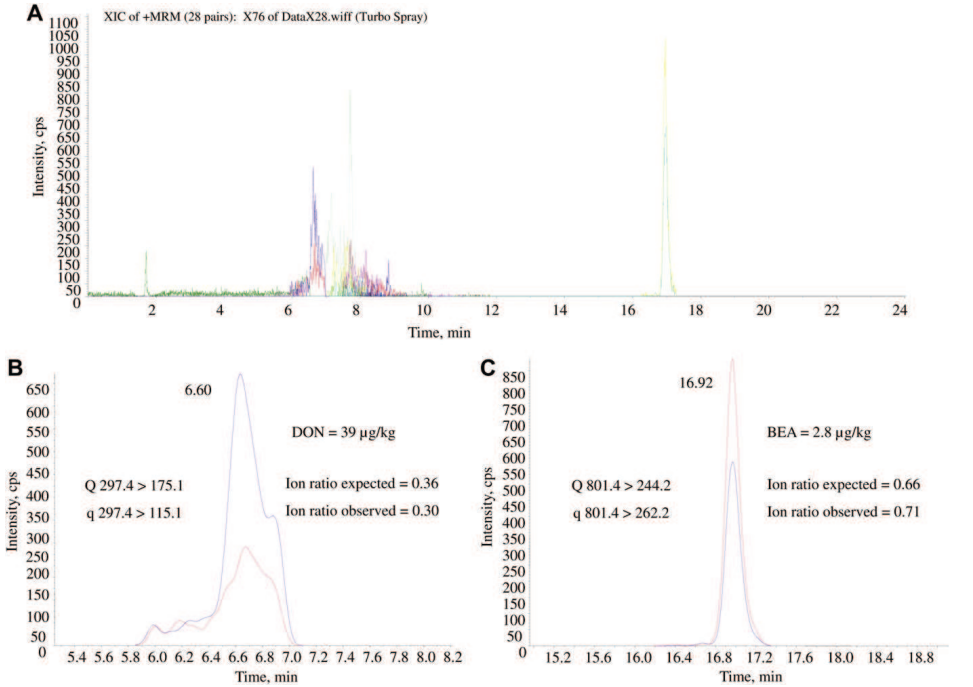

Fig. 2. Chromatogram of tiger-nut from unknown origin in which was detected DON and BEA (A). (B) and (C) show calculated concentrations and q/Q ratios for DON and BEA.

based soft drink, suggesting that the elaboration process does not minimize the concentration of these mycotoxins.

It is the first time, to our knowledge, that BEA and DON are detected in tiger-nut. Scarce literature is available about the production of BEA, however, it is known that DON is characteristic to cereals as wheat or corn. However the authors suggest some reasons for what DON can be presented in this tuber: (i) the fungus can survive on residue left on the field from the previous season's crop, providing an inoculums source for the new crop (Kroes et al., 2002) or/and (ii) the same food industry process different kind of cereals. As it has been studied, tiger-nuts are a favourable substrate so as to produce *fusarium* toxins (Mateo & Jiménez, 2005).

4. Conclusions

The analytical methodology developed was efficiently used to control 14 mycotoxins in tiger-nuts. A total of 83 samples were analyzed and 26 samples were positive for some of the mycotoxins studied. Although the results highlight the importance to monitor the presence of mycotoxins, it is important to keep in mind that the concentrations determined were at trace levels or lower that the limits established for these mycotoxins in other similar products as unprocessed cereals. However, nowadays the annual production of tiger-nuts is increasing in order to use as food, to elaborate tiger-nut milk (horchata) or to use it as animal feed. This means that the daily intake of these compounds is being increased by one or another way, for what it is important to quantify mycotoxins in tiger-nuts in order to evaluate the risk of the consumers.

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4.6 Application of an HPLC-MS/MS method for mycotoxins analysis in commercial baby food.

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Analytical Methods

Application of an HPLC–MS/MS method for mycotoxin analysis in commercial baby foods

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ABSTRACT

This article describes the validation of an analytical method for the detection of 21 mycotoxins in baby food. The analytical method is based on the simultaneous extraction of selected mycotoxins by matrix solid-phase dispersion (MSPD) followed by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) using a hybrid triple quadrupole-linear ion trap mass spectrometer (QTRAP®). Information Dependent Acquisition (IDA), an extra confirmation tool for samples that contain the selected mycotoxins, was used. The matrix effects were evaluated, and the corrections for the matrix effects were performed using two calibration approaches: external matrix-matched calibration and internal standard calibration. Matrix-matched calibration was ultimately used for accurate quantification, and the recoveries obtained were generally higher than 70%. The analytical method was applied to the analysis of 35 samples of commercial baby foods. No sample exceeded the maximum limit (ML) fixed by the European Union for these mycotoxins in baby food. However, this survey highlighted the occurrence of mycotoxins in cereal-based infant foods.

1. Introduction

Although breast milk is the major food source for the period of infancy, the gradual replacement of exclusive milk feeding by complementary foods from the fourth month of life onwards is very important, according to paediatric guidelines (Briefel, Reidy, Karwe, & Devaney, 2004; Forrest & Riley, 2004). Cereals are one of these complementary foods.

Mycotoxins are widely regarded as the natural toxins that can cause the most serious contamination of these cereals (FAO, 2004). This situation becomes more worrisome because several of these mycotoxins are stable throughout the processing of the foodstuffs and can survive intact in the final products (Bullerman & Bianchini, 2007). Infants and children are considered to be more susceptible to these toxins than adults because of their lower body weight, higher metabolic rate, and lower ability to detoxify the mycotoxins (Sherif, Salama, & Abdel-Wahhab, 2009).

Bearing in mind the risks associated with mycotoxin intake by infants, the European Union has set a very low limit for the presence of mycotoxins in infant food (EU, 2006a, 2007, 2010). However, validated methods for the analysis of mycotoxins in baby food are scarce and there is currently a growing interest in the development of reliable detection systems for mycotoxins in this food commodity.

The analytical methods that are developed and reported in the literature are not usually directly applied to baby food, but the methods that have been developed achieve good performance at low concentration levels and are also sufficient for baby food analysis (Beltrán, Ibáñez, Sancho, & Hernández, 2009; Ren et al., 2007). Only a few papers specifically focus on baby food (D'Arco, Fernández Franzón, Font, Damiani, & Mañes, 2008; Lombaert et al., 2003). In Table 1 Supplementary data, an overview of several validated LC methods for the analysis of mycotoxins in baby food is presented.

Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) is the technique of choice for multi-mycotoxin analysis because of its versatility, specificity, and selectivity. Until recently, triple quadrupole (QqQ) LC–MS/MS equipment has been the most widely employed equipment for the performance of quantitative mycotoxin analysis (Beltrán et al., 2009; D'Arco et al., 2008; Ren et al., 2007). Although the sensitivity, selectivity and efficiency of QqQ are excellent, the qualitative information needed to support the structural elucidation of the compounds is lost (Hernández et al., 2005). This liability could be overcome with the hybrid mass spectrometer QTRAP®, which is appropriate for both quantification and confirmation (Gros, Petrovic, & Barceló, 2009; Martínez Bueno et al., 2007).

In previous research (Rubert, Soler, & Mañes, 2010, 2011), matrix solid-phase dispersion (MSPD) extraction procedures have been developed and reported for the legislated mycotoxins. As a follow-up to these previous studies, the objective of this work was the development of a fast, selective and sensitive mycotoxin

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analytical method based on MSPD extraction followed by LC–MS/MS using a 3200 QTRAP® instrument applied to mycotoxins in baby food. To our knowledge, an MSPD method (followed by QTRAP® mass spectrometry) is a technique that has scarcely appeared in the literature as a routine analytical technique in the mycotoxin field and baby food analysis appears to be an unresolved issue for the analysis of these natural contaminants.

2. Experimental section

2.1. Reagents and chemicals

Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). The dispersant used for MSPD was octadecyl silica (C_{18}) (50 μm), bonded silica from Analisis Vinicos S.L. (Tomelloso, Spain).

Deionised water (>18 MΩ cm^{-1} resistivity) was purified using the Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, MA, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade formic acid (purity >98%), and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

The standards of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), sterigmatocystin (STER), α-zearalenol (ZOL), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), fusarenon X (FUS-X), neosolaniol (NEO), diacetoxyscirpenol (DAS), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and beauvericin (BEA) were purchased from Sigma Aldrich (Madrid, Spain). T-2 and HT-2 toxins, aflatoxin M_1 (AFM_1) and deepoxy-deoxynivalenol (DOM-1) stock solutions (in acetonitrile) were obtained from Biopure referenzsubstanzen GmbH (Tulln, Austria). Fumonisin B_3 (FB_3) was supplied by the PROMEC unit (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa).

The stock solutions of aflatoxins (AFs) and OTA at a concentration of 500 μg mL^{-1} were prepared in acetonitrile and stock solutions of STER, ZOL, ZEN, NIV, DON, 3-ADON, 15-ADON, FUS-X, NEO, FB_1, FB_2 and BEA were also prepared at a concentration of 500 μg mL^{-1} but in methanol. Stock solutions of FB_3, DAS, T-2 and HT-2 at a concentration of 100 μg mL^{-1} were prepared in acetonitrile. The internal standards (ISs) were AFM_1 (for AFs) at 0.05 μg mL^{-1} and DOM-1 (for trichothecenes) at 0.150 μg mL^{-1}. Both of these solutions were prepared by dilution of individual stock solutions in methanol.

All solutions were kept in secure conditions at −20 °C.

All other working standard solutions were prepared immediately before use by diluting the stock solution with methanol:water (50:50) (v/v).

2.2. Samples

Baby food samples were purchased and kept at −20 °C under dark and dry conditions. A wide range of brands and retailers, including pharmacies, supermarkets and smaller shops of Valencia (Spain), were covered to ensure that the survey was a representative study. The entire commercial sample was homogenised and a subsample of 200 g of the retail packing was collected in a plastic bag and kept at −20 °C in a dark and dry place until analysis. A total of 35 commercial baby foods, including breakfast foods, savoury products and dessert-cereal products, were bought and analysed between March 2010 and July 2010. Samples were classified according to the way the product is presented as the following: (i) powdered baby food (i.e., multi-cereals, rice, maize, wheat, oats, with fruits, with nuts, with honey, with chocolate), (ii) puréed baby food (cereals and fruit) and (iii) liquid "ready-to-eat" baby food (cereals, fruit juice and milk).

2.3. Extraction procedure

Sample preparation was performed according to a previous study (Rubert et al., 2011). Baby food subsamples (200 g) were mixed thoroughly using an Oster® food processor (Professional Series Blender model BPST02-B00). Portions of 1 g were placed into a glass mortar (50 mL) and gently blended with 1 g of C_{18} for 5 min using a pestle to obtain a homogeneous mixture. This homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column and eluted dropwise with 15 mL of a mixture of acetonitrile:methanol (50:50) (v/v) and 1 mM ammonium formate by applying a slight vacuum. The extract was then transferred to a 25 mL conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 mL with a mixture of methanol:water (50:50) (v/v) and filtered using a 13 mm/0.22 μm nylon filter purchased from Membrane Solutions (Texas, USA) before the injection of the prepared samples into the LC–MS/MS system.

For fortified samples (a sample enriched with a known amount of the analyte to be detected) (EU, 2002), 1 g of "blank" sample (sample in which it was corroborated that no analyte was present) was spiked with 0.2 mL of a working mixture of the mycotoxins at the appropriate concentration and 0.05 mL of the IS mixture at an appropriate concentration as described above. Spiked samples were then left to stand for 3 h at room temperature before the extraction to allow the solvent to evaporate and to establish equilibration between the spiked mycotoxins and the baby food samples. Ten replicates were prepared at each spiking level.

2.4. Instrumentation

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

The analyses were performed using the Turbo V® ionspray in positive ionisation mode (ESI+). The operating conditions for the analysis were the following: ion spray voltage, 5500 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 55 and 65 psi, respectively; probe temperature (TEM), 500 °C. Nitrogen served as the nebuliser and collision gas. SRM experiments were performed to obtain the maximum sensitivity for the detection of target molecules. The optimisation of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) was performed by flow injection analysis for each compound; entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively, for all analytes. The MS was operated in

selected reaction monitoring (SRM) mode and with the resolution set to unit resolution for Q1 and Q3. For increased sensitivity and selectivity, MS/MS data acquisition was also performed in the SRM mode. For LC–MS/MS analysis, scheduled SRM (sSRM) was used with a 45 s SRM detection window and 2 s of target scan time. Scheduled SRM is defined as SRM with the amount of time for detection that surrounds the retention time for each transition. Analyst® version 1.5.1 software (Applied Biosystems/AB Sciex) was used to control all components of the system and also for data collection and analysis.

In addition, to obtain additional confirmation, especially when trace concentration levels were required, IDA experiments were performed with SRM as the survey scan and the EPI mode and MS^3 mode were operated.

2.5. Validation of the method

Quantification of each compound was performed using two SRM transitions and monitoring the SRM ratio. Enhanced product ion (EPI) scan (as an extra information tool) was used for confirmation of the positive mycotoxin findings. To evaluate matrix effects, two strategies were studied: external matrix-matched calibration and internal standard calibration. The matrix effect (ME) for each analyte is defined as the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A) and was calculated for baby food. The ratio (B/A × 100) is defined as the absolute matrix effect (ME%). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%.

Finally, the matrix-matched calibration was used for reliable quantitative determinations. The linearity in the response was calculated using matrix-matched curves prepared by spiking three different baby food presentations and analysing them in triplicate at six concentration levels within the analytical range: from the limit of quantification (LOQ) to 100 times this LOQ.

All results were calculated comparing the area obtained for a blank extract spiked before the extraction (fortified samples) to the results obtained from a blank extract spiked after the extraction (matrix-matched sample). This experiment was repeated 10 times within a day for an intra-day precision test and additionally performed once each day, for 5 consecutive days, for the inter-day test.

The recovery experiments were conducted by spiking the blank baby food in 10 replicates at two concentration levels (LOQ and 10 times LOQ). In this way, intra-day and inter-day parameters of the method were determined at LOQ and 10 times LOQ concentration levels by repeating the analysis of the baby food samples in 10 replicates on the same day and for 5 non-consecutive days.

Limits of detection (LODs) and limits of quantification (LOQs) were defined as the concentration at which the signal-to-noise (S/N) obtained was close to 3 and 10, respectively. These limits were calculated by Analyst version 1.5.1 software (Applied Biosystems/AB Sciex) and both parameters were determined by the analysis of decreasing concentrations of the spiked baby food.

3. Results and discussion

3.1. Determination by LC–QTRAP-MS/MS

In the field of mycotoxin analysis, several methods have been described in the literature using the hybrid triple quadrupole-linear ion trap mass spectrometer (Berthiller, Schuhmacher, Buttinger, & Krska, 2005). In this study, good sensitivity was obtained for selected mycotoxins when the ESI+ mode was applied: the base peak observed was $[M+H]^+$ for all the mycotoxins studied except for trichothecene type A, which formed the stable ammonium adduct as has been widely reported in the literature. Table 2 Supplementary data shows the optimum parameter values for each analyte and the two most relevant SRM transitions: according to the EU regulations (EU, 2002), the first transition was used for quantification purposes, whereas the second transition was used to confirm the presence of target compounds in the sample.

In this work, the mycotoxin patulin has not been included for two reasons. On the one hand, this mycotoxin has been detected mainly in apples and apple products. In the selected baby food samples, this fruit was contained in several samples only as a minor ingredient. It was therefore assumed that if patulin were present, the compound would be present only at trace concentration levels. On the other hand, one of the main aims of this method was to detect as many mycotoxins as possible in a single run. Patulin has traditionally been detected by ESI in negative ionization mode (Kataoka, Itano, Ishizaki, & Saito, 2009). Including patulin in our experiments meant performing another analysis, thereby increasing analysis time.

To compare the performance of the two operating modes of the QTRAP® (triple quadrupole and triple quadrupole linear ion trap), the IDA method was developed. Several experiments were conducted. The first experiment was an SRM method, including the most abundant transitions of the target compounds. The intensity threshold was set at 500 cps and when the intensity of the ions rose to the minimum 3 EPI scans (dependent scans), the ions were released at different collision energies (20, 35 and 50 eV). The inclusion of this IDA experiment provided an unequivocal identification of the mycotoxins in the matrix.

Fig. 1 shows an example of one IDA experiment for the determination of BEA in eight baby food cereal samples. Fig. 1A depicts the Total Ion Chromatogram (TIC) of the sSRM transitions recorded. To isolate each compound separately, the relevant SRM transition can be extracted (XIC) from the TIC (Fig. 1B). Finally, Fig. 1C shows the EPI spectrum obtained for BEA, where the residual precursor ion and two main fragments were present.

3.2. Investigation of matrix effects

One of the main problems encountered in quantitative LC–MS/MS analysis is the existence of matrix effects. Although sampling plans and performance features that control requirements for methods in mycotoxin analysis have been regulated (EU, 2006b; FAO, 2004), there is still a need for a specific performance criterion to overcome these matrix effects in the field of mycotoxin analysis. Other contaminants such as pesticides or veterinary antibiotics have specific guidelines (Document SANCO, 2000, 2003, 2009) that recommend matrix-matched calibration as the optimal option to eliminate these interferences and obtain accurate results.

In this work focused on mycotoxins, different techniques applied in other fields (pesticide and antibiotic analysis) have been evaluated to meet the established performance requirements in mycotoxin analysis (EU, 2002, 2006b). The validation of the method should be conducted in accordance with the performance criteria of the analytical method selected (EU, 2002). External matrix-matched calibration and internal standard calibration were therefore compared to evaluate matrix effects in baby foods.

To obtain more information about the influence on the MS responses of coeluting substances originating from the baby food, the samples were classified based on the way the products were presented: powdered baby food, puréed baby food and liquid "ready-to-eat" baby food. Although all these samples included cereals as a major ingredient, some of them contained other minor ingredients such as fruits, chocolate, honey or nuts.

Because validation for each sample is normally not feasible, various recovery trials for the different "test" samples were performed

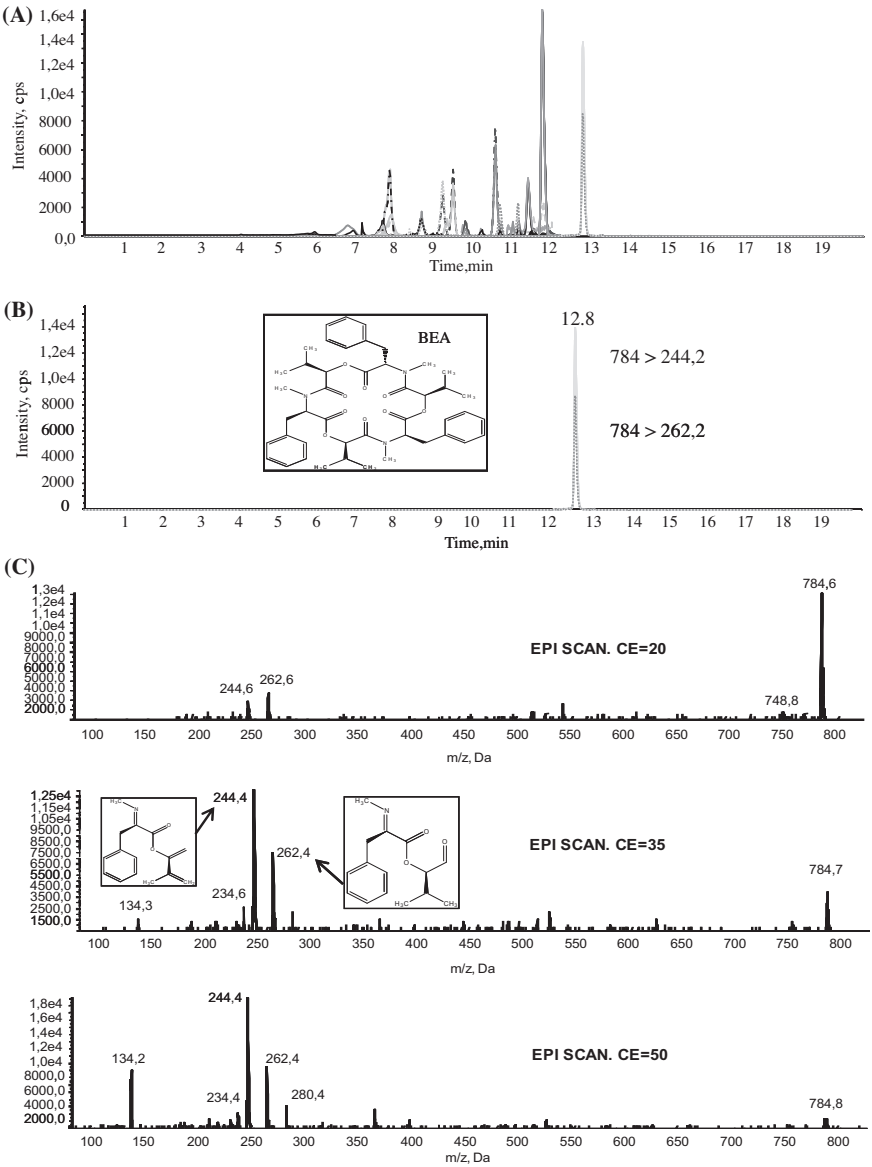

Fig. 1. (A) Total ion chromatogram of SRM, (B) extracted ion chromatogram of BEA and (C) EPI spectrum of BEA simultaneously obtained by IDA in the QTRAP system.

to cover the potential matrix effects. For a preliminary study, (i) eight cereals powdered baby food (eight different cereals), (ii) six fruits and cereals puréed baby food (cereals and fruits) and (iii) cereals and apple liquid baby food (cereals and apple juice) were selected to constitute representative samples to evaluate the matrix effects (ME%) on different baby food groups.

Statistically significant variability was observed for the three different baby food presentations. For the powdered baby food, the matrix suppression was prominent for AFs (36–56%). In the case of puréed and liquid baby food, the highest signal suppression was observed for trichothecenes. Slight enhancement due to the matrix effect was observed only for ZEN in liquid baby food, although it was in the acceptable range (70–120%) (EU, 2002).

Based on the large differences in the matrix effect values within and between baby foods, these matrix effects depended not only upon the cereal composition but also upon its presentation. Based on these results, it became necessary to evaluate matrix effects as a part of the method validation to ensure the reliability of results.

A great number of approaches to evaluate and compensate for these matrix effects have been tested. However, the only way to ensure high accuracy in the results is the use of isotopically labelled internal standards. Ideally, each analyte would be corrected by its own isotope-labelled molecule. Achieving this ideal situation is problematic in a multi-residue method because of the commercial unavailability of several compounds and because of the economic restrictions on the acquisition of a large number of these isotopically labelled compounds. The evaluation of different systems able to compensate for matrix effects is particularly important (Rubert et al., 2011; Sforza, Dall'Asta, & Marchelli, 2006).

In this work, the trichothecenes and AFs were quantified using two common ISs (Berthiller et al., 2005; Ren et al., 2007): DOM-1 and AFM_1, respectively. These two ISs were used to compensate for the matrix suppression effect of trichothecenes (DOM-1) and AFs (AFM_1) in powdered baby food. Only DOM-1 was used in the puréed and liquid baby food because AFs were not suppressed in these baby foods. Matrix effects were only partly corrected by the addition of these ISs because, as previously mentioned, matrix effects were also analyte dependent.

Matrix-matched calibration curves were therefore prepared in baby food extracts, showing good linearity between the LOQ and the 100 times LOQ concentration levels, with a correlation coefficient $\geqslant 0.9900$. The compensation for the matrix effect was evaluated by comparison of these curves with the curves obtained using methanol–water standards as described in the experimental section. Matrix-matched standard calibration was demonstrated to be capable of compensating completely for the matrix effects observed in this study (EU, 2002).

Because of the variability of the matrix influence for different compounds among the samples investigated and presented in this study, the matrix-matched calibrations were used for effective quantification to avoid any over- or under-estimation of residues. Both signal enhancement and suppression were observed when matrix effects were studied.

3.3. Validation of the method

Parameters such as recovery, repeatability, reproducibility and linearity over the working range were evaluated for each mycotoxin. In Tables 1 and 2, the validation parameters obtained are summarised, with the maximum levels (MLs) fixed by the EU for baby food and the IARC classification. Recalling the dependence of mycotoxin behaviour upon sample presentation and cereal composition and the similarity of this dependence to matrix effects, validation parameters were determined using 10 replicates for each of the three different sample presentations (powdered, puréed and liquid baby food).

After some assays, the authors decided to use the eight baby food cereals as a model for the powdered baby food. Cereals and six fruits were used as a model for puréed baby food and milk and cereals and fruits were used as a model for the liquid "ready-to-eat" baby food. When other baby foods were analysed using these samples for matrix-matched calibration, all the results followed the EU guidelines (EU, 2002), achieving acceptable values (Table 2).

Signal-to-noise ratios (S/Ns) of three or above were considered acceptable for LOD and a S/N of 10 or above was considered acceptable for LOQ (Table 1). The LOQs obtained were lower than the ML set by the EU except in the case of AFB_1 and AFM_1. In both of these cases, the objective was to determine these mycotoxins at concentrations that were as low as possible, although AFM_1 in the powdered baby food was the internal standard (IS).

Different criteria for recovery, reproducibility and repeatability have been established to evaluate the suitability for quantitative analysis. In this study, the Commission Regulation (EC) No. 401/2006 of 23 February 2006 specifying the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs (EU, 2006b) was applied.

Table 2 presents a summary of the recoveries and repeatability obtained exclusively for powdered baby food. Puréed and liquid baby food recoveries and repeatability are summarised in Table 3 Supplementary data. Recovery values for the mycotoxins ranged from 68% to 101%, from 69% to 94% and from 68% to 86% (between intra-day and inter-day) for powdered, puréed and liquid baby food, respectively. Relative standard deviations for this procedure were lower than 19%, 15% and 17% for powdered, puréed and liquid baby food, respectively. These results demonstrated that the developed methodology yielded acceptable recoveries from different baby food presentations for all the mycotoxins under study. These results were in good agreement with the performance criteria of Commission Regulation (EC) No. 401/2006 (EU, 2006b).

3.4. Application to commercial samples

The method that had been developed was applied to the evaluation of mycotoxins in commercial baby food samples acquired in different local markets. A total of 35 samples were analysed: 27 powdered baby food samples, 3 samples of puréed baby food and 5 samples of liquid baby food. Table 3 lists all of the results obtained in this study from the analysis of commercial baby food samples. Fig. 2 presents a chromatogram of a spiked sample and a chromatogram of BEA-positive samples with the confirmation criteria.

Internal quality control was conducted for every batch of samples to verify that the analytical system was under control. This internal quality control included a matrix-matched calibration, a reagent blank, a matrix blank and a spiked blank at the LOQ concentration level to check the reliability of the proposed method. For accurate quantification, calibration was performed using external matrix-matched standards in "model" matrices, as explained in the previous section.

The specific mycotoxin in the positive samples was identified by searching in the appropriate retention time window (defined as the retention time ± three standard deviations of the retention time of a blank sample spiked at LOQ for each mycotoxin), and confirmation was conducted by comparison of the signal intensity ratios of the two transitions (quantification and qualification) to the two transitions obtained using fortified blank samples. In addition, IDA experiments for positive mycotoxin samples were performed as an extra confirmation tool.

Positive results (i.e., baby food samples containing mycotoxins) were found for a total of 17 baby food samples. BEA (15 positive samples out of 35 total samples) and DON (9 positive samples out of 35 total samples) were the most commonly detected mycotoxins.

The literature describes the occurrence of trichothecenes in different foodstuffs (Gottschalk, Barthel, Engelhardt, Bauer, & Meyer, 2009; Lombaert et al., 2003). In this study, multi-cereal powdered baby food contained DON and ZEN, while NIV was present in

Table 1
LODs and LOQs obtained in different baby food. IARC classification and maximum levels (ML) for mycotoxins in baby food according to EC1881/2006 Commission regulation.

Compound	IARC classification	ML (EU)	Powdered baby food		Puréed baby food		Liquid baby food	
		Baby foods (μg kg^{-1})	LOD (ng g^{-1})	LOQ (ng g^{-1})	LOD (ng g^{-1})	LOQ (ng g^{-1})	LOD (ng g^{-1})	LOQ (ng g^{-1})
NIV	3		35	100	70	200	50	175
DON	3	200	20	60	45	140	30	125
3-ADON	n.c.		4	16	4	16	4	16
15-ADON	n.c.		5	15	5	15	5	15
FUS-X	3		10	30	15	45	18	55
DOM-1	n.c.		8	25	12	40	15	50
NEO	n.c.		7	20	10	35	15	50
DAS	n.c.		2	5	8	25	4	15
HT-2	n.c.		3	10	6	20	10	30
T-2	3		0.8	2	1.2	4	2	6
FB_1	2B	200[a]	22	70	25	80	35	90
FB_2	2B		25	72	32	100	40	90
FB_3	n.c.		23	75	30	95	25	80
ZEN	3	20	2	8	3	10	3	9
ZOL	n.c.		5	18	6	20	6	18
BEA	n.c.		0.5	1.8	0.5	2	0.5	1.5
AFB_1	1	0.1	0.05	0.10	0.05	0.10	0.05	0.10
AFB_2			0.4	1	0.3	1	0.3	1
AFG_1			0.35	1	0.25	0.75	0.25	0.8
AFG_2			0.45	1	0.35	1	0.35	1
AFM_1	2B	0.025	0.08	0.25	0.03	0.1	0.015	0.05
STER	2B		2.5	8	3.5	12	4	15
OTA	2B	0.5	0.1	0.3	0.15	0.5	0.15	0.5

n.c., not classified. Group 1 IARC: the agent is carcinogenic to humans. Group 2A IARC: the agent is probably carcinogenic to humans. Group 2B IARC: the agent is possibly carcinogenic to humans. Group 3 IARC: the agent is not classifiable as to its carcinogenicity to humans.
[a] Expressed as the sum of fumonisins (FB_1 + FB_2).

wheat-based baby food at trace levels (lower than the LOQ range). These results were reported by Gottschalk et al. (2009).

The maximum level of DON is fixed by the EU at a ML of 200 ng g^{-1}, and no sample exceeded this limit. This mycotoxin has been widely reported in the literature; Lombaert et al. (2003) evaluated 367 cereal-based infant foods and the authors affirmed that DON was the most frequently detected mycotoxin (63% of the samples).

A concentration level for BEA is not legislated by the EU, and it is therefore not possible to determine the suitability of the products containing BEA for the infant population. Other authors have confirmed the presence of BEA in baby food (Mahine et al., 2011), making the inclusion of BEA in a multi-mycotoxin analysis in baby food necessary. The high incidence of this mycotoxin in wide range of samples demonstrates the problem posed by these "emergent" mycotoxins where the concentration levels are not fixed by the EU, but these "emergent" mycotoxins are found to be present in the food chain.

ZEN, AFG_2, OTA, FB_1 and FB_2 were also detected in several samples, although in all cases, the concentrations were lower than the ML established by the EU. These mycotoxins have also been described in the literature (Beltrán et al., 2011; D'Arco et al., 2008; Lombaert et al., 2003).

Table 2
Recoveries (%) and repeatability (RSD, %) given in brackets for the selected mycotoxins in powdered baby food at LOQ and 10 LOQ concentration levels.

Compound	Powdered baby food			
	Low level		High level	
	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]
NIV	85 (9)	80 (11)	77 (10)	78 (13)
DON	88 (10)	81 (8)	85 (9)	82 (9)
3-ADON	71 (4)	77 (6)	69 (5)	79 (4)
15-ADON	70 (7)	78 (4)	68 (6)	77 (5)
FUS X	76 (5)	70 (4)	74 (4)	73 (8)
DAS	74 (7)	71 (9)	79 (2)	73 (8)
HT-2	79 (9)	75 (7)	78 (9)	74 (6)
T-2	71 (7)	70 (5)	70 (3)	73 (10)
NEO	75 (5)	77 (7)	73 (6)	77 (9)
FB_1	98 (17)	101 (16)	93 (15)	94 (12)
FB_2	97 (12)	95 (14)	96 (18)	93(15)
FB_3	98 (13)	99 (18)	97 (19)	99 (18)
ZEN	76 (6)	75 (7)	73 (4)	74 (9)
ZOL	78 (9)	78 (6)	75 (9)	79 (6)
BEA	71 (9)	73 (5)	69 (8)	74 (11)
AFB_1	73 (6)	77 (8)	70 (8)	78 (12)
AFB_2	77 (9)	74 (7)	75 (9)	76 (14)
AFG_1	72 (7)	74 (6)	69 (10)	77 (16)
AFG_2	78 (6)	79 (7)	75 (9)	78 (17)
STER	71 (6)	72 (4)	70 (7)	70 (6)
OTA	78 (7)	73 (5)	72 (8)	76 (9)

Low level: LOQ level. High level: 10 times LOQ level.
[a] Number of replicates: 10.
[b] Different days: 5.

Table 3
Occurrence of mycotoxins in 35 analysed baby food.

Mycotoxin	Sample (total positive samples)	Range concentration (μg kg^{-1})
NIV	Wheat-based powdered baby-food (2)	<LOQ
DON	Multicereal powdered baby-food (5)	70–210
	Cereal and fruit liquid baby food (1)	
	Wheat-based powdered baby-food (2)	
	Cereal and fruit purée baby food (1)	
OTA	Cereal liquid baby food (1)	0.35–0.5
	Oat-based powdered baby-food (1)	
FB_1	Corn-based powdered baby-food (1)	75–100
	Multicereal powdered baby-food (2)	
FB_2	Corn-based powdered baby-food (1)	75
AFG_2	Corn-based organic powdered baby-food (1)	1.2
ZEN	Multicereal powdered baby-food (1)	10–15
	Corn-based powdered baby-food (1)	
BEA	Multicereal powdered baby-food (3)	5–100
	Wheat-based powdered baby-food (2)	
	Corn-based powdered baby-food (2)	
	Rice-based powdered baby-food (4)	
	Cereals and fruits purée baby food (1)	
	Soy-based powdered baby-food (1)	
	Cereal and fruit liquid baby food (1)	
	Oat-based powdered baby-food (1)	
AFM_1	Cereal and fruit liquid baby food (1)	<LOQ
STER	Rice-based powdered baby-food (2)	10–50

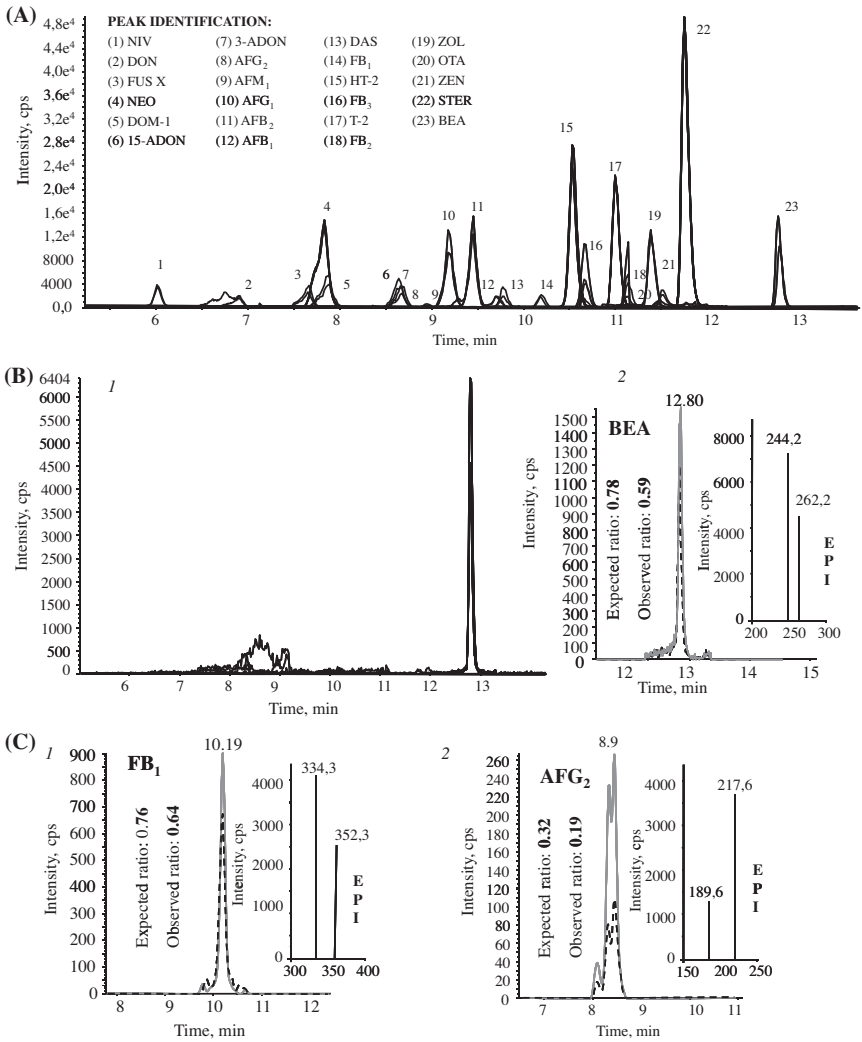

Fig. 2. (A) Typical chromatogram of spiked sample at LOQ concentration level. (B) 1. Chromatogram of BEA positive rice-based baby food; 2. Confirmation by the accomplishment of *Q*/*q* ratios and EPI scan. (C) Confirmation of positive samples: 1. FB_1; and 2. AFG_2.

OTA is the related mycotoxin present at the highest concentrations reported in the literature in cereals and derivative products (Duarte, Pena, & Lino, 2010). In this work, however, OTA was detected in oat-based powdered baby food and in multi-cereal liquid "ready-to-eat" baby food.

The analytical method that was developed detected FB_1 and FB_2 simultaneously in one maize-based powdered baby food but detected only FB_1 in one multi-cereal powdered baby food. In the two positive fumonisin samples, the FBs were detected at levels below the ML fixed by the EU. The maize is one of the raw materials most commonly contaminated by fumonisins (Anfossi et al., 2010; D'Arco et al., 2008).

AFG_2 was detected in one maize-based organic powdered baby food. This result indicates that more attention should be paid to post-harvest conditions to minimise the content of these toxins (Frenich, Vidal, Romero-González, and Aguilera-Luiz (2009)).

STER was presented in two powdered baby food samples, and similarly BEA, this mycotoxin has no ML established by the EU. It

is therefore not possible to determine the safety of the products found to contain this mycotoxin.

4. Conclusion

The analytical method has been validated for three different baby food presentations (powdered, puréed and liquid), obtaining satisfactory accuracy and precision for the analyte/matrix concentrations studied, supporting the applicability of the method and considering the maximum allowable levels of mycotoxins established by the EC for these food stuffs. Present research fails to attain the required sensitivity only for AFB_1 in baby food analysis according to the very low concentration levels required by the EC. However, the LOQ attained for this mycotoxin was very close to this established low concentration level, allowing quantification of AFB_1 at trace levels.

In this work, the authors suggest the application of the commonly accepted matrix-matched calibration approach as an attempt to resolve matrix effects in the mycotoxin field when other methods are unattainable or not available. Consensus on the evaluation of these matrix effects is obviously needed.

The survey clearly demonstrated the regular presence of low levels of mycotoxins in cereal-based infant foods. Considering the potential negative health impact of the presence of the analysed toxins, baby foods must be controlled to contribute to a broader exposure assessment of the health effects associated with the presence of mycotoxins in food given to babies and infants.

The main obstacle in this study was the absence of regulations for some of the mycotoxins. Regulations are necessary for deciding whether the presence of the observed concentration levels is tolerable or not. The toxicological effects of consuming these contaminated products over a period of time are difficult to predict and to evaluate. Consumption of these contaminated products over a prolonged period of time is a concern because these products are directed at the infant population.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.12.035.

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4.7 Study of mycotoxin calibration approaches on the example of trichothecenes analysis from flour.

Study of mycotoxin calibration approaches on the example of trichothecenes analysis from flour

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1. Introduction

The analysis of mycotoxins is changeling as they are usually present in minute concentrations in complex matrices, and they may occur in various combinations produced by a single or by several fungal species. The fact that most mycotoxins are toxic at very low concentrations requires sensitive and reliable methods for their detection (Capriotti et al., 2010), because of the fact that EU directives are restrictive (EU, 2006; EU, 2007).

LC-MS/MS is becoming the procedure of choice for simultaneous determination of mycotoxins from food (Turner et al., 2009; Rasmussen et al., 2010). However, it is well known that matrix effects are one of the main drawbacks of LC-MS/MS methods. These undesirable effects typically cause a loss of method accuracy, precision and sensitivity leading to incorrect quantification and also to problems for accurate confirmation (Marín et al., 2009).

Traditional calibration methods, such as external standard, internal standard or standard addition method, can be used for the quantification purposes; each calibration method presents different advantages and disadvantages (Ouyang and Pawliszyn, 2008).

Matrix-matched calibration has been wide-used to compensate matrix effects. The advantage of this method is that matrix effects can be compensated since all samples will be affected to the same extent. It is appropriate when the sample composition is unknown and complex. This procedure has been used for different organic contaminants in many different matrices (Rasmussen et al., 2010; Rubert et al., 2010, 2011).

Although the use of matrix-matched calibration standards has been recommended by SANCO (SANCO, 2009), different calibration approaches have been reported in the literature for effective quantification analysis of mycotoxins by LC-MS/MS.

The use of matrix-matched calibration or internal standard (IS) calibration can minimize the variations between samples (Cuadros-Rodríguez et al., 2007; Lattanzio et al., 2011). The use of appropriate IS can overcome ion

suppression/enhancement. However, this ideal analysis is difficult or expensive to do, because it is necessary two homologous analytes.

The objective of the present work was to evaluate the applicability of different calibration strategies to compensate or minimize matrix effects. An example is given for evaluating the results obtained using matrix solid-phase dispersion (MSPD) extraction for the analysis of eight trichothecenes from flour. To calculate reliable concentrations, three approaches were compared: (i) matrix-matched calibration, (ii) analogue internal standard calibration and (iii) deuterated internal standard calibration.

2. Material and methods

2.1. Reagents and materials

HPLC grade solvents, acetonitrile, methanol and water were supplied by Thermo Fischer (Dublin, Ireland). Analytical grade reagent formic acid (purity > 98%) and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain). Solid-phase used for MSPD was octadecysilica (C_{18}) (50 µm) bonded silica from Análisis Vínicos S.L. (Tomelloso, Spain).

The standards of nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON), Fusarenon X (FUSX), neosolaniol (NEO), diacetoxyscirpenol (DAS), 3-ADON-d_3 and DON-d_1 were purchased from Sigma Aldrich (Madrid, Spain). T-2 and HT-2 toxin, deepoxy-desoxynivalenol (DOM-1) stock solutions (in acetonitrile) were obtained from Biopure referenzsubstanzen GmBH (Tulln, Austria). The individual stock solutions of NIV, DON, 3-ADON, 15-ADON, FUS-X, NEO were prepared at 500µg mL^{-1} in acetonitrile. On the other hand, stock solutions of DAS, T-2 and HT-2 at concentration of 100µg mL^{-1} were prepared in acetonitrile. All standards were kept at -20ºC. DOM-1, DON-d_1, 3-ADON-d_3 and NEO were used as internal standard compounds at 0.150 µg mL^{-1} and they were prepared by dilution of individual stock solutions in acetonitrile and kept at -20ºC. All other working

standard solutions were prepared immediately before use by diluting the stock solution with methanol: water (50:50, v/v).

Certified reference material (BRM 003004) was purchased from Biopure referenzsubstanzen GmBH (Tulln, Austria).

2.2. Samples

Commercial wheat flour samples were purchased from different stores and supermarkets of Cork (Ireland) and Valencia (Spain). The composition of wheat flour was completely wheat and these matrices were used for the calibration approaches. All blank samples (in which it was corroborated before the analysis that no analyte was present) were stored at -18 °C prior to mycotoxin analysis.

2.3. Sample preparation

An MSPD validated method (Rubert, 2011) was used for the extraction. Samples (200 g) were prepared using a food processor and mixed thoroughly. Portions of 1 g were mixed with 1 g of C_{18} for 5 min using a pestle, to obtain a homogeneous mixture. The homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 15 ml of acetonitrile: methanol (50:50, v/v) 1 mM ammonium ammonium formate by applying a slight vacuum. Then, the extract was transferred to a 25 ml conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 ml of methanol:water (50:50, v/v), consecutively it was filtered through a 0.20 µm Millex-GN nylon filter (Millipore, Carrightwohill, Ireland) and collected into a vial before a prior to be injected into the LC-MS/MS system.

2.4. Liquid chromatography tandem mass spectrometry

The LC-MS/MS system consists on a Finnigan Surveyor CTC (Autosampler ThermoFischer Scientifics), a Finnigan Surveyor LC quaternary Pump (Accelera, ThermoFischer Scientifics) and a Finnigan TSQ Quantum Discovery MAX triple quadrupole mass spectrometer (Thermo Fischer Scienctific, Hemel Hempstead, UK). Chromatographic separation was performed on a Luna HST C_{18} column

(100mm×3.00mm I.D., 2.5µm particle size) from Phenomenex. The mobile phase was a gradient of 5mM ammonium formate in water (pH 5.6) (A) and 5mM ammonium formate in methanol (B). The gradient elution program started with 10% B, and increased linearly to 90% B in 12 min, and kept at 90% B for 2 min, and then returned to the initial composition (10% B) in 1 min and held for 7 min to re-equilibrate the column prior to the next injection. The flow rate was 300 µL min^{-1}. The autosampler was set at 10ºC and column temperature was set at 35 ºC. An injection volume was 10µL.

All mycotoxins were detected using heated electrospray (H-ESI) source. First, mycotoxins standard solutions (10µg mL^{-1}) were infused (10 µL min^{-1}) with a syringe pump. The optimizations of MS parameters were performed by flow injection analysis for each compound and the values are summarized in *Table 1*. Ion source parameters were optimized for each compound using the quantum tune application of Xcalibur 2.0.7 software. The source was operated in the positive ESI mode; spray voltage, 4500 V; vaporizer temperature, 300 ºC; ion transfer capillary temperature, 350 ºC; with both the sheath gas pressure set to 40, auxiliary gas pressure 55 arbitrary units and ion sweep gas was set to 0 arbitrary. Skimmer offset was set to -3 V and the collision gas pressure was 1.5 mTorr. Data processing was performed using the Xcalibur (Version 2.0.7) software (ThermoFischer Scientifics). Mass spectral data were acquired in SRM mode in a single time segment with 20 ms dwell time for each transition. Collision energy and tube lens offset voltages were optimized for each mycotoxin using the automated optimization procedure in syringe infusion mode provided by the manufacturer. *Table* 1 shows the two monitored transitions for each mycotoxin and the parameters optimized.

2.5. Mycotoxin solutions for external matrix-matched calibration

Calibration solutions for external matrix-assisted curve (eight-point calibration) were prepared in blank sample extracts (it was corroborated before the analysis that no analyte was present) obtained following MSPD extraction method (section 2.2).

Concentration levels between limit of quantification (LOQ) and 100 times this LOQ were added to aliquots before drying it down. Then the residue was dissolved with 1 ml of methanol:water (50:50, v/v) by vortex for 1 min.

2.6. Standard solution for internal calibration

For the analysis of naturally contaminated samples, internal standards (ISs), deuterated and analogues, were used. The deuterated internal standards used for quantification were DON-d_1 and 3-ADON-d_3 at concentration level of 0.150 µg mL^{-1}. On the other hand, DOM-1 and NEO were used as analogue internal standard compounds at the same concentration level (0.150 µg mL^{-1}).

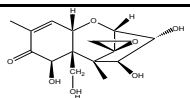
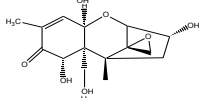
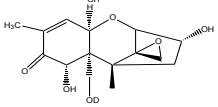
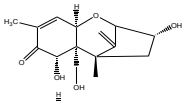
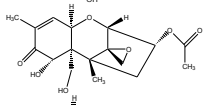
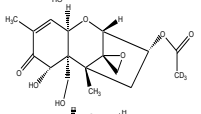
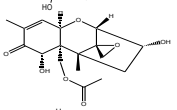
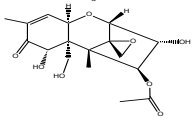
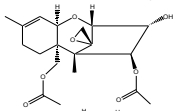
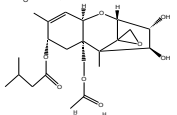
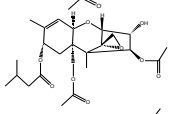
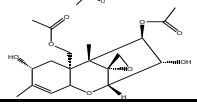
In any case, internal standards were added to the working solutions at adequate concentration levels

2.7. Method performance

A previously developed MSPD extraction procedure was applied (Rubert, 2010). In order to evaluate the applicability of the external and internal calibration approaches, performances such as recoveries, repeatability, detection limits and matrix effects were studied.

The criteria applied to identify mycotoxins was: (i) a signal for each of the two SRM transitions of the analyte had to be equal in the sample and in the standard or matrix matched (ii) the ratio between the relative (to the I.S. when it was used) retention time of the analyte in the sample and that of this analyte in standard solution should be within ± 2.5% tolerance. When IS was not used the measured retention time of the suspected peak had to correspond to the measured retention time of the standard. At the end, (iii) the peak area ratio of the confirmation transition against the quantification one should be within the tolerance fixed by the EU criteria (EC, 2002).

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

Mycotoxin	Structure	Precursor Ion(m/z)	Product Ion	Collision Energy (eV)	Turbo Lens
NIV		313 $[M+H]^+$	175^Q 115^q	54 30	100
DON		297 $[M+H]^+$	115^Q 127^q	56 49	61
DON-d_1		298 $[M+H]^+$	248^Q 175^q	25 14	51
DOM-1		281 $[M+H]^+$	118^Q 134^q	27 31	93
3-ADON		339 $[M+H]^+$	231^Q 278^q	14 13	63
3-ADON-d_3		342 $[M+H]^+$	202^Q 212^q	18 16	52
15-ADON		339 $[M+H]^+$	115^Q 127^q	55 35	92
FUSX		355 $[M+H]^+$	336^q 175^Q	28 12	123
DAS		384 $[M+NH_4]^+$	307^Q 105^q	11 33	113
HT-2		442 $[M+NH_4]^+$	263^Q 215^q	13 12	102
T-2		484 $[M+NH_4]^+$	215^Q 305^q	20 13	102
NEO		400 $[M+NH_4]^+$	185^Q 215^q	19 18	95

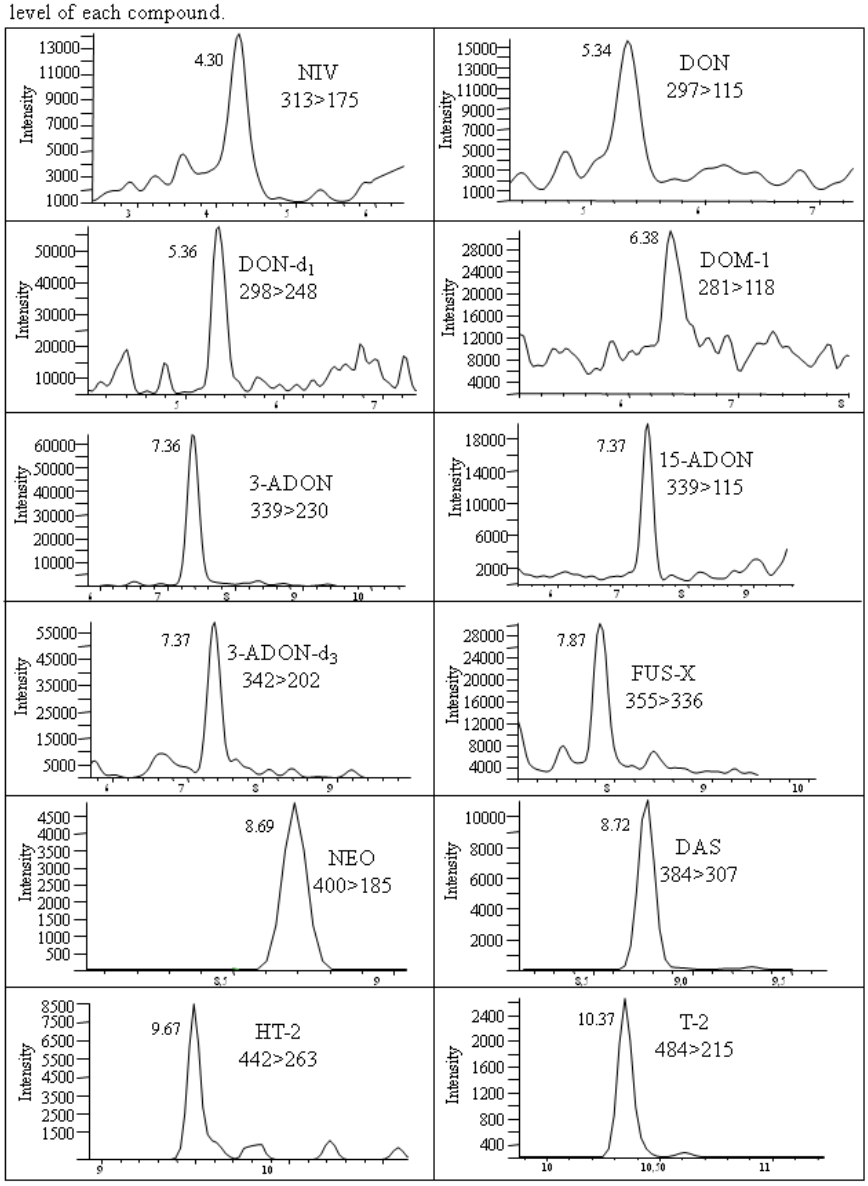
The calibration curves were evaluated by constructing an eight calibration points between LOQ and 100 times LOQ analysing them in triplicate. The calibration curves were prepared in solvent and in matrix extracts. To asses matrix effects (ME) the slope of matrix matched wheat flour (B) and the slope of standards in methanol-water (A) were calculated. Thus, the ratio (B/A x 100) is defined as the absolute matrix effect (ME %). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%.

In this study, absolute recovery was calculated by comparing the mean area response of extracted samples (spiked before extraction) to that of blank spiked samples (spiked after extraction) at each concentration level. Relative recovery was assessed by the ratio comparing the mean area response of extracted samples (spiked after extraction) at each concentration level with mean area of neat IS solution and the mean are response of blank spiked samples (spiked after extraction) at each concentration level to those obtained by IS solution.

Spiking the sample in ten replicates at two concentration levels; LOQ concentration level and 100 times LOQ concentration level carried out the recovery experiments. In the same way, intra-day and inter-day repeatability of the method were calculated by carrying out repeating the analysis of wheat flour, during the same day and five non-consecutive days respectively, at LOQ and 100 times LOQ in ten replicates and expressed as relative standard deviation (RSD).

Limit of detection (LOD) is defined as the concentration with a signal-to-noise (S/N) of 3 while limit of quantification (LOQ) is defined as the concentration with a signal-to-noise (S/N) of 10. These limits were calculated by Xcalibur software; both parameters were determinate by analysis decreasing concentration of the spiked wheat flour. *Figure 1* shows a chromatogram after MSPD extraction at 2 times LOQ level.

Figure 1. LC–MS/MS chromatogram obtained from an extract of wheat flour at 2 times LOQ level of each compound.



2.8. Recovery experiments

To determine the recoveries obtained by optimized MSPD, wheat flour samples were spiked in ten replicates with a standard mixture of mycotoxins at concentration levels between LOQ and 100 times this LOQ. The spiked samples were homogenized vigorously in order to enable better contact of mycotoxins with the matrix. The samples were left to stand 3 hours at room temperature before the extraction to allow the evaporation of the solvent and to establish equilibration between the mycotoxins and flour. Consecutively, spiked samples were extracted and treated by the previously described protocol (Rubert, 2011).

3. Results and discussion

3.1. Matrix effects

Before validating the extraction procedure, it is necessary to check the presence of matrix effect. Matrix effects were estimated as it was previously explained in the section 2.7 method performance. All the results are summarized in *table 2*.

From this *Table 2*, it is evident that signal of analytes were affected by suppression (ME% <100%) since the range varied from 59% to 79% confirming that matrix effects pose a problem with this extraction procedure of trichothecenes. However, when comparing the slope of extracted samples (spiked before extraction) to that of blank spiked samples (spiked after extraction), the matrix effects were compensated obtaining values ranged between 69 % to 85 %. This compensation was improved using IS: matrix effects ranged between 76 % to 111 % and 78 % to 108 % by comparing with analogue IS and deutered IS respectively.

Cuadros-Rodriguez (Cuadros-Rodríguez et al., 2007) reported that the matrix produces a change in the signal which can be: (i) constant and independent of the analyte amount, (ii) variable and proportional, (iii) the combination of both type of effects. In this study, after to inject different amounts of matrix, it could be concluded that the matrix effects were constant and independent of the analyte amount presented in the extract (data not shown).

The linear dynamic range was also calculated (*table 2*); the calibration curves for mycotoxins were linear over the analytical concentration range (LOQ and 100xLOQ) and correlation coefficients were >0.9899.

In order to demonstrate specificity and ruggedness during 3 months, the tolerance of the relative ion abundances varied no more than 16%, and retention time no more than 2%. This meets requirements reported in the 2002/657/EC Decision (EC, 2002).

Table 2. Evaluation of matrix effects in wheat flour. Concentration range used for standard in solvent, matrix-matched calibration and spiked wheat flour.

Compound	Linear dynamic range (µg/kg)	Absolute matrix effects[a]	Corrections		
			Matrix-matched[b]	Analogue IS	Deuterated IS
NIV	110-11000	66	81	88[1]	93[3]
DON	45-4500	69	77	87[1]	90[3]
3-ADON	18-1800	62	69	95[1]	107[4]
15-ADON	20-2000	60	71	95[1]	108[4]
FUSX	30-3000	70	74	76[1]	78[3]
DAS	6-600	69	85	108[2]	
HT-2	10-1000	79	79	100[2]	
T-2	6-600	72	82	111[2]	
DOM-1 (IS)		72			
NEO (IS)		63			
DON-d_1 (IS)		64			
3-ADON-d_3 (IS)		59			

ME% [(a)]: slope matrix matched wheat flour/slope standard in solvent x 100
Matrix-matched % [(b)]: slope spiked wheat flour/slope matrix matched wheat flour x 100
Analogue IS: DOM-1[1] and NEO[2]
Deuterated IS: DON-d1[3] and 3-ADON-3d[4]

3.2. Calibration approaches and reliable quantifications

Matrix effects produce the presence of systematic errors during the measurement step. In order to avoid these errors, the applicability of three calibration approaches was evaluated in this study.

3.2.1 Matrix-matched multi-level calibration

Matrix-matched calibration standards have been recommended by SANCO in order to account for matrix effects (SANCO, 2009). In this study, matrix matched calibration allowed to obtain absolute recoveries ranged from 69% to 85% (*Table* 3). The repeatability or intra-day precision of the method expressed in terms of RSDs by analyzing 10 replicates at two concentration levels (LOQ and 100 times LOQ) ranged from 4% to 8%. The reproducibility or inter-laboratory reproducibility obtained by analysing five non-consecutive days at two levels varied from 4% to 14%.

3.2.2. Internal standard calibration

The addition of internal standards is largely used, however, not all the labelled analogues of trichothecenes were accessible to us because either they were not commercially available or they were excessively expensive. In this research different internal standard were evaluated; concretely analogue IS and deuterated IS.

3.2.2.1. Analogue internal standard addition

DOM-1 and NEO were used as analogues IS (these mycotoxins have similar structure to that mycotoxins to calibrate) (Table 1); DOM-1 was used to quantify type B trichothecenes while type A trichothecenes were quantified using NEO. After comparing the mean area response of extracted samples (spiked after extraction) with mean area of analoguet IS solution and the mean are response of blank spiked samples (spiked after extraction) to those obtained by analogue IS, matrix effects varied from 76% to 111% (Table 2). The calibration curves were linear over LOQ and 100 times LOQ range and correlation coefficients were >0.9933. The relative recoveries ranged between 83% and 112% for intra-day

repeatability (RSD < 8%), as well as, acceptable values were obtained for inter-day reproducibility between 84% and 114% (RSD < 11%) (Table 3).

Table 3. Analytical parameters: LOD, LOQ and absolute and relative recoveries, relative standard deviation (RSD) in brackets (%) in wheat flour spiked at LOQ concentration level (low level) and 100 times LOQ concentration level (high level).

Mycotoxin	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	Absolute recovery (%)				Relative recovery (%)							
			Low level		High level		Analogue IS				Deuterated IS			
							Low level		High level		Low level		High level	
			Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[b]	Intra-day[a]	Inter-day[a]
NIV	35	110	72 (6)	74 (4)	73 (6)	75 (9)	83 (8)[1]	84 (7)[1]	85 (3)[1]	87 (8)[1]	82 (7)[3]	82 (8)[3]	83 (5)[3]	82 (8)[3]
DON	15	45	82 (5)	79 (8)	85 (5)	79 (10)	90 (2)[1]	89 (5)[1]	93 (2)[1]	91 (7)[1]	98 (3)[3]	98 (4)[3]	100 (3)[3]	98 (7)[3]
3-ADON	6	18	72 (4)	78 (6)	73 (5)	77 (7)	109 (5)[1]	111 (8)[1]	103 (6)[1]	104 (7)[1]	101 (3)[4]	109 (3)[4]	100 (2)[4]	103 (4)[4]
15-ADON	7	20	71 (5)	77 (8)	71 (8)	76 (9)	112 (6)[1]	114 (9)[1]	109 (4)[1]	106 (9)[1]	112 (7)[4]	111 (9)[4]	110 (5)[4]	112 (9)[4]
FUSX	10	30	76 (5)	70 (9)	77 (5)	71 (9)	105 (6)[1]	103 (6)[1]	106 (3)[1]	101 (5)[1]	104 (3)[3]	104 (5)[3]	103 (4)[3]	102 (9)[3]
DAS	2	6	78 (8)	77 (10)	81 (5)	77 (14)	89 (3)[2]	89 (7)[2]	91 (4)[2]	87 (11)[2]				
HT-2	3	10	73 (4)	69 (5)	75 (5)	71 (7)	107 (4)[2]	104 (6)[2]	110 (6)[2]	109 (8)[2]				
T-2	2	6	80 (8)	79 (8)	83 (5)	81 (11)	105 (7)[2]	108 (8)[2]	107 (6)[2]	108 (9)[2]				

Analogue IS: DOM-1[1] and NEO[2]
Deuterated IS: DON-d_1[3] and 3-ADON-d_3[4]
Low level: LOQ level
High level: 100 times LOQ level
[a] Number of replicates:10
[b] Different days: 5

3.2.2.2. Deutered internal standard addition

On the other hand, DON-d_1 and 3-ADON-d_3 were used as deuterated IS. In this case labelled deuterated of type A trichothecenes were not commercially available. For this reason, type A trichothecenes were not calibrated in this section. In our study DON-d_1 was used to quantify DON, NIV and FUSX and 3-ADON-d_3 was used to quantify 3-ADON and 15-ADON, although, strictly, the method should be applied for the signal correction of DON and 3-ADON, since each mycotoxin should be calibrate with it deuterated IS.

The corrections of matrix effects varied from 78% to 108% (*Table 2*). The calibration curves were linear over LOQ and 100 times LOQ and correlation coefficients were >0.9981. Deuterated IS calibration allowed to obtain relative recoveries ranged from 82% to 112% with RSD <9% (*Table 3*). The repeatability of the method expressed in RSDs obtained by analyzing 10 replicates at LOQ level and 100 times LOQ level was ranging from 3% to 7%, while the reproducibility obtained by analysing five non-consecutive days at LOQ level and 100 times LOQ level varied from 4% to 9%.

Although the calibration approach should have been reasonably applied for the signal correction of DON and 3-ADON, the results were acceptable for all studied compound (*Table 2* and *3*). The accurate results obtained with fix and deuterated internal standard were according to Directive (EC) 2002/657/CE (EC, 2002).

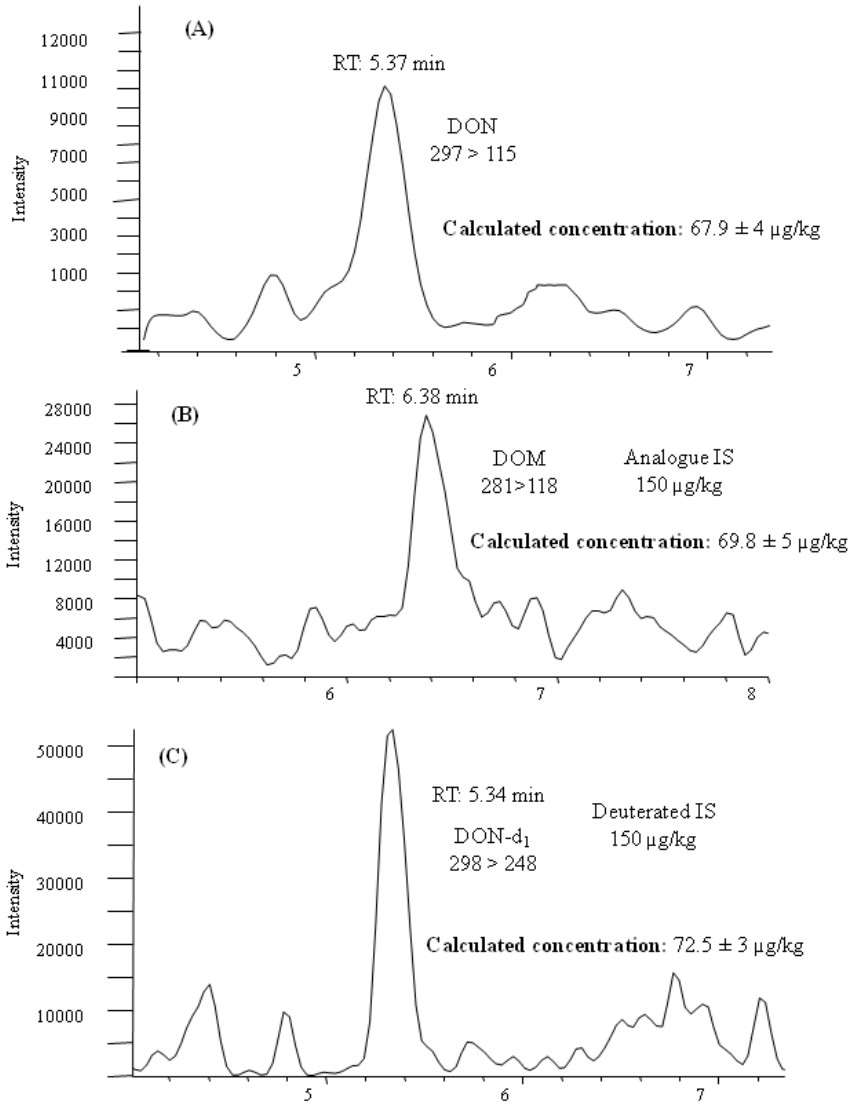
3.3. Trueness of the method

The trueness of the method was demonstrated by 3 ways: (i) recoveries, (ii) quality control material and (iii) certified reference material.

Respect to the recoveries, as it has been described before, recovery values were according to European guidelines. *Figure 2* shows a chromatogram obtained after MSPD extraction of wheat flour fortified at 75 µg/kg of DON and IS in appropriate concentration. The calculated concentration for each approach calibration is showed also, demonstrating acceptable values.

Finally, certified reference material (BRM 003004) was used. Calculated concentration was 969±18 µg/kg (n=6) when matrix-matched was used. In the event, trueness was demonstrated for analogue and deuterated IS using the same reference certified material (BRM 003004). DON was therefore quantified by DOM-1 and DON-d_1. When DOM-1 was used for calculating concentration, this was 973±15 µg/kg (n=6). DON-d1 allowed obtaining reliable data. The calculated concentration was 999±11 µg/kg (n=6). These calculated concentrations were satisfactory according to the certificated values 1062±110 µg/kg.

Figure 2. Chromatogram obtained after MSPD extraction in wheat flour fortified at 75 µg/kg of DON and ISs. Reliable quantification of DON using matrix-matched (A) , analogue IS (B) and deuterated IS (C) (n=5).



4. CONCLUSION

The extraction method, known as MSPD, is easy, cheap, fast and a robust method for type A and B trichothecenes analysis. One of its main advantages is that extraction and clean-up are in the same step. However, not all the interfering components are eliminated. The presence of these components can cause errors leading to inaccurate results. These problems should be solved by using appropriate calibration method.

Internal standard addition is the wide-used calibration technique for the quantification of mycotoxins. The mycotoxins and IS should exhibit the same behaviour in extraction and purification steps, as well as identical or very similar retention time during the separation. In this study, best accurate results were obtained when deuterated internal standards were used.

Because they are expensive and there is unavailability of isotopically labelled standard for each target mycotoxin and, moreover, this addition decrease the sensitivity as increase the number of selected transitions, the structural analogue internal standard addition method may represent another possibility. DOM-1 and NEO helped slightly to relax matrix effects. However, since the analogues and mycotoxins are not identical, different co-elution with different matrix components can occurs and one cannot assume the same underlying mechanism, when comparing signal enhancement or suppression observed for different mycotoxins.

When matrix-matched calibration was used, a practically full compensation of matrix effects is achieved. Moreover, the trueness was demonstrated by certified reference material for internal and external calibration. The problem is that an appropriate blank is necessary.

If European guideline does not fix an adequate form to calibrate, the analyst should decide the best option according to the possibilities.

Conflict of interest

The authors declare that there are no conflicts of interest.

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4.8 Analysis of mycotoxins in barley using ultra high liquid chromatography high resolution mass spectrometry: Comparison of efficiency and efficacy of different extraction procedures.

Analysis of mycotoxins in barley using ultra high liquid chromatography high resolution mass spectrometry: Comparison of efficiency and efficacy of different extraction procedures

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Abstract

The effectiveness of four extraction methods (modified QuEChERS, matrix solid-phase dispersion (MSPD), solid-liquid extraction (SLE) and solid-phase extraction (SPE) clean-up) were evaluated for simultaneous determination of 32 mycotoxins produced by the genus *Fusarium*, *Claviceps, Aspergillus*, *Penicillium* and *Alternaria* in barley by ultra high pressure liquid chromatography coupled to ultra-high resolution mass spectrometry (UHPLC-Orbitrap® MS). The efficiency and efficacy of extraction methods were evaluated and compared in number of extracted mycotoxins and obtained recoveries. From the one point of view, QuEChERS procedure was fast and easy, as well as it was able to successfully extract all selected mycotoxins. On the other hand, SLE method, MSPD and SPE clean-up method did not extract adequately all selected mycotoxins and recoveries were not suitable enough. Thereby, method employing QuEChERS extraction connected with UHPLC-Orbitrap® MS was developed to quantify 32 mycotoxins in barley within this study. Analytical method was validated and recoveries ranged from 72 to 101% for selected mycotoxins with only one exception nivalenol (NIV) and deoxynivalenol-3-glucoside (D3G), which were lower than 67%. Relative standard deviations (RSD) were lower than 17.4% for all target mycotoxins. The lowest calibration levels (LCLs) ranged from 1 to 100 µg/kg. Validated method was finally used for monitoring mycotoxins in Czech barley samples, when only *Fusarium* toxins representatives were detected in 53% of samples and the mycotoxins with the highest incidence were enniatins.

Keywords: *QuEChERS; MSPD; Clean-up; Solid-liquid extraction; Mycotoxins: Orbitrap*

1. Introduction

Cultivated for over 10.000 years, barley is one of the oldest domesticated grain crops. There are different varieties of barley, which have been developed during a long time. Actually barley is the world's fourth most important crop and an important staple in many countries. The largest commercial producers of barley are Canada, United States, Russia, Germany, France and Spain [1, 2]. Moreover, in countries like Czech Republic, barley is a crop with a great economical importance, with a cultivation area of about 400 000 ha [3]. The use of barley is predominantly focused to the production of malt by malting process intended for beer production. Even though, the malt is also used for the manufacture of distilled spirits, such as whisky, as well as syrups, coffee substitutes, and some other cereal-based foods. Moreover, malt or barley derivatives are used for feed production. Thereby, this product is commonly consuming by humans and animals [4].

Normally, the plant of barley can easily grow in different climatic regions. Unfortunately, this particular capacity does vulnerable to be colonised by various toxinogenic fungi, some of them can be able to produce mycotoxins [5, 6].

These toxins can cause both acute and chronic effects for humans and animals [7, 8]. For this reason, well-known mycotoxins, such as aflatoxins (AFs), ochratoxin A (OTA) and some *Fusarium* toxins have been classified by International Agency for Research on Cancer (IARC) and regulated by European Union [9-11]. On the other hand, there are other mycotoxins, such as enniatins, beauvaricin or ergot alkaloids, which have not been classified nor legislated up to now. The starting point of the monitoring of mycotoxins began to be focused on legislated mycotoxins [12, 13], but step-by-step the range was extended also to emerging mycotoxins. In fact, several recent works have been focused only on these new and emerging mycotoxins [14-16].

In all the contexts, liquid chromatography tandem mass spectrometry is commonly used for mycotoxins analysis [17]. Most often triple quadrupole (QqQ) has been widely accepted as the main tool in the identification and quantification of mycotoxins owing to its superior sensitivity, specificity and efficiency [12, 13, 15-17]. However, liquid chromatography coupled to ultra-high resolution mass spectrometry (HPLC-Orbitrap®) has been also included recently for routine mycotoxin analysis showing acceptable sensitivity and unambiguous identification [18-20].

The applicability of liquid chromatography triple quadrupole linear ion trap (HPLC-MS/MS) and HPLC-Orbitrap® has been recently evaluated for the analysis of mycotoxins in baby food. The comparison has highlighted that both instruments were complementary for determination of mycotoxins [21]. Orbitrap® technology has been therefore applied for routine analysis demonstrating some advantages: accurate mass, robust, sensitivity and unambiguous identification. In this research, different extraction procedures have been carefully studied using Orbitrap® technology. This issue has been normally carried out using QqQ analysers in the mycotoxin field [22, 23]. However, in this work the use of Orbitrap® MS technology demonstrated to be effective and a powerful tool for routine validation.

Overcome the drawbacks of detection, one of the main problems in a multi-mycotoxins analysis is to develop a method with rapid and simple extraction and purification step of these analytes from various food matrices, caused predominantly by great differences in physicochemical properties of these compounds. In fact, the extraction and the clean-up are the critical steps since they both determine the recoveries for all mycotoxins under investigation [24]. The varied structures of these mycotoxins make the extraction difficulties in using one standard extraction technique in order to detect different genera of toxins. Many extraction procedures have been already described in the literature, such as solid-liquid extraction (SLE) and liquid-liquid extraction (LLE), commonly linked with

mass spectroscopy [17]. For example, the classic solid-liquid extraction (SLE) with or without clean-up methods have been mainly applied for cereals and derivates [24-27].

The last trends have been attractive alternatives, such as modified QuEChERS or matrix solid-phase dispersion (MSPD), which have been used for cereals and derivates [13, 22, 28-30]. These extractions have been demonstrated as reliable methods and they have been successfully applied to different matrices.

Thereby, the main aim of this work was to develop a robust analytical method for the simultaneous extraction and determination of 32 mycotoxins in barley. In this way, different extraction methods (SLE, solid-phase extraction (SPE) clean-up method, QuEChERS and MSPD) were compared and evaluated, as well as the selected procedure was applied to common agricultural samples. Analysis was carried out using ultra performance liquid chromatography coupled with Exactive Orbitrap® MS (UPLC–Orbitrap® MS). Finally, by comparison of existing methods results, it was able to optimize an analytical method according to the EU Commission Decision 2002/657/EC guidelines [31].

2. Materials and methods

2.1. Chemicals and reagents

Certificated standards of 3-acetyldeoxynivalenol (3-ADON), 15-acetydeoxynivalenol (15-ADON), deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), fusarenon-X (FUSX), nivalenol (NIV), HT-2 toxin (HT-2), T-2 toxin (T-2), diacetoxyscirpenol (DAS), neosolaniol (NEO), aflatoxin B1 (AFB_1), aflatoxin B2 (AFB_2), aflatoxin G1 (AFG_1), aflatoxin G2 (AFG_2), ochratoxin A (OTA), fumonisin B1 (FB_1), fumonisin B2 (FB_2), fumonisin B3 (FB_3), sterigmatocystin (STER), zearalenone (ZEA), Penitrem A were supplied by

Biopure (Tulln, Austria). Standards of beauvericin (BEA), altenuene, alternariol, ergocornine, ergocryptine, ergocrystine and ergosine were obtained from Sigma–Aldrich (Steinheim, Germany). On the other hand, enniatins A1 (ENA_1), A (ENA), B (ENB) and B1 (ENB_1) were purchased by Enzo Life Science (Lausen, Switzerland).

Acetonitrile and methanol, both HPLC-grade, were supplied by Merck (Darmstadt, Germany). Deionized water was prepared from a Milli-Qsystem (Millipore, Bedford, MA, USA). Anhydrous magnesium sulphate, sodium chloride and ammonium formate and ammonium acetate (≥99% purity), were from Sigma–Aldrich (Steinheim, Germany).

Solid-phase used for MSPD was octadecy-silica (C18-E) (50 μm) bonded silica from Phenomenex (Torrance, USA). Oasis HLB 150 mg sorbent cartridges were from Waters Corp. (Milford, MA, USA).

2.2. Barley samples

A total of 15 spring barley samples were examined for selected mycotoxins. Samples were purchased from Czech farmers as a part of national projects. Barley samples were kept under the dark and dry conditions.

2.3. Extraction procedures

2.3.1. Matrix Solid Phase Dispersion (MSPD)

Sample preparation was partially performed according to a previous research [32]. Barley samples were homogenized by mixing them thoroughly. Homogenized and representative 1 g portions were weighed and placed into a glass mortar (50 ml) and gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain a homogeneous mixture. This mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 1 mM ammonium formate in 10 ml of

acetonitrile/methanol (50/50, v/v) by applying a slight vacuum. Then, an aliquot (1 mL) of extract was filtered through a 22 μm nylon filter prior to injection into the UPLC–MS/MS system.

2.3.2. QuEChERS

Modified QuEChERS procedure was employed to extract mycotoxins from the examined matrix [33, 34]. Homogenized and representative portions of 2 g were weighed into a 50 mL PTFE centrifuge tube, and then 10 mL of 0.1% formic acid in deionised water were added. The mixture was mixed during 3 min and waited for the next step during 10 min. Afterwards, 10 ml acetonitrile were added, and consecutively the mixture was vigorously shaken (3 min). The following step, 4g $MgSO_4$ and 1g of NaCl were added and then the mixture was shaken 3 min again. Once the extraction was finished, the sample was centrifuged (5 min, 11.000 rpm, 20 °C). Then, an aliquot (1 mL) filtered through a 22 μm nylon filter before their injection into the UPLC–MS/MS system.

2.3.3. Solid-liquid extraction (SLE)

The classical SLE method was partially performed according to previous work [35]. Representative portions of 2g samples were accurately weighed and transferred to PTFE centrifuge tube (50 mL). Samples were extracted by shaking with 10mL acetonitrile/water/acetic acid (79:20:1, v/v/v) on an automatic shaker (IKA Laboratortechnik, Germany) for 90 min, and then centrifuged (5 min, 11,000 rpm, 20 °C). Afterwards, the supernatant extract was two-fold diluted with HPLC-grade water, taking an aliquot of 0.5 mL and diluting to 1 mL. After that the sample was filtered through a 0.22mm filter, consecutively the sample was injected.

2.3.4. Solid-phase extraction (SPE) clean-up method

The previous SLE extract was used for clean-up method. The extraction procedure was used according to Vendl et al. [36]. C_{18}-SPE clean-up procedure was performed with Oasis HLB cartridges (150 mg) from Waters (Milford, MA, USA). 2 mL of SLE extract were diluted with 30 mL of water in order to obtain a required maximum concentration of 5% organic solvent. The columns were pre-washed with 10 mL of acetonitrile, and further conditioned with 10 mL of 5% acetonitrile in deionized water. Consequently, diluted sample was loaded onto C_{18} cartridge. After that, SPE columns were washed with 10 ml of 5% acetonitrile in water. The cartridges were then dried for 30 min. In the last step, the mycotoxins were eluted by adding of 5 ml acetonitrile. Then, the extract was transferred into a 15 ml conical tube and evaporated to dryness at 35 °C with Rotavapor (model?). The residue was reconstituted to a final volume of 1 ml with methanol/water (50:50, *v/v*) and filtered through a 0.22 μm Millex-GN nylon filter, before the injection.

2.4. Ultra High Preassure Liquid Chromatography Orbitrap® MS

The detection method has been optimized in a previous research [19]. An Accela U-HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was used for the separation of target analytes. It was equipped with an Acquity UPLC HSS T3 analytical column (100 mm × 2.1 mm i.d., 1.8 μm; Waters, Milford, MA, USA) held at 40°C for the separation of sample components. As the mobile phase, 5 mM ammonium formate and 0.1% acid formic in water (A) and methanol (B) was used. The gradient was as follows: start with 5% B, linear increase to 50% B in 6 min, for next 4 min another linear increase to 95% B, keep up to 15 min, switching to 5% B in 15.1 min, and column equilibration for 3 min before the next injection start. The flow rate was 300 μL min^{-1}. The injection volume was 5 μL and the partial loop was used as an injection technique.

The operation parameters of the single-stage Orbitrap® mass spectrometer (Exactive; Thermo Fisher Scientific, Bremen, Germany) optimised for the heated electrospray interface (HESI-II; Thermo Fisher Scientific, Bremen, Germany) were as follows: sheath gas/aux gas: 35/10 arbitrary units, capillary temperature: 250°C, heater temperature: 250°C, capillary voltage: +60/–50 V, and spray voltage +4/−3.1 kV.

The system was operated in the full spectral acquisition mode in the mass range of m/z 100–1000 at resolving power settings of 50 000 FWHM at fixed acquisition rate of 2 spectrum s^{-1}. The method was developed in positive and negative ionisation mode. The external mass axis calibration without the use of the specific lock mass was employed. For the mass accuracy estimation, mass at the apex of the chromatographic peak obtained as the extracted ion chromatogram was used. The calculated (exact) masses of analytes ions have been summarised in a previous work [19].

3. Results and discussion

3.1. Selection of Orbitrap® MS ionization mode.

Most of the published studies concerned with determination of multiple mycotoxins have used an electrospray ionisation (ESI) source for ionisation, however, a recent work has compared between ESI and atmospheric pressure chemical ionisation (APCI) for multiple mycotoxins detection using Orbitrap® MS [19]. The authors concluded that using APCI enhancement in detectability of *Fusarium* toxins was archived, with the exception of OTA, which showed better ionisation efficiency under ESI conditions. However several limitations of APCI source were noted; on the one hand fumonisins did not show ionisation efficiency under APCI conditions at all. On the other hand, these compounds require acidic

conditions, which limit the ionisation of other mycotoxins, mainly type B trichothecenes.

To keep in mind these premises, a compromise between sensitivity and identification was evaluated. 32 target mycotoxins were simultaneously detected by ESI. For this reason, this ionisation mode was selected and LCLs were accepted knowing that they were higher than APCI except for OTA and fumonisins.

3.2. Optimization of proposed extraction methods

The proposed extraction methods have been partially performed. In this work, some parameters were evaluated again, as well as they were improved in order to extract selected mycotoxins. The efficiency and efficacy were evaluated and compared in number of compounds extracted and recoveries obtained.

For example, efficiency of MSPD extractions depends on type and amount of dispersing phase, the amount of sample and nature and volume of the eluting solvents [32]. In our study, the solid support was studied comparing between octy-silica (C_8) and octadecy-silica (C_{18}). At the end, C_{18} demonstrated to be the ideal support for multi-mycotoxins analysis when MSPD is used since the obtained recoveries were highest. However, in this study the eluting solvent could be reduced to 10 ml MeOH/ACN (50/50, v/v) demonstrating similar effectiveness to 20 ml (data not shown).

On the other hand, SLE was partially used according to previous work [35]. This extraction was used in different ways. Firstly, it was used as clean-up using SPE cartridges, secondly without clean-up using diluted-and-shoot method. The SLE procedure without clean-up step used as diluted-and-shoot method demonstrated to be effective, crude extract and different diluted extracts (1+1, 1+2, 1+4) were evaluated (data not shown). At the end, the 1+1 diluted extracted showed acceptable recoveries for selected mycotoxins. Focusing on clean-up method, C_{18}

cartridge was studied according to a previous work [36], in order to extract all selected mycotoxins.

Modified QuEChERS was evaluated according to previous works [33, 34]. This extraction offers different alternatives, for example QuEChERS could be modified and it is an important advantage [37]. In our research the selected extraction did not require a clean-up step using (PSA) due to the low lipid content of the matrix, as well as by the presence of fumonisins which have an acidic nature, increasing the risk of their binding on the sorbent.

3.2. Comparison of proposed extraction procedures

MSPD, QuEChERS, SLE and SPE clean-up methods are commonly used for mycotoxins analysis. Even so, these methods have advantages and drawbacks. Among the four evaluated methods, QuEChERS is the fastest and cheapest procedure, because of pre-concentration and clean-up steps were not necessaries, as well as the glass decontamination. QuEChERS procedure was able to extract 10-15 samples in 1 hour and thirty min, whereas MSPD and SLE methods took twice as long and clean-up method three times as long. For example, the time consuming could depend on the glass decontamination in MSPD and SPE steps or waiting time in SLE. Moreover, the cost of SPE columns, solid-phases, salts, solvents or the working time is important in order to decide the best option.

Overview of recoveries data for MSPD, QuEChERS, SLE and SPE clean-up methods is summarised in *Table 1*; recovery study was carried out by spiking selected mycotoxins in blank barley at 250 μg kg^{-1}. In *Table 1* could be observed that MSPD, QuEChERS and SLE showed an acceptable range of recoveries which were higher than 60% for most of selected compounds. However, SPE clean-up method presented low recoveries, which ranged from <50% to 80%. Recoveries were higher than 65.4% for type A and B trichothecenes, aflatoxins and fumonisins. Moreover, they were not upper than 58.5% for Penitrem A and some

mycotoxins, such as ergot alkaloids. In our research HLB cartridges showed an unsuccessful efficiency for target mycotoxins. In other studies, C_{18} cartridges were compared with other specific columns and their recoveries were improved successfully for some compounds [26, 36]. However, in our research C_{18} was used looking for a wide number of mycotoxins, but it was rejected due to low recoveries. Obviously, owing to the poor recoveries obtained, SPE clean-up method was rejected for the study.

Table 1. Preliminary recovery studies for selected mycotoxins. Blank barley was spiked at 250 μg/kg for targeted mycotoxins.

Recovery, (average, n=5)		Extraction methods			
		MSPD	QuEChERS	SLE	Clean-up
Percent of 32 mycotoxins	<50%	2	0	1	6
	50-60%	1	0	0	10
	60-70%	11	2	5	14
	70-80%	13	17	2	2
	80-90%	5	12	17	0
	90-100%	0	1	7	0
	100-110%	0	0	0	0

The following step was to evaluate deeply the recoveries obtained by other techniques, as it shows in *Table 2*. Delving into every detail, it was observed that MSPD was able to extract all selected mycotoxins; recoveries ranged from 66.7 to 87.1%, but Penitrem A, D3G and some ergot alkaloids showed lower recoveries and high RSDs (%). SLE showed the highest recoveries, as well as, all selected mycotoxins were completely extracted. The problem was that Penitrem A and fumonisins showed lower recoveries. QuEChERS did not present the highest recoveries, but this method was able to extract adequately all selected mycotoxins. The recoveries ranged from 64.1 to 93.4% without exception. Thereby, the recoveries obtained were into acceptable range and RSDs were lower than 20% (*Table 2*).

To sum up, modified QuEChERS was selected for further studies in order to take advantage its potential for simultaneous extraction of selected compounds. The data comparison showed that QuEChERS offered acceptable range of recoveries and low RSDs. Furthermore, QuEChERS gave low time consuming during the extraction procedure, as well as it was easier and cheaper than MSPD, SLE and SPE clean-up. For these reasons, QuEChERS was the most efficient and effective extraction procedure evaluated.

3.3. Validation of the QuEChERS procedure

Validation of the method was performed according to following directive and guide on that subject [38, 39]. The following parameters were studied: confirmation of identity, specificity/selectivity, linearity, lowest calibration level (LCL), precision as repeatability and within-lab reproducibility, process efficiency and recovery.

Confirmation of identity was based on the following criteria: (i) the measured accurate mass of $[M+H]^+$, $[M+NH4]^+$ or $[M-H]^-$ and $[M+HCOOH]^-$ must fit the theoretical accurate mass with a mass tolerance set at ±5 ppm and (ii) the retention time window was set to ±2% from that of a calibration standard.

The LCLs were determined as previous works [19, 40]. *Table 3* gives LCLs for target mycotoxins in barley. The LCLs ranged between 1 to 100 μg kg^{-1} for ENB and NIV, respectability. Based on LCLs obtained values the method proved to be sensitive and it allows us to assess the compliance of all the mycotoxins and matrix with the Commission Regulation No. 1881/2006 [10].

Table 2. Recovery data for MSPD, QuEChERS and SLE method in blank barley at 100 µg kg-1 except type B trichothecenes and fumonisins which were spiked at 250 µg kg-1. In brackets are given % RSD (n=5).

Toxin classification	Mycotoxin	Extraction method		
		MSPD	QuEChERS	SLE
Fusarium toxins	NIV	68.2 (14)	65.2 (12)	69.1 (16)
	D3G	60.1 (22)	64.1 (16)	67.2 (18)
	DON	77.9 (9)	87.9 (9)	83.1 (6)
	3-ADON	72.1 (18)	85.1 (12)	83.2 (14)
	15-ADON	70.9 (21)	83.9 (11)	88.2 (19)
	FUSX	67.9 (15)	81.1 (14)	83.1 (7)
	NEO	71.1 (12)	76.7 (5)	87.2 (5)
	DAS	76.5 (8)	86.1 (6)	92.8 (20)
	HT-2	71.5 (12)	88.2 (10)	93.3 (15)
	T-2	75.3 (20)	93.4 (12)	92.3 (18)
	ZEA	66.7 (16)	71.8 (13)	91 (10)
	FB_1	87.1 (15)	83.3 (8)	61.1 (19)
	FB_2	86.1 (13)	88.1 (7)	64.2 (12)
	FB_3	81.3 (17)	82.8 (9)	60.7 (11)
Ergot alkaloids toxins	ENA	68.6 (11)	77.3 (12)	81.1 (15)
	ENA_1	69.1 (8)	74.4 (11)	80.2 (13)
	ENB	74.1 (11)	76.1 (12)	85.1 (16)
	ENB_1	67.1 (6)	70.5 (12)	90.1 (10)
	BEA	69.3 (19)	72.8 (15)	80.1 (20)
	Ergosine	60.1 (21)	74.1 (9)	88.1 (4)
	Ergocornine	47.3 (18)	76.4 (11)	91.1 (13)
	Ergocryptine	56.7 (23)	71.9 (16)	90.6 (18)
	Ergochristine	63.4 (27)	76.6 (10)	79.6 (11)
Aspergillus toxins	AFB_1	73.1 (14)	81.9 (9)	82.1 (12)
	AFB_2	76.6 (17)	81.2 (10)	85.1 (13)
	AFG_1	81.1 (18)	78.2 (12)	83.2 (14)
	AFG_2	71.7 (16)	75.3 (11)	80.1 (17)
	STER	73.5 (20)	85.3 (7)	81.5 (24)
Penicillium, Claviceps *Aspergillus* and *Alternaria* toxins	OTA	68.9 (12)	86.9 (7)	79.2 (10)
	Penitrem A	42.5 (13)	73.4 (10)	46.1 (16)
	Altenuen	76.5 (15)	85.7 (14)	83.1 (8)
	Alternariol	81.1 (18)	89.4 (9)	83.3 (26)

Table 3. Validation modified QuEChERS method. Lowest calibration Levels (LCLs), matrix effects (ME), percentage recovery and repeatability (% RSD) at three levels used for validation, and Inter-day precision (% RSD) at medium level.

Mycotoxin	LCL (µg kg^{-1})	ME[a]	Intra-day[c]			Inter-day[d]
			Low level 25 µg kg^{-1}	Medium level 50 µg kg^{-1}	High level 100 µg kg^{-1}	Medium level 50 µg kg^{-1}
NIV	100	86.1	62.8 (6.8)[b]	66.3 (5.5)[b]	67.1 (5.2)[b]	9.1[b]
D3G	25	68.9	61.8 (8.8)[b]	63.9 (7.5)[b]	65.1 (7.2)[b]	6.1[b]
DON	5	81.2	86.9 (6.7)[b]	87.1 (5.9)[b]	90.2 (8.2)[b]	8.2[b]
3-ADON	50	88.1	95.9 (16.1)[b]	83.9 (8.5)[b]	81.1 (9.1)[b]	12.5
15-ADON	50	85.1	92.8 (13.4)[b]	80.1 (7.9)[b]	79.3 (10.2)[b]	14.4[b]
FUSX	100	77.8	89.1 (7.8)[b]	91.5 (6.9)[b]	90.1 (9.9)[b]	7.7[b]
NEO	1	109.2	83.9 (11.7)	77.5 (9.1)	77.7 (8.9)	11.1
DAS	1	111.9	93.9 (6.1)	88.3 (7.2)	86.7 (9.3)	9.6
HT-2	1	99.4	101.1 (10.1)	95.5 (9.7)	93.2 (9.9)	12.9
T-2	1	123.1	95.1 (10.1)	95.5 (9.1)	93.4 (9.5)	10.9
ZEA	1	91.2	81.8 (7.8)	77.8 (6.9)	79.8 (10.1)	10.3
FB_1	50	112.1	83.9 (6.4)[b]	89.6 (7.9)[b]	84.9 (6.9)[b]	8.6[b]
FB_2	10	103.4	81.7 (5.8)[b]	87.2 (8.5)[b]	82.3 (7.9)[b]	9.1[b]
FB_3	10	107.1	85.9 (4.7)[b]	89.1 (9.4)[b]	87.1 (8.3)[b]	12.6[b]
ENA	5	77.9	84.1 (6.2)	81.8 (6.3)	80.1 (6.3)	7.1
ENA_1	1	82.9	80.1 (5.2)	79.8 (7.2)	80.4 (5.7)	6.9
ENB	1	88.1	84.1 (7.1)	86.5 (5.6)	83.4 (6.1)	7.5
ENB_1	1	78.1	79.1 (11.9)	78.9 (8.1)	77.6 (7.9)	11.8
BEA	1	110.1	78.2 (14.6)	74.1 (10.1)	73.1 (10.2)	17.4
Ergosine	1	110.1	78.8 (10.8)	76.3 (7.1)	74.8 (6.9)	9.3
Ergocornine	2.5	66.1	76.3 (11.1)	74.1 (9.1)	70.1 (12.2)	9.9
Ergocryptine	2.5	86.3	74.8 (8.8)	76.6 (4.4)	71.9 (5.9)	7.9
Ergochristine	2.5	69.8	76.8 (10.1)	82.2 (9.6)	78.4 (11.2)	11.3
AFB_1	1	82.3	74.7 (5.2)	75.7 (8.6)	86.1 (9.1)	10.8
AFB_2	1	71.2	73.7 (9.2)	77.1 (12.1)	81.2 (3.1)	11.9
AFG_1	1	68.9	71.4 (14.1)	79.5 (4.5)	78.9 (6.4)	8.1
AFG_2	1	98.1	72.3 (11.8)	75.7 (7.4)	76.3 (7.1)	10.2
STER	2.5	128.1	86.5 (5.3)	81.8 (3.8)	85.2 (5.9)	5.6
OTA	10	103.9	96.3 (4.2)	87.9 (1.5)	88.8 (4.1)	4.3
Penitrem A	50	114.1	88.8 (10.4)[b]	84.8 (7.4)[b]	79.8 (5.8)[b]	7.9[b]
Altenuene	2.5	109.8	92.7 (7.2)	80.9 (7.9)	79.8 (9.3)	8.3
Alternariol	2.5	111.1	94.1 (7.7)	92.4 (7.1)	89.8 (8.4)	8.1

[a] ME%: (slope matrix matched calibration/slope standard in solvent) x 100
[b] The spiking levels of type B trichothecenes, fumonisins and Penitrem A were 150, 300, 600 µg kg^{-1}.
[c] Number of replicates: 10.
[d] Different days: 5

It is well known that the presence of matrix components in the extract (co-eluting compounds), which can affect the ionization of the compounds when ESI is used producing the so-called matrix effects (ME). There are different ways, which could be applied to compensate matrix effects. Although the best way to compensate the matrix effect is the use of isotope internal standards, these compounds are not available for some of the studied mycotoxins, as well as they are expensive for routine analysis. Another form to avoid matrix effects is the use matrix-matched calibration curves for effective quantitative determinations of mycotoxins in barley. The ME was calculated for each mycotoxin in barley, as the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A); the ratio (B/A x 100) is defined as the matrix effect. A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%. In this way, the linearity in the response was calculated using standard solutions and matrix-matched solutions were prepared by spiking barley in triplicate at six concentrations levels into the analytical range: from LOQ to 100 times this LOQ. Type A trichothecenes, altenuene, STER and BEA showed great signal enhancement. However, matrix suppresses the response for AFG_1, D3G, ergocristine and ergocornine. Thereby, matrix-matched calibration was used.

Linearity was then evaluated. Peak area was selected as response and good linearity within LCL and 100 times LCL was found with determination coefficients higher than 0.9922 in all the cases.

Trueness was evaluated through recovery studies. Recoveries (n=10), they were carried out spiking barley at three levels (*Table 3*). The precision of the method, expressed as relative standard deviation (%RSD), was estimated by the repeated analysis (n=10) of a spiked barley at these levels during the same day (intra-day) and on different five days (inter-day).

Recoveries ranged from 71.4 to 101.1% for all mycotoxins assayed at concentration levels evaluated (*Table 3*), except for NIV and D3G, which were lower than 67.1%. Good recoveries were therefore obtained throughout the developed QuEChERS method. Precision of the overall method was studied by performing intra-day and inter-day precision experiments, showing the results in *Table 3*. It can be observed that repeatability, expressed as RSD was lower than 16.1% for intra-day experiments and for inter-day precision, RSDs were always lower than 17.4% for three spiked levels.

Thus, the method was successfully validated according to the criteria specified in Commission Decision 2002/657/EC for quantitative confirmation method [16]. Furthermore, the specificity of the methods was demonstrated by the analysis of blank barley and spiked samples.

3.3. Analysis of barley samples

Developed analytical method was applied for testing of 15 barley samples from Czech Republic. Within this monitoring, several *Fusarium* toxins were identified (*Table 4*). In our research *Alternaria*, *Aspergillus*, *Claviceps* and *Penicillium* toxins were not detected, although several works have demonstrated the presence of these toxins in barley [41, 42].

In this research, enniatins were commonly detected in barley samples, as well as type A and B trichothecenes. By contrast, the presence of trichothecenes has been commonly related in barley [43, 44], but the presence of enniatins have not been commonly cited up to now [45]. At the end, in total 7 samples out of 15 tested samples the co-occurrence of *Fusarium* mycotoxins was presented, but in all cases the calculated concentrations were lower than those established by European directives [10]. However, the calculated concentrations for enniatins were considerable.

Table 4. Occurrence of target mycotoxins in barley.

	DON	ENB	ENB_1	ENA	ENA_1	HT-2	T-2
Sample 1	33.1	87.7	139	100.5	108.9	78.5	30.5
Sample 2	43.3	2029	1821	340	698	26.2	8.8
Sample 3	38.1	95.6	101.1	75.6	93.5	30.5	<LCL
Sample 4	49.1	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 5	25.1	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 6	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 7	31.2	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 8	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 9	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 10	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 11	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 12	36.5	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 13	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL
Sample 14	<LCL	19.4	28.5	21.9	25.3	<LCL	<LCL
Sample 15	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL	<LCL

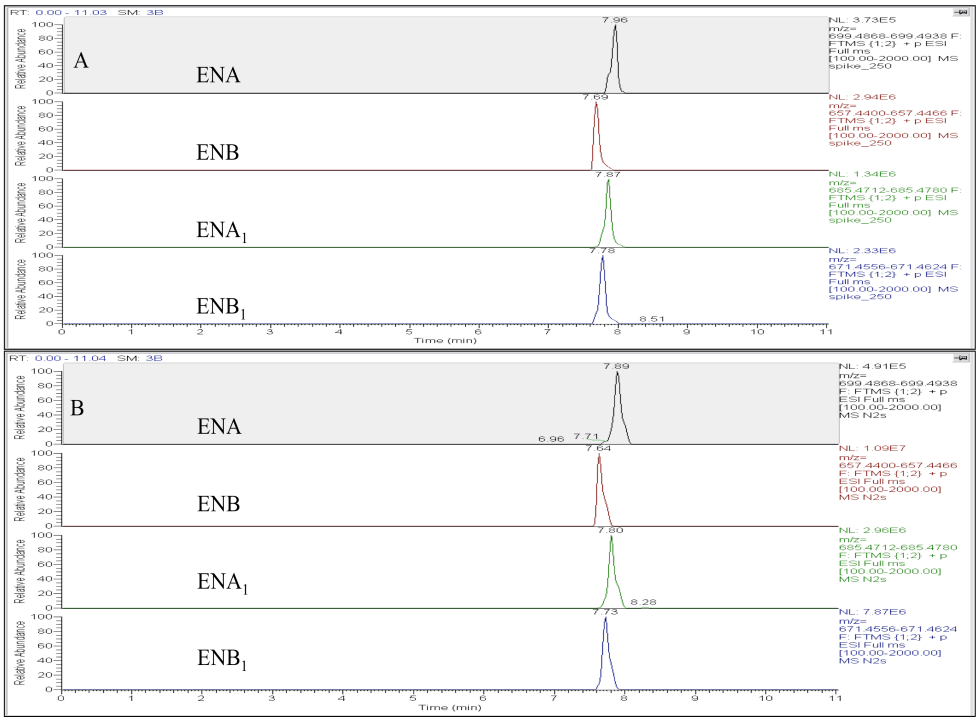
<LCLs: Lower than LCL level

Figure 1 shows a chromatogram of a spiked barley at 250 µg kg^{-1} and a positive sample in which enniatins were identified: ENA, ENA_1, ENB and ENB_1. Thereby, *Fusarium* mycotoxins were identified and quantified in barely samples. It is important to keep in mind that it was difficult to find samples without enniatins for validation study.

The confirmation of positive samples was carried out, according to previous criteria cited above. Furthermore, an internal quality control was carried out for every batch of samples to check if the system was under control, and it implied a matrix-matched calibration, a matrix blank and a spiked barley sample at low concentration level. This quality control is very important to guarantee accuracy of the analysis. Mycotoxin analysis in the raw material could assure the quality of the

product, as well as its derivate. In this form, it could minimise the public health risk.

Figure 1. Chromatograms for enniatin A, A1, B, B1 spiked at 250 μg kg^{-1} (A). Positive barley, sample 2, extracted ion chromatograms for enniatins (B).



4. Conclusion

The simultaneous extraction of 32 mycotoxins from barley was difficult because of the varied structures of these compounds, as well as, it was a compromise between sensitivity and detection. The selection of the extraction procedure depends on the group of mycotoxins to be extracted. Moreover, if the number of mycotoxins is extended different extraction procedures should be carefully studied and compared. In fact, the extraction is the critical step because of it has to extract selected compounds and to reach acceptable recoveries.

The efficiency and efficacy of modified QuEChERS demonstrated to be superior to SLE, MSPD, and SPE clean-up method. The developed method could extract selected compounds from barley at low cost, reducing time consuming and increasing throughput.

The validated UHPLC-Orbitrap® MS was confirmed to be an accurate, precise, and sensitive methodology for the detection of 32 mycotoxins in barley samples. This instrument allowed analyses of target mycotoxins, but ultra-high resolution mass spectrometry could have been used to identify non-target mycotoxins.

Finally, the validated method was used to analyse commercialize barley samples, detecting *Fusarium* toxins at low concentrations. To sum up, in our research it has been demonstrated the applicability of QuEChERS for this type of organic contaminants as well as the excellent sensitivity obtained using liquid chromatography ultra-high resolution mass spectrometry.

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4.9 Application of hybrid linear ion trap-high resolution mass spectrometry to the analysis of mycotoxins in beer.

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Application of hybrid linear ion trap-high resolution mass spectrometry to the analysis of mycotoxins in beer

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This paper reports the application of liquid chromatography electrospray ionization ion trap–orbitrap mass spectrometry for the determination of 18 mycotoxins (aflatoxins, fumonisins, trichothecenes, ochratoxin A, sterogmatocystin, beauvaricin, zearalenone and zearalenol) in beer. The extraction procedure was carried out by solid phase extraction (SPE): SPE columns were conditioned with acetonitrile/methanol and water. Beer was loaded onto the column which was washed with water. In these conditions, the recoveries were more than 65% and the relative standard deviation (RSD) were below 18%. The lowest limits of quantification (LLOQ) ranged from 9 to 155 ng ml^{-1}. Matrix-matched calibration was performed for each beer and reliable results were obtained from selected mycotoxins. The method was applied to the analysis of 25 commercial beers. Taking advantage of the hybrid capabilities, the presence of other mycotoxins were checked; enniatins (A, A_1, B and B_1) and fusaproliferin were studied in all the tested samples. The survey detected the presence of zearalenone in one stout beer sample.

Keywords: LC/MS; mycotoxins; beer

Introduction

During the 1970s, considerable efforts were directed toward perfecting high resolution instruments. Fortunately, nowadays when liquid chromatography (LC) is combined with a high resolution instrument, it can support a wide range of applications from routine compound identification in complex mixtures (Thurman et al. 2006). One of these high resolution instruments is LTQ Orbitrap MS; it combines a high resolution mass spectrometer, as Orbitrap analyzer, with an external accumulation device, such as a linear ion trap, making possible multiple levels of fragmentation (MS^n) for the elucidation of analyte structures. The use of the LTQ Orbitrap allows high-quality accurate mass and acquisition of MS^n spectra. Fourier transformation of the acquired transient allows wide mass range detection with high resolving power, mass accuracy, and dynamic range. The LTQ Orbitrap MS has two main advantages: high sensitivity in full scan and the possibility of determination of accurate mass of product ions (Makarov and Scigelova 2010).

Over the last few years, the use of this instrument has been restricted to proteomic or metabolic field (Bennett et al. 2011). In fact, literature is scarce on its application in the analysis of molecules with relative low molecular weights, such as pesticides (Edison et al. 2011), veterinary drugs and doping control (van der Heeft et al. 2009; Peters et al. 2010) or toxins. Hogenboom et al. (2009) applied Orbitrap technology to search for target compounds and "unknown" compounds in different water samples. This study demonstrated the presence of target compounds as pharmaceuticals, benzotriazoles and illicit drugs, and they identified "unknown" compounds in groundwater sample and landfill soil sample. Škrabáková et al. (2010) validated the use of high mass resolution in full-scan mode for the determination of six azaspiracids in mussel tissue extracts.

In the field of mycotoxins analysis, Herebian et al. (2009) carried out a first approach for determining 31 mycotoxins in grain and they compared QqQ technology with Orbitrap instrument. They concluded that one of the major advantages of the high resolution full scan method is the possibility of screening unknown compounds. However, best sensitivity was obtained with triple quadrupole (QqQ).

Improving the results, Vaclavik et al. (2010) used Orbitrap technology to determine 11 mycotoxins in cereals and the same group extended the analysis to 32 mycotoxins in beer (Zachariasova et al. 2010).

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In the first study, QuEChERS was used for extraction, while in the second study the extraction was carried out via classical liquid–liquid extraction.

Therefore, expanding upon this previous work, the objectives of the present study were (i) to develop and validate an analytical procedure based on the applicability of the LTQ Orbitrap for the simultaneous extraction of aflatoxins, fumonisins, trichothecenes, OTA, ZEN, ZON and BEA in beer; (ii) to apply the validated method to the routine analysis of 25 different Irish beer samples collected in Cork (Ireland); (iii) to apply the method to acquire data essential for the identification of other "non-target" mycotoxins that may be present in beer, as enniatins (A, A_1, B and B_1) and fusaproliferin.

Materials and methods

Reagents and chemicals

HPLC-grade solvents, acetonitrile, methanol and water were supplied by ThermoFisher (Dublin, Ireland). Analytical-grade reagent formic acid (purity >98%) and ammonium formate was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). All solvents were passed through a 0.45-μm cellulose filter purchased from Scharlau (Barcelona, Spain).

Stock solution

The certificate standards of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), ochratoxin A (OTA), sterigmatocystin (STER), α-zearalenol (ZOL), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 3-acetyldeoxynivalenol-3deuterated (3-ADON-d_3), diacetoxyscirpenol (DAS), neosolaniol (NEO), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and beauvericin (BEA) were purchased from Sigma Aldrich (Madrid, Spain). T-2 and HT-2 toxin and aflatoxin M_1 (AFM_1) stock solutions (in acetonitrile) were obtained from Biopure referenzsubstanzen GmBH (Tulln, Austria). Fumonisin B_3 (FB_3) was supplied by the PROMEC unit (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa).

The individual stock solutions of aflatoxins (AFs) and OTA with concentration 500 μg ml^{-1} were prepared in acetonitrile and STER, ZOL, ZEN, NIV, DON, 3-ADON, FB_1, FB_2 and BEA were prepared at the same concentration in methanol. Stock solutions FB_3, DAS, T-2 and HT-2 at concentration of 100 μg ml^{-1} were prepared in acetonitrile. As internal standard (I.S.) compounds, AFM_1 at 0.05 μg ml^{-1} and 3-ADON-d_3 and NEO at 0.150 μg ml^{-1} were used; working solutions were prepared by dilution of individual stock solutions in methanol. All the standard solutions were kept in safe conditions and at −20°C.

All other working standard solutions were prepared immediately before use by diluting the stock solution with methanol/water (50 : 50, v/v).

Oasis HLB SPE (200 mg) cartridges were purchased from Waters (Milford, MA, USA). An extraction manifold from Waters connected to a Büchi Vac V-500 (Flawil, Switzerland) vacuum system was used for SPE experiments.

Samples

Beer samples were purchased from different stores and pubs in Cork (Ireland) and kept at −20°C in a dark dry place. Bottled samples were purchased in supermarkets and conserved. On the other hand, pint (568 ml) samples were collected in sterile beakers from each individual beer and stored until analysis. Samples with undetectable levels of mycotoxins were used for spiking and recovery studies.

Extraction procedure

The extraction procedure was developed according to Romero-González et al. (2009). Beer was degassed by sonication for 25 min. The Oasis HLB cartridges were conditioned with 5 ml of acetonitrile/methanol (50 : 50, v/v) and 5 ml of water. Then, 10 ml of beer was loaded onto the C_{18} cartridge and the SPE columns washed with 5 ml of water. The cartridges were then dried for 30 min. In the final step, the mycotoxins were eluted by adding 4 ml of acetonitrile/methanol (50 : 50, v/v). Then, the extract was transferred into a 15-ml conical tube and evaporated to dryness at 35°C under a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 ml with methanol/water (50 : 50, v/v), filtered through a 0.20-μm Millex-GN nylon filter (Millipore, Carrightwohill, Ireland) and collected in a vial.

HPLC-Orbitrap XL

Chromatographic separations were carried out using an Accela LC system (Thermo Scientific, Hemel Hempstead, UK), equipped with a reversed-phase analytical column (Gemini C_{18}, 150 mm, 2 mm I.D., 5 μm; Phenomenex) maintained at 35°C. As mobile phase, 5 mM ammonium formate and 0.1% formic acid in water (A) and 5 mM ammonium formate in methanol (B) were used. The gradient was as follows: at the start 5% solvent B was used: the percentage of solvent B was linearly increased up to 95% in 10 min and then the percentage of solvent B was linearly

decreased to 80% over 5 min. The column was equilibrated to initial conditions for 5 min. The flow rate was 200 µl min^{-1} and the injection volume was 10 µl.

The LC system was connected to a hybrid LIT–FT mass spectrometer (LTQ Orbitrap XL; Thermo Scientific, Bremen, Germany), operating in positive ion mode. The linear ion trap (LTQ) part of the hybrid MS system was equipped with heated electrospray interface (H-ESI). Full-scan accurate mass spectra (mass range from 90 to 900 Da) were obtained at high resolution (100,000 FWHM) and processed using Xcalibur v.2.0 software. The electrospray source conditions were: source voltage 4 kV, heated capillary temperature 275°C, capillary voltage 30 V, sheat gas and auxiliary gas 35 and 30 (arbitrary units), respectively.

The mass spectrometer was operated in a data-dependent-acquisition (DDA) mode in which both MS and MS^n spectra were acquired specifying parent masses of target compounds. In this mode, the acquisition software probed the MS spectra in real-time on a full scan and allowed accurate mass. The accurate mass is capable of finding true unknowns since the method does not require any pre-selection of masses. The instrument is initially set to operate in full-scan mode until a parent ion appears to preset the instrument, which switches into the product-ion mode (MS^n). The mass resolution was set at 100,000 FWHM for both screening and quantitative analysis. The products ions were generated in the LTQ trap at an optimized collision energy setting of mycotoxins. The scan-type settings are presented in Table 1. No exclusion list was used. The total cycle time depends upon the resolution; at a resolution of 100,000 FWHM, the total cycle time is about 1 s. The results were used to create a (full-scan) accurate mass MS and an MS^n database to enable identification of compound in future screening.

The Orbitrap instrument was calibrated using a solution containing caffeine, MRFA, and Ultramark 1621, according to the manufacturer's instructions. Operation of the entire LC/MS instrumentation was controlled using Xcalibur software (Thermo Scientific).

Data processing

LTQ Orbitrap MS for target compound analysis is related to its high mass resolution potential, corresponding to a mass assignment accurate to four decimal places. The ability to determine the m/z of an ion to within a maximum deviation of 5 ppm allows the determination of a unique elemental composition based on the mass defect of the constituent atoms (van der Heeft et al. 2009). The ability to closely match the measured/theoretical mass with the observed mass greatly increases the reliability of identification (Table 1).

The total list of accurate masses detected in a sample was corrected manually for masses also found in matrix. Based on the accurate mass, the elemental composition of the peaks of interest was calculated using the elemental composition tool within the Xcalibur software.

Table 1. Ion formation, elemental formula, theoretical and measured m/z and error mass (ppm) for selected mycotoxins (Data-dependent acquisition parameters for ion trap acquisition).

	Full scan FTMS					Data-dependent acquisition (DDA)		
Compound	Ion formation	Elemental formula	Theoretical m/z	Measured m/z	Error (ppm)	Retention time	Parent mass	CE
NIV	$[M+H]^+$	$C_{15}H_{21}O_7$	313.1287	313.1289	0.63	4.99	313.13	18
DON	$[M+H]^+$	$C_{15}H_{21}O_6$	297.1338	297.1337	−0.33	6.35	297.13	20
3-ADON	$[M+H]^+$	$C_{17}H_{23}O_7$	339.1445	339.1444	−0.29	8.50	339.14	18
DAS	$[M+NH_4]^+$	$C_{19}H_{30}O_7N$	384.2023	384.2021	−0.52	8.48	383.20	19
HT-2	$[M+NH_4]^+$	$C_{22}H_{36}O_8N$	442.2441	442.2445	0.90	10.81	442.24	18
T-2	$[M+NH_4]^+$	$C_{24}H_{38}O_9N$	484.2547	484.2545	−0.55	11.34	484.25	18
FB_1	$[M+H]^+$	$C_{34}H_{60}O_{15}N$	722.3963	722.3962	−0.13	10.45	722.39	54
FB_2	$[M+H]^+$	$C_{34}H_{60}O_{14}N$	706.4014	706.4017	0.42	11.62	706.40	25
FB_3	$[M+H]^+$	$C_{34}H_{60}O_{14}N$	706.4014	706.4016	0.28	11.06	706.40	25
ZEN	$[M+H]^+$	$C_{18}H_{23}O_5$	319.1546	319.1547	0.31	12.19	319.15	20
ZOL	$[M+H]^+$	$C_{18}H_{25}O_5$	321.1702	321.1705	0.93	11.95	321.17	20
AFB_1	$[M+H]^+$	$C_{17}H_{13}O_6$	313.0711	313.0712	0.31	9.78	313.07	35
AFB_2	$[M+H]^+$	$C_{17}H_{15}O_6$	315.0869	315.0868	−0.31	9.63	315.08	35
AFG_1	$[M+H]^+$	$C_{17}H_{13}O_7$	329.0661	329.0662	0.30	9.30	329.06	29
AFG_2	$[M+H]^+$	$C_{17}H_{15}O_7$	331.0818	331.0816	−0.60	9.10	331.08	32
STER	$[M+H]^+$	$C_{18}H_{13}O_6$	325.0712	325.0711	−0.30	12.43	325.07	35
OTA	$[M+H]^+$	$C_{20}H_{19}O_6NCl$	404.0901	404.0903	0.43	12.23	404.09	17
BEA	$[M+NH_4]^+$	$C_{45}H_{61}O_9N_4$	801.4439	801.4437	−0.24	14.01	801.44	50

In this study, the calculated elemental compositions with a maximum deviation of 5 ppm from the measured exact mass were considered. To ensure correct mass calibration, the exact mass of the MS internal standard compounds were checked regularly. To obtain an elemental composition, all compounds were studied by chemical structure. Thus, theoretical mass was calculated by using ChemDraw 11.0 (CambridgeSoft) and www.chemspider.com.

Accurate mass screening for non target mycotoxins

Full-scan accurate mass measurements were compared with theoretical exact masses of known emergent mycotoxins, such as enniatins A, B, A_1, B_1 and fusaproliferin (Jestoi et al. 2004, 2009; Jestoi 2008).

Method validation

Confirmation of the identity was carried out by comparing the retention time and fragmentation pattern of the compound with that of a certified standard and quantification by accurate mass (European Community 2002). Linearity was assessed using standard solutions and matrix-matched calibrations by analyzing in triplicate six concentrations levels; matrix-matched calibration was prepared for each beer brand. To evaluate matrix effects, two strategies were studied: matrix-matched calibration and internal standard addition. The recovery experiments were carried out by spiking the sample in five replicates at two concentration levels: LLOQ level and 100 times LLOQ level (Table 2). In the same way, precision of the method was determined in fortified beer at LLOQ level and 100 times LLOQ level and calculated as RSD of measurements in quintuplicate, in the same day and five non-consecutive days.

Other analytical parameters, such as limits of detection (LODs) and lowest limits of quantification (LLOQs), were calculated by Xcalibur 2.0 software; both parameters were determined by decreasing concentration levels in fortified beer. When the signal-to-noise ratios (S/N) of 3 or above were considered acceptable for LOD. LLOQ was defined as the lowest concentration that could be quantified with coefficient of variation (%CV) and the absolute value of mean relative error (%MRE) [(spiked concentration−nominal concentration/nominal concentration)×100] of less than or equal to 20% (Table 2).

Results and discussion

LC-LTQ Orbitrap optimisation

All ion source tune parameters were optimized manually by infusing mycotoxin standards and monitoring the $[M+NH_4]^+$ ion for trichothecenes type A and BEA, also $[M+H]^+$ ion for the other mycotoxins (Table 1). This optimisation was carried out by adjusting the parameters until no precursor ion fragmentation was observed. In this study, mass resolution was set at 100,000 FWHM and the mean values of the mass measurements were all lower than 3 ppm of their theoretical m/z values, as shown in Table 1.

Table 1 also presents the data-dependent acquisition settings. MS^n measurements were performed to obtain information of main fragment ions generated in the linear ion trap (nominal mass product ions) within the same analysis. In addition, the accurate masses of these product ions were linked with precursor compound masses.

Confirmation of the identity was done by comparing the retention time, accurate mass and MS^n fragmentation pattern of the compound to that of a reference standard.

SPE optimisation

The extraction was carried out according to Romero-González et al. (2009). However, in this study, the range of mycotoxin analyzed was extended and, to improve the recovery values, some parameters were studied. With this aim, blank beer was fortified at twice the LLOQ level, being this level the reference concentration level.

The organic extraction solvent was the parameter optimized due to the wide-range of mycotoxins analyzed with different polarities. Then, the use of 4 ml of methanol and 4 ml of acetonitrile were compared. Methanol provided good recoveries for fumonisins (FB_1, FB_2, and FB3) and trichothecenes type B (DON, NIV, 3-ADON); however, the recoveries obtained for trichothecenes type A and AFs were lower than 50%. On the other hand, acetonitrile reached acceptable recovery values for AFs and trichothecenes Type A (>65%). For this reason, it was decided to use a solvent mixture elution of acetonitrile/methanol. The proportion acetonitrile/methanol (40 : 60, v/v) proposed by Romero-González et al. (2009) allowed acceptable recoveries between 63% for STER to 95% for OTA; however, in this study the mixture acetonitrile/methanol (50 : 50, v/v) improved absolute recoveries for trichothecenes Type A (108% DON), AFs (>66% AFB_2) and BEA (>66%), as shown in Table 2.

Validation of the method

To evaluate the ability of the developed method for quantitative analysis of the selected mycotoxins in beers, the analytical performance of the proposed method was studied and validated in terms of linearity, limits of detection and quantification, precision and

Table 2. Matrix effect, lowest limits of quantification (LLOQs) and recovery values (RSD %) in brackets calculated at two concentration levels (ng ml^{-1}). Mean level for all four matrices is presented with results showing values for stout/red ale/ale/pale lager beers.

				Intra-day precision		Inter-day precision	
Compound	Matrix effect (%)	LOD (ng ml^{-1})	LLOQ (ng ml^{-1})	Low level (LLOQ)	High level (100 × LLOQ)	Low level (LLOQ)	High level (100 × LLOQ)
NIV	90/98/94/98	50/45/44/40	155/135/131/120	77/80/84/91 (10)	78/82/84/89 (10)	73/81/80/90 (10)	70/81/84/89 (11)
DON	83/105/85/94	20/18/16/16	60/54/48/48	83/84/87/108 (11)	80/81/86/103 (9)	78/83/81/97 (11)	81/81/83/100 (9)
3-ADON	43/55/48/59	30/27/25/25	90/80/75/75	83/84/87/108 (11)	82/86/85/102 (8)	80/84/85/101 (12)	82/85/85/102 (9)
DAS	107/101/102/102	13/13/12/10	40/40/35/30	93/96/95/103 (9)	95/96/98/101 (5)	92/95/95/102 (10)	98/98/98/103 (9)
HT-2	59/63/53/65	7/9/7/7	20/27/20/20	71/75/77/79 (9)	73/78/79/79 (6)	72/78/79/81 (10)	76/80/81/85 (11)
T-2	63/69/63/68	5/5/4/4	15/15/12/12	68/76/74/92 (10)	71/79/78/99 (8)	68/69/71/83 (10)	68/72/72/89 (11)
FB_1	115/111/99/112	35/32/30/32	105/95/90/95	80/85/87/92 (11)	77/79/80/89 (9)	81/81/82/89 (12)	81/80/80/89 (10)
FB_2	100/125/111/109	32/32/30/30	95/95/90/90	85/89/87/88 (10)	80/82/83/85 (8)	86/88/87/88 (15)	80/81/80/86 (14)
FB_3	116/112/119/123	32/32/32/32	95/95/95/95	82/85/79/91 (12)	78/80/75/81 (11)	83/83/81/88 (15)	77/79/76/78 (11)
ZEN	87/79/82/83	12/10/12/10	36/30/36/30	75/77/85/88 (10)	72/74/85/83 (8)	77/77/81/85 (11)	71/70/81/82 (7)
ZOL	90/88/89/93	10/10/8/8	30/30/24/24	75/81/85/91 (8)	72/78/78/89 (5)	73/83/82/90 (9)	73/75/76/88 (5)
AFB_1	56/60/59/61	5/4/3/3	15/12/9/9	77/79/73/78 (5)	79/79/78/81 (5)	78/79/76/80 (7)	77/79/77/78 (9)
AFB_2	55/56/65/64	4/3/3/3	12/10/9/9	66/76/71/81 (8)	72/79/75/83 (6)	66/71/72/82 (10)	71/78/72/80 (9)
AFG_1	51/57/67/65	5/4/4/4	15/12/12/12	70/73/71/79 (7)	76/75/77/79 (7)	70/70/70/79 (7)	72/71/72/81 (8)
AFG_2	56/56/69/61	4/5/4/4	12/15/12/12	68/69/72/77 (9)	70/76/76/79 (6)	68/68/70/75 (9)	71/75/74/78 (9)
STER	66/67/69/71	7/6/6/5	21/18/18/15	69/79/72/81 (6)	72/80/76/84 (7)	65/73/76/80 (6)	71/77/75/87 (9)
OTA	77/71/75/79	5/4/4/4	15/12/12/12	70/77/75/94 (10)	78/82/85/97 (9)	71/72/78/89 (12)	79/80/81/90 (8)
BEA	55/66/59/64	4/5/5/4	12/15/15/12	69/75/91/99 (15)	71/73/88/93 (13)	66/77/88/93 (18)	70/73/90/95 (16)

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

reproducibility of the technique. Validation experiments were performed with four types of beer brands available in Ireland: (i) stout beer, (ii) red ale beer, (iii) ale beer and (iv) pale lager beer. The analytical performance of the methodology for beer samples is summarized in Table 2.

Limits of detection (LODs) for selected mycotoxins ranged from 3 ng ml^{-1} (AFB_1 and AFB_2 in ale and pale lager beers) to 50 ng ml^{-1} (NIV in stout beers). Lowest limits of quantification (LLOQs) ranged from 9 ng ml^{-1} (AFB_2 in ale and pale lager beers) to 155 ng ml^{-1} (NIV in stout beers). Although these limits did not differ significantly between the four matrices, in the case of stout and red ale beer seemed to be slightly higher. It is assumed that this phenomenon is caused by the greater roasting of the malt or barley and the appearance of different new compounds that could interfere with the selected mycotoxins increasing these limits.

Electrospray ionization is usually susceptible to matrix-related signal suppression, which is believed to result from the competition between the analyte ions and matrix components. Therefore, the presence of co-extracted matrix components may affect analyte quantification. For this purpose, five calibration sets (standard in pure solvent, and matrix-matched standards of stout, red ale, ale and pale lager beer) at six concentration levels, between LLOQ and 100 times LLOQ, were prepared. As seen in Table 2, matrix effects were calculated in percentages as the ratio of matrix-matched calibration slope and solvent calibration slope (Zachariasova et al. 2010). A value of 100% indicates that there is no absolute matrix effect. There exists signal enhancement if the value is >100% and signal suppression if the value is <100%. The matrix effects did not differ significantly between the matrices selected. Severe suppression occurred for aflatoxins, STER, trichothecenes type A (T-2 and HT-2) and trichothecenes type B (3-ADON).

The best way to compensate the matrix effect is the use of isotopic internal standards. Unfortunately, the labelled analogues or internal standard (I.S.) of these mycotoxins were inaccessible to us owing to their cost (Romero-González et al. 2009). Thus, other approaches were evaluated.

The first option was the addition of established internal standards used in the mycotoxin field, such as AFM_1, NEO and 3-ADON-d_3. AFM_1 and NEO are analogue molecules of AFs and trichothecenes type A, respectively, since they are metabolites of these mycotoxins. STER and AFs were calibrated with AFM_1, since STER is supposed to be a precursor of the AFs and AFM_1 is a metabolite of AFB_1. 3-ADON-d_3 is the deuterated analogue of 3-ADON. With this calibration approach, no significant improvement was achieved, probably due to the fact that the optimal SPE-HPLC-LTQ-Orbitrap conditions for internal standards were different from those of the mycotoxins.

A second calibration alternative was tested according to Council Decision 2002/657/EC (European Community 2002) and document SANCO/10684 (2009): external matrix-matched calibration. The use of matrix-matched standards compensated the suppression signal effects, achieving an accurate quantification, as reported the literature (Bacaloni et al. 2005; Romero-González et al. 2009; Zachariasova et al. 2010). Matrix-matched calibration curves were prepared in the four extract beers, showing good linearity between LLOQ and 100 times LLOQ concentration levels (linear range), with a correlation coefficient $\geq$0.9861.

The inter-day precision and intra-day repeatability of the method were also evaluated on extracted beer samples at two different concentration levels (LLOQ and 100 times LLOQ). The RSD values for intraday analyses were in the range 5–15% and the RSD for inter-day values were between 6 and 18%, showing good reproducibility of the technique. The recovery ranges in low and high spiked levels were 66–108% for the intraday test and 65–103% for the inter-day test.

In light of these results, the developed method gave acceptable recoveries and these results were in good agreement with the performance criteria of Commission Regulation (EC) No. 401 of 23 February 2006 (European Community 2006) laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs.

Analysis of commercialized Irish beers

In this study, the analyzed samples were divided into four main categories: stout, red ale, ale and pale lager. Twenty-five samples were analysed: six stouts, three red ale, six ale and 10 pale lager beers. The first step was to identify target compounds (selected mycotoxins) in Irish beers. Red ale, ale and pale lager beers were analysed by LTQ Orbitrap XL; in these alcoholic beverages selected mycotoxins were not present. However, ZEN was identified and quantified (37.8 ng ml^{-1}) in one stout beer. Figure 1 depicts a full scan of the stout beer sample where ZEN was identified by retention time (RT) (Figure 1a). After that, extracted ion chromatogram was checked for selected compound and ZEN was observed at appropriate RT of 12.19 (Figure 1b). This compound was unambiguously identified by an accurate mass and pathway study. The measured m/z was 319.1547 and the calculated error (ppm) was 0.31 (Figure 1c). To improve the identification of the mycotoxin, the ion trap spectrum was acquired and the characteristic pathway for ZEN was obtained. This mycotoxin

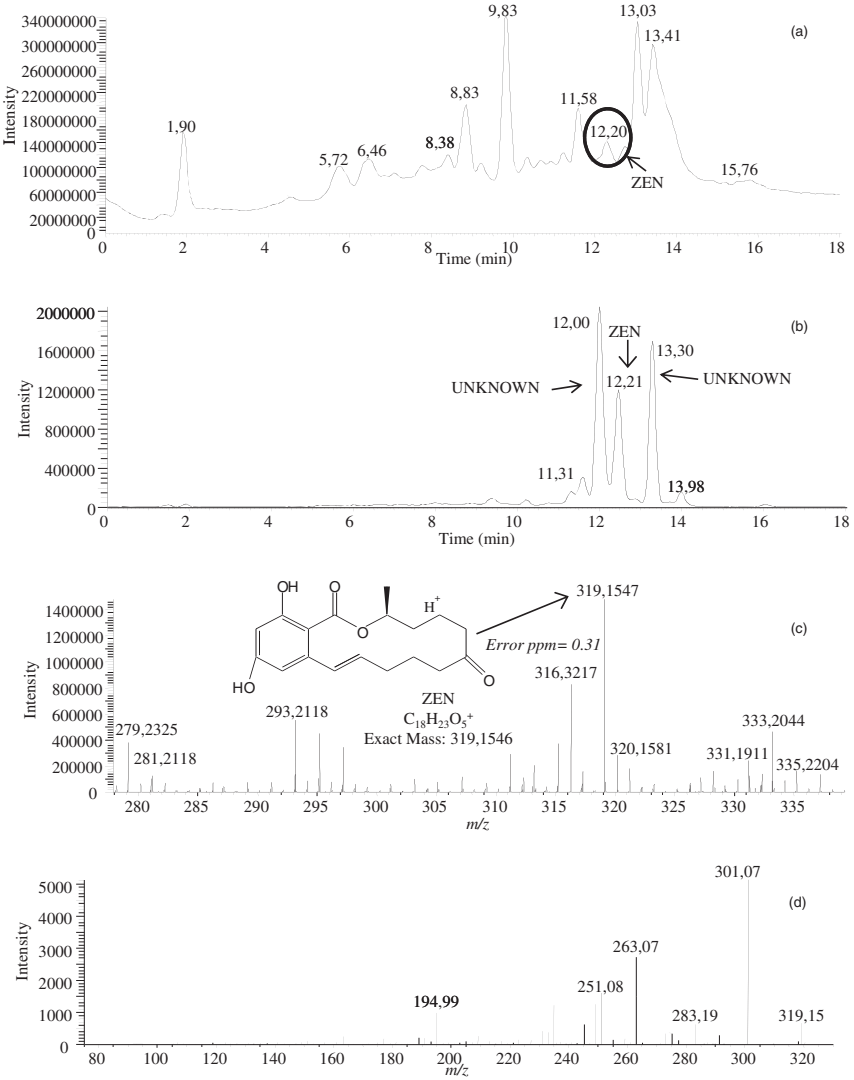

Figure 1. Chromatogram of positive stout beer sample: (a) Full scan MS 100–900 *m*/*z*. (b) Extracted ions *m*/*z* 319.15. (c) Accurate mass ZEN. (d) Confirmation by ion trap (MS_2).

(ZEN) lost one molecule of water in the first fragmentation of ion trap (MS^2); this fragment has been also reported by Frenich et al. (2009).

To our knowledge, this is the first time that ZEN has been identified unambiguously by accurate mass and fragmentation study in beer. Previous works that

screened ZEN in beer (Maragou et al. 2008; Romero-González et al. 2009) did not identify this mycotoxin in this food commodity.

The second step was to screen "non-target" mycotoxins in Irish beer samples: enniatins A, B, A_1, B_1 and fusaproliferin. The structures of these emergent mycotoxins were studied (www.chemspider.com) and the exact mass was calculated (ChemDraw 11.0). Several studies about emergent mycotoxins have reported two possibilities for molecular ion formation; $[M + H]^+$ and $[M + NH_4]^+$ (Uhlig and Ivanova 2004; Jestoi et al. 2009). For this reason, exact mass for these ions were calculated and scanned on each sample by Xcalibur 2.0 software. In this study, these emergent mycotoxins were not identified.

Conclusions

The SPE-LC-LTQ-Orbitrap-MS method reported in this paper was able to determine 18 mycotoxins in beer providing high sensitivity, high recoveries and achieving low LODs and LLOQs. Moreover, this technology allowed a screening for five emergent mycotoxins, non-target compounds which could be checked.

The developed method was applied for the analysis of selected mycotoxins in 25 different Irish beer samples. The results confirmed the presence of ZEN in one sample of stout beer.

Even though the detected concentration level was low, exposure depends of the quantity of beer consumed over a prolonged period of time, highlighting the necessity of mycotoxin control in the beer production chain.

To the best of our knowledge, this work reports for the first time the application of LTQ-Orbitrap technology to routine monitoring of mycotoxins in commercial beers, demonstrating its potential by an accurate mass and pathway study.

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4.10 Survey and distribution of mycotoxins in European beers using different LC-MS analysers.

Survey and distribution of mycotoxins in European beers using different LC-MS analysers

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ABSTRACT

In this work, an existent solid-phase extraction (SPE) procedure was used to study the occurrence of mycotoxins in different European beers. HPLC-QqQ-MS/MS and ultra high resolution mass analyser have been optimised for the analysis of 18 mycotoxins: the methods were validated according to the EU Commission Decision 2002/657/EC guidelines. In this sense, matrix-matched calibration was performed for each type of beer, obtaining an accurate quantification. The recoveries ranged from 63 to 91% and repeatability and reproducibility expressed as RSD were lower than 17%. While HPLC-LTQ-Orbitrap® was used for unambiguous identification of target mycotoxins, as well as screening of un-known compounds, such as enniatins, fusaproliferin and deoxynivalenol-3-glucoside, the quantification was carried out using HPLC-QqQ-MS/MS. Finally, the presence of ochratoxin A, fumonisins and toxins T-2 and HT-2 in lager, stout and red ale beers respectively were confirmed and quantified.

KEYWORDS*:* Mycotoxins; Triple quadrupole; Orbitrap; Beer; SPE

1. INTRODUCTION

Beer is the oldest alcoholic beverages and a cereal-based product worldwide consumed. It invention has been argued to be responsible for humanity's ability to develop technology, concretely food technology. Nowadays, brewing and fermentation of starches, mainly derived from cereal grains, such as barley, wheat, maize and rice produce beer. The fermentation generates substances such as carbohydrates, mainly sugars or starches, which produce a benefit beverage (Bamforth, 2002).

The cereal grains could be contaminated by intentioned addition or natural contaminants. For example, metals have been detected in cereal and beer (Donadini, Spalla, & Beone, 2008), and pesticides have been also identified in this matrix (Navarro, Pérez, Navarro, Mena, & Vela, 2007; Bolaños, Romero-González, Frenich, & Vidal, 2008). On the other hand, natural contaminants as mycotoxins have been recently identified in cereals, hop and beer (Romero-González, Vidal, Aguilera-Luiz, & Frenich, 2009; Běláková, Benešová, Mikulíková, & Svoboda, 2011). Theses contamiants or its residues could reach the consumers, and moreover, a frequent consume of the contaminated product could suppose a risk for the health of the consumers. For this reason, maximum levels (MLs) for mycotoxins have been established (EU, 2006; EU 2010), besides some of them have been also classified by IARC (IARC, 1993).

Beer is a complex matrix; it composition contains water, carbohydrates, protein substances, mineral salts and alcohol. The alcohol (ethanol) is fermentation sub-product and it can strongly influence the extraction of mycotoxins. Because of it complexity and the presence of the alcohol, extraction procedures for mycotoxins from beer have been carefully developed.

At the beginning of the decade, the developed mycotoxins methods in beer were focused on one mycotoxin as OTA (Visconti, Pascale, & Centonze, 2000; Bacaloni et al., 2005; Araguás, González-Peñas, & López De Cerain, 2005; Aresta, Palmisano, Vatinno, & Zambonin, 2006) or on the detection of a group of

mycotoxins as fumonisins (Shephard et al., 2005), trichothecenes (Suga, Mochizuki, Harayama, & Yamashita, 2005) or aflatoxins and OTA detection (Ventura et al., 2006) .

Nowadays, HPLC-MS/MS methods have allowed the development of fast multi-residue methods in foodstuff (Desmarchelier et al., 2010; Sulyok, Krska, & Schuhmacher, 2010; Ediage, Di Mavungu, Monbaliu, Peteghem, & De Saeger, 2011; Rubert, Soler, & Mañes, 2011a). Focusing on beer, several methods have been also developed using HPLC-MS/MS (Rudrabhatla, & Wood, 2007; Romero-Gonzalez et al., 2009). Triple-quadrupole detector has therefore demonstrated to be robust and sensible for the routine analysis of mycotoxins in beer.

However, the last tends in organic contaminant analysis have been to take advantage of the ultra-high resolution mass spectrometry, which allows qualitative and quantitative analysis for target, non-target and unknown compounds. For example, this technology has been recently applied to mycotoxin analysis in beer (Zachariasova et al., 2010; Rubert, Mañes, James, & Soler, 2011). In these cases, Orbitrap® technology demonstrated to be useful for routine analysis, since effective quantification and unambiguous identification were obtained.

Thereby, the main aim of this work was to develop a rapid, easy and sensitive method to identify and quantify 18 mycotoxins in beer. For that purpose, a previous SPE method was used to extract selected mycotoxins. HPLC-QqQ-MS/MS method was optimized to identify selected mycotoxins, as well as to obtain lowest levels of quantification (LOQs). On the other hand, hybrid linear ion trap-high resolution mass spectrometry was used to unambiguous identification of target compounds studying the fragmentation and accurate mass. Owing to the capacity of Orbitrap® technology to acquire data essential for the identification of other ‘‘non-target’’ mycotoxins that may be present in beer, several “emergent” mycotoxins as such enniatins (ENs): ENA, ENA1, ENB, ENB1, fusaproliferine (FUS) and deoxynivalenol-3-glucoside (D3G) were tested.

Finally, the developed methods were applied to monitoring commercial beers for main manufacture countries in Europe.

2. MATERIAL AND METHODS

2.1. Chemical and standards

The certificate standards of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), Ochratoxin A (OTA), sterigmatocystin (STER), α-zearalenol (ZOL), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), diacetoxyscirpenol (DAS), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2) and beauvericin (BEA) were purchased from Sigma Aldrich (Madrid, Spain). T-2 and HT-2 toxin stock solutions (in acetonitrile) were obtained from Biopure referenzsubstanzen GmBH (Tulln, Austria). Fumonisin B_3 (FB_3) was supplied by the PROMEC unit (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa). The individual stock solutions were prepared as a previous work (Rubert et al., 2011b).

HPLC grade solvents, acetonitrile, methanol and water were supplied by ThermoFischer (Dublin, Ireland). Analytical grade reagent formic acid (purity > 98%), ammonium formate was obtained from Panreac Quimica S.A.U. (Barcelona, Spain). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Oasis HLB SPE (200 mg/6 ml) cartridges were purchased from Waters (Milford, MA, USA) and Strata-X 33 μm Polymeric Reversed Phase cartridges (200mg/6 ml) were purchased from Phenomenex (Phenomenex, Torrance, CA, USA). An extraction manifold from Waters connected to a Büchi Vac V-500 (Flawil, Switzerland) vacuum system was used for SPE experiments.

2.2. Samples and sampling

A total of 49 beer samples were purchased from different stores, supermarkets and specialized beer stores from Cork (Ireland) and València (Spain) and kept at -20°C in a dark and dry place. Several imported beers from Belgium, Czech Republic,

Germany, Italy and Poland were also included in the study. The samples with undetectable levels of mycotoxins were used for spiking and recovery studies.

2.3. Extraction Procedure

Sample preparation was performed as described in a previous study (Rubert et al., 2011). Beer was degassed by sonication for 25 min. The Oasis HLB cartridges were conditioned with 5 ml of acetonitrile/methanol (50:50, v/v) and 5 ml of water. Consequently, 10 ml of beer was loaded onto C_{18} cartridge. After that, SPE columns were washed with 5 ml of water. Then the cartridges were dried for 30 min. In the last step, the mycotoxins were eluted by adding 4 ml of acetonitrile/methanol (50:50 v/v). Then, the extract was transferred into a 15 ml conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 ml with methanol/water (50:50, v/v), filtered through a 0.20 μm Millex-GN nylon filter (Millipore, Carrightwohill, Ireland) and collected into a vial.

2.4. Instrumental parameters

2.4.1. Liquid chromatography triple quadrupole tandem mass spectrometry

The HPLC-QqQ-MS/MS system consists on a Finnigan Surveyor CTC (Autosampler ThermoFischer Scientifics), a Finnigan Surveyor LC quaternary Pump (Accelera, ThermoFischer Scientifics) and a Finnigan TSQ Quantum Discovery MAX triple quadrupole mass spectrometer (ThermoFischer Scienctific, Hemel Hempstead, UK). Chromatographic separation was performed with a reversed-phase analytical column (Gemini C_{18}, 150 mm, 2 mm i.d, 5 μm; Phenomenex). The mobile phase was a gradient of H_2O 5mM ammonium formate and 0.1% formic acid (A) and methanol 5 mM ammonium formate (B) and the gradient conditions were as follows: 0–10 min, linear from 5 to 95% B; 10–15 min, isocratic 95% B. Ten minutes were used to equilibrate the column with initial conditions. The flow rate was 200 μl min^{-1}. The autosampler was set at 10ºC and column temperature was set at 35 ºC, 10 μl was injection volume.

All mycotoxins were detected using heated electrospray (H-ESI) source. The first step was to infuse each mycotoxin standard solution (10 μg/ml) with a syringe pump (10 μl/min). Ion source parameters were optimized for each compound using the quantum tune application of Xcalibur 2.0.7 software. The source was operated in the positive heated ESI mode; spray voltage, 4500 V; vaporizer temperature, 300 ºC; ion transfer capillary temperature, 350 ºC; with both the sheath gas pressure set to 40, auxiliary gas pressure 55 arbitrary units and ion sweep gas was set to 0 arbitrary. Skimmer offset was set to -3 V and the collision gas pressure was 1.5 mTorr. Data processing was performed using the Xcalibur (Version 2.0.7) software (ThermoFischer Scientifics). Mass spectral data were acquired in SRM mode in a single time segment with 20 ms dwell time for each transition. Collision energy and tube lens offset voltages were optimised for each mycotoxin using the automated optimisation procedure in syringe infusion mode provided by the manufacturer. *Table 1* shows the two monitored transitions for each mycotoxin.

2.4.2. HPLC-LTQ-Orbitrap XL

The analytical method has been previously optimized and explained by Rubert et al. (2011b). The LC system was connected to a hybrid LTQ Orbitrap XL (Thermo Scientific), operating in positive ion mode. The column and the chromatographic separation used were the same as HPLC-QqQ-MS/MS method (section 2.4.1.).

The linear ion trap (LTQ) part of the hybrid MS system was equipped with heated electrospray interface (H-ESI). Full-scan accurate mass spectra (mass range from 90 to 900 Da) were obtained at high resolution (100,000 FWHM) and processed using Xcalibur v.2.0 software. The electrospray source conditions were: source voltage 4 kV, heated capillary temperature 275 °C, capillary voltage 30 V, sheat gas and auxiliar gas 35 and 30 (arbitrary units), respectively.

2.5. Method Validation

Trueness, precision and linearity of the HPLC-QqQ-MS/MS method were evaluated. Linearity was evaluated using standard solutions and matrix-matched calibrations by analyzing in triplicate six concentrations levels (linear range from

LOQ to 100 times LOQ); matrix-matched calibration was prepared for each type of beer. In order to evaluate matrix effects, matrix-matched calibration and standard calibration in solvent were used. Recovery experiments were carried out by spiking the samples in five replicates at two concentration levels; LOQ level and 100 times LOQ level. Precision of the method was determinate in fortified beers at LOQ level and 10 times LOQ level and calculated as RSD (%) of measurements in quintuplicate the same day and five non-consecutive days. The LODs were determined as the lowest mycotoxin concentration whose qualified transition (q) presented a signal-to-noise ratio (S/N) ≥ 3. The LOQs were determined as the minimum detectable amount of analyte with a S/N ≥ 10 for the quantified transition (Q) transition.

Table 1. ESI-MS/MS parameters for selected mycotoxins.

Mycotoxin	Retention Time (min)	Precursor Ion(m/z)	Product Ion	Collision Energy (eV)	Turbo Lens
Nivalenol	5.05	313.1 $[M+H]^+$	175^Q 115^q	54 30	100
Desoxynivalenol	6.40	297.1 $[M+H]^+$	115^Q 127^q	56 49	61
Diacetoxyscirpenol	8.47	384.2 $[M+NH_4]^+$	307^Q 105^q	11 33	113
3-acetyldeoxynivalenol	8.53	339.1 $[M+H]^+$	321^Q 230^q	13 13	63
Aflatoxin G_2	9.10	331.1 $[M+H]^+$	189^Q 245^q	42 30	120
Aflatoxin G_1	9.25	329.1 $[M+H]^+$	243^Q 200^q	36 31	120
Aflatoxin B_2	9.65	315.1 $[M+H]^+$	259^Q 243^q	40 35	137
Aflatoxin B_1	9.75	313.1 $[M+H]^+$	241^Q 213^q	39 45	132
Fumonisin B_1	10.44	722.4 $[M+H]^+$	334^Q 352^q	36 37	115
HT-2	10.78	442.1 $[M+NH_4]^+$	263^Q 215^q	13 12	102
Fumonisin B_3	11.00	706.4 $[M+H]^+$	336^Q 354^q	37 31	134
T-2	11.30	484.1 $[M+NH_4]^+$	215^Q 305^q	20 13	102
Fumonisin B_2	11.60	706.4 $[M+H]^+$	336^Q 318^q	40 37	116
Zearalenol	11.98	321.1 $[M+H]^+$	303^Q 189^q	15 22	69
zearalenone	12.20	319.1 $[M+H]^+$	283^Q 187^q	23 29	69
Ochratoxin A	12.25	404.1 $[M+H]^+$	239^Q 102^q	29 69	80
Sterigmatocystin	12.45	325.1 $[M+H]^+$	281^Q 310^q	38 29	98
Beauvericin	14.10	801.4 $[M+NH_4]^+$	244^Q 262^q	25 27	164

3. RESULTS AND DISCUSSION

The present study is a step forward over previous published method (Rubert et al., 2011b). On the one hand, an extraction method based on a solid-phase extraction (SPE) was evaluated with different cartridges. On the other hand, HPLC-LTQ-Orbitrap XL validated method was used in order to indentify unambiguously target mycotoxins, as well as to investigate the presence of non-target mycotoxins.

3.1. Optimization HPLC-QqQ-MS/MS

The optimization procedure was according to previous works (Rubert et al., 2011a). Preliminary experiments were conducted with the purpose of finding the best instrumental conditions that allowed unambiguous identification of target compounds in commercial beer samples at trace levels. Tuning of the instrument was performed for each mycotoxin using a standard solution in solvent as it was indicated above. At the end, all target mycotoxins were analysed using ESI positive mode as it is shown in *table 1*.

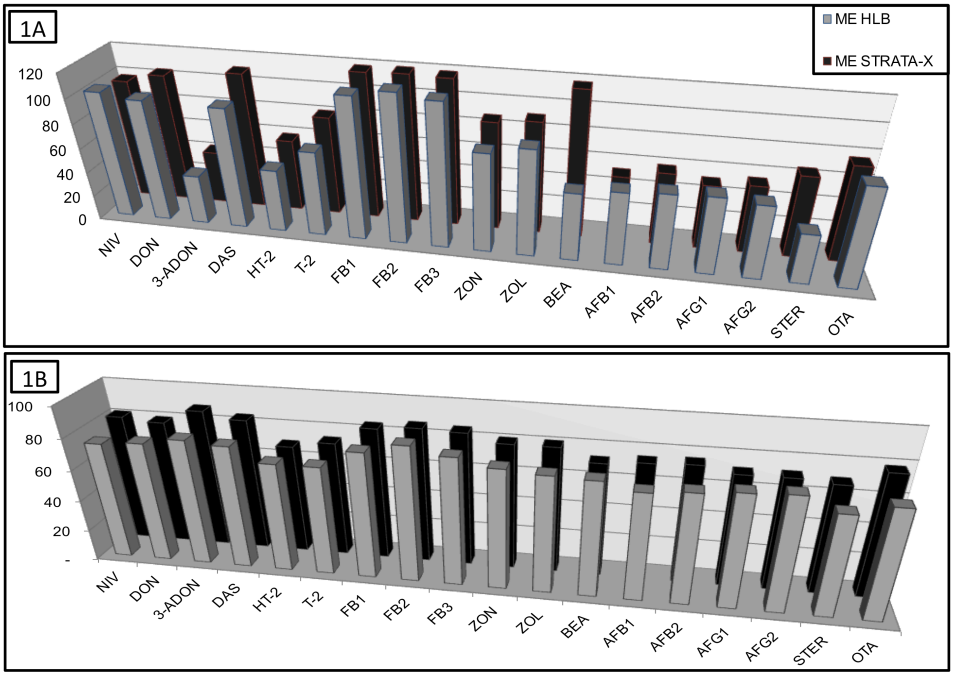
3.2. SPE cartridges selection

In this research, two different manufactures of C_{18} SPE columns were tested; Oasis HLB and Strata-X. In order to select the best cartridge, the extraction procedure was studied fortifying at 100 ng mL^{-1} for each mycotoxin as reference level (*figure 1*).

First of all, matrix effects were evaluated for each mycotoxin in pale lager beer using the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A); the ratio (B/A x 100) is defined as the matrix effect (ME%). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%. The *figure 1A* shows the matrix effects observed for each mycotoxin in pale lager beer as a model matrix study. Both columns presented similar behaviour for type A and B trichothecenes, AFs, HT-2, T-2 and 3-ADON: high signal suppression was calculated for these mycotoxins. However, BEA and STER presented different behaviour for each cartridge; whereas, Oasis

HLB showed higher suppression for BEA and STER, Strata-X did not present suppression for either mycotoxins or showing lower suppression than Oasis HLB. Secondly, SPE columns were compared by their recoveries (*Figure 1B*). In this comparison did not observed significant difference between them. For example, Strata-X recoveries ranged from 68% to 89% and Oasis HLB recoveries were from 65% to 87%. This study revealed that both SPE columns offered similar data, even so the validation of the method using HPLC-MS/MS was realised using Oasis HLB according to previous work.

Figure 1. Comparison of SPE cartridges. Figure 1A shows the matrix effects (ME) study for selected mycotoxins in pale lager beer using different SPE columns. Figure 1B shows recoveries at 100 ng mL^{-1} (n=5) for pale large beer using different SPE columns.



3.3. Validation of SPE-HPLC-QqQ-MS/MS method

The analytical performance of the proposed method was studied and validated in terms of linearity, LODs and LOQs, precision and reproducibility of the method (*Table 2*). Validation experiments were performed using four types of beer brands available in Europe: (i) stout beer, (ii) red ale beer, (iii) ale beer and (iv) pale lager

beer. Analytical performance of the methodology for beer samples is summarized in table 2.

Limits of detection (LODs) for selected mycotoxins were ranged between 0.3 ng ml^{-1} (AFB_1 and AFB_2 pale lager beer) to 15 ng mL^{-1} (NIV in stout beers). Limits of quantification (LOQs) were between 0.9 ng mL^{-1} (AFB_2 pale lager beer) to 45 ng mL^{-1} (NIV in stout beer).

Matrix effects were calculated as explained above (section 3.2). For this purpose, six points (standard in pure solvent, and matrix-matched standards of stout, red ale, ale and pale larger beer) at six concentration levels between LOQ and 100 times LOQ were prepared. Matrix effects did not differ significantly between the matrices selected. Severe suppression occurred for AFs, STER, type A trichothecenes (T-2 and HT-2) and type B trichothecenes (3-ADON), which ranged from 55% to 68% for the four selected beers. These results were according to previous works (Zachariasova et al., 2010; Rubert et al., 2011b). Matrix-matched calibration curves were prepared in the four different extract beers, showing good linearity between LOQ and 100 times LOQ concentrations level (linear range), with a correlation coefficient ≥ 0.9922 for all the varieties.

The *figure 2* shows the intra-day recoveries for the four types of beer spiked at 100 times LOQ level using the selected cartridge. Recoveries were into acceptable range, although these recoveries obtained at LOQ concentration level and 100xLOQ concentration level varied slightly depending on the type of beer. For example stout beer showed the lowest range between 63-87%. However, red ale and ale ranged from 68 to 91% and 67 to 87%, respectively. On the other hand, pale lager beer showed a range between 69 to 91%. The intra-day and inter-day tests were also evaluated on spiked beer extracts at two different concentration levels (LOQ and 100 times LOQ). The RSD values for intra-day analyses were lower than 15.8% and the inter-day values were lower than 17.1%.

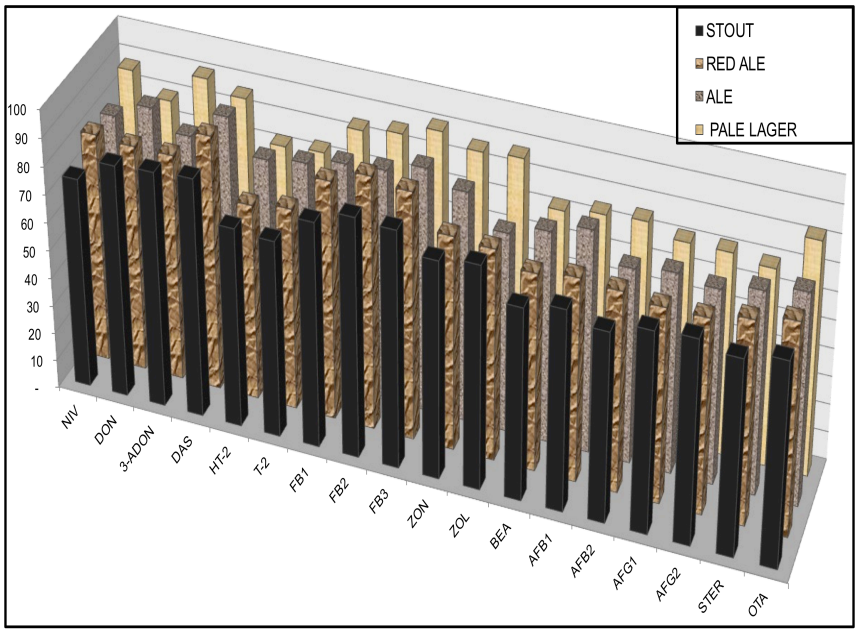
Then, SPE-HPLC-QqQ-MS/MS method was successfully validated according to the criteria specified in Commission Decision 2002/657/EC for quantitative confirmation method (EU, 2002).

Table 2. Limits of detection (LOD), limits of quantification (LOQs), inter-day and intra-day test calculated as relative standard deviations (RSD%) calculated at two concentration levels (ng mL-1), mean level for all four matrices is presented. Results show values for stout/red ale/ale/pale lager beers.

			Intra-day[a]		Inter-day[b]	
Compound	LOD ($ng\ ml^{-1}$)	LOQ ($ng\ ml^{-1}$)	Low level (LOQ)	High level (100xLOQ)	Low level (LOQ)	High level (100xLOQ)
NIV	15/10/12/10	45/30/35/30	9.6	11.3	9.4	11.2
DON	15/12/12/12	40/35/35/35	10.1	7.8	10.1	8.9
3-ADON	10/10/10/10	30/25/25/25	12.1	7.8	11.2	8.6
DAS	8/5/5/5	25/15/12/12	8.8	6.5	9.1	8.2
HT-2	7/9/7/6	20/27/18/15	7.1	8.1	10.3	11.2
T-2	2/2/1.5/1	6/6/4/3	8.9	7.8	11.5	10.2
FB_1	25/22/20/22	70/65/60/65	10.3	12.3	10.8	9.6
FB_2	25/22/20/22	70/70/60/65	10.2	15.8	14.9	13.5
FB_3	20/20/20/20	60/60/60/60	12.5	11.1	15.1	11.1
ZEN	8/7/7/7	25/20/23/20	9.6	10.8	11.7	7.2
ZOL	7/5/6/6	20/15/12/12	8.4	7.5	8.7	4.7
AFB_1	0.5 /0.3/0.3/0.3	1.5/1.2/1/1	5.1	6.5	7.1	9.6
AFB_2	0.5/0.3/0.3/0.3	1.5/1/0.9/0.9	7.9	8.6	9.4	9.2
AFG_1	0.5/0.3/0.3/0.3	1.5/1/1/1	6.4	6.7	6.6	9.1
AFG_2	0.4/0.5/0.4/0.4	1.2/1.5/1.2/1.2	7.8	6.9	5.4	8.4
STER	1/1/1/1	3/3/3/3	5.8	9.7	5.7	8.1
OTA	1/0.75/0.75/0.75	3/2.5/2.5/2.5	11.1	8.9	11.6	8.7
BEA	1/1/1/1	3/2.5/3/2.5	15.4	12.1	17.1	16.2

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

Figure 2. Recoveries obtained at 100 times LOQ level (n=5) for stout, red ale, ale and pale lager beer using Oasis HLB SPE columns.



3.4. Analysis of commercialized European beers

In this research, the analysed samples were divided into four main categories: stout, red ale, ale and pale lager beers. Forty-nine samples were analysed: 10 stouts, 10 red ale, 10 ale and 19 pale lager beers. The samples came from Belgium, Czech Republic, Germany, Italy, Ireland, Poland and Spain. The first step was to identify target mycotoxins in these European beers, and with this aim red ale, ale and pale lager and stout beers were analysed by HPLC-QqQ-MS/MS. The confirmation of positive samples was carried out according to the European Commission (EU, 2002); the ion ratio of the primary and secondary product ions were monitored and compared, the ion abundances were therefore compared with those calculated for fortified samples. *Table 3* shows the co-occurrence and calculated concentrations for detected mycotoxins. Fumonisins B_1 and B_2 were identified and quantified in 32% of samples. On the other hand, HT-2 and T-2 toxins were found in 8% of

samples. The co-occurrence of *Fusarium* mycotoxins were mainly observed in pale lager beer, maybe because of type of raw cereal used or the production process. However, OTA was identified in six samples (12%) of red ale, ale and pale lager.
The developed analytical method demonstrated to be effective for quantifying and qualifying these samples positives for OTA, T-2, HT-2 and fumonisins using a fast and easy multi-mycotoxin SPE extraction procedure.

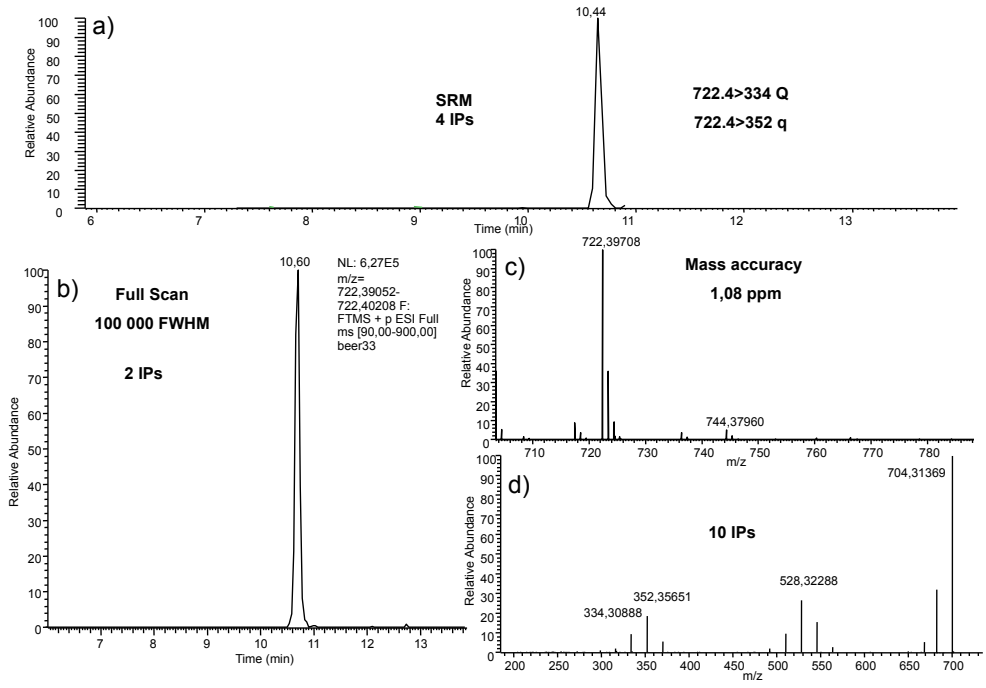
Table 3. Calculated concentration (ng mL^{-1}) for pale lager, stout, ale and red ale beers (n=49).

Samples	OTA		FB1		FB2		HT-2		T-2	
	Positives	Range	Positives	Range	Positives	Range	Positives	Range	Positives	Range
Stout			2/10	75.1-127	2/10	87-98				
Red ale	1/10	5.1	1/10	92.3	1/10	96.1				
Ale	1/10	3.2								
Pale lager	4/19	2.7-6.9	7/19	71.2-118	7/19	71-87	3/19	15.1-20	3/19	4-12.1
Total	6/49		10/49		10/49		3/49		3/49	

Recently, an extensive monitoring of European beer has been carried out using four different extraction procedures and four detection methods (Bertuzzi, Rastelli, Mulazzi, Donadoni, & Pietri, 2011). Regarding the results, our results showed lower incidence than Bertuzzi et al. (2011), as well as our levels were lower than this previous work: the authors demonstrated an elevated incidence of OTA (67.9%), DON (66%) and fumonisin B_1 (97%) and B_2 (57.6%). On the other hand, Romero-Gonzalez et al. (2009) detected traces of Fumonisin B_2, T-2, and HT-2 toxins, and aflatoxin B_1 in three normal beer and one special beer (n=15) using a developed SPE-HPLC-QqQ-MS/MS
Therefore, one-way to guarantee the data obtained was to confirm all the samples using LTQ-Orbitrap® XL. The use of LTQ-Orbitrap® XL had two objectives; the

first aim was the confirmation of identity based on the following criteria (EURACHEM, 1998; EU, 2002): (i) the retention time was set to ±2% from that of calibration standard. (ii) Accurate mass must fit the theoretical accurate mass with a tolerance set at ±5 ppm and (iii) fragment ions obtained by ion-trap must coincide with those of a calibration standard within ±15%, as well as the fragment ions must be present with their relative abundances. *Figure 3* shows a positive for FB_1 in pale lager beer. This figure depicts the chromatogram obtained using HPLC-QqQ-MS/MS (*figure 3a*), as well as a full scan of pale lager beer where FB_1 was found and confirmed by the retention time (*figure 3b*). After that, extracted ion chromatogram for FB_1 was checked for selected compound using windows. This compound was unambiguously identified by accurate mass and pathway study. The measured *m/z* was 722.39708 and the calculated error (ppm) was 1.08 (*Figure 3c*). The ion trap spectrum was acquired, and characteristic pathway from FB_1 was obtained (*Figure 3d*). This workflow was carried on for each positive and this form the positive compounds were confirmed. The number of identification points was studied for each mass analyser. For mycotoxins a minimum of 3 IPs are required according to Commission Decision 2002/657/EC (EU, 2002). Focus on triple quadrupole was able to reach 4 IPs working on SRM mode, thereby this instrument fulfilled the criterion established. However, Full Scan obtained by Orbitrap® did not permit to obtain IPs enough (N=2), although the instrument worked at ultra high mass resolving power settings of 100.000 FWHM and the mass error exceeding slightly 1 ppm. Obviously, when LTQ-Orbitrap® was used combining Full Scan and DDA the number of IPs was increasable. The *figure 3* shows 2 IPs for the Full Scan precursor ion and 10 IPs for the transition ions, addend 12 IPs. Thereby, it was obtained an unambiguous identification. Previously, Rubert et al. (2012) had experimentally confirmed FB_1 in baby food using LTQ-Orbitrap® obtaining similar FB_1 pathway and mass accuracy.

Figure 3. Positive pale lager beer. This figure shows a HPLC-QqQ-MS/MS chromatogram for positive FB_1 (a), the Full Scan obtained by LTQ-Orbitrap® (b), accurate mass FB_1 (c) and confirmation by LIT (d).



The second aim reached using LTQ-Orbitrap® was that all the samples could be analysed screening non-target and unknown compounds in the European selected beers. Non-target compounds were "emergents" mycotoxins such as enniatins A, B, A_1, B_1, FUS and D3G. The structures of these emergent mycotoxins were studied (www.chemspider.com) and the exact mass was calculated (ChemDraw 11.0).

Several studies about emergent mycotoxins and masked mycotoxins related two ion formation possibilities: $[M+H]^+$ and $[M+NH_4]^+$ (Uhlig, & Ivanova, 2004; Jestoi, Rokka, Järvenpää, & Peltonen, 2009; Kostelanska et al., 2011). For this reason, exact mass for these possibilities were calculated and scanned on each sample by Xcalibur 2.0 software.

Finally, in our research study these emergent mycotoxins and masked mycotoxins were not identified in European beers.

4. CONCLUSION

In conclusion, the SPE-HPLC-QqQ-MS/MS method was able to determine simultaneously 18 mycotoxins in beer proving a sensitive and robust technique. The triple quadrupole was therefore applied for the analysis of target mycotoxin in 49 different beer samples. Thereby, the presence of fumonisins B_1, B_2, HT-2, T-2 and OTA in the selected beers could be demonstrated.

In our research, the use of liquid chromatography triple quadrupole mass analyser and linear ion trap-high resolution mass spectrometry provided an unambiguous identification reaching required IPs. Moreover, the LTQ-Orbitrap® allowed a screening of non-target mycotoxins owing to Full-Scan could be carefully studied looking for it.

To sum up, the survey and distribution of mycotoxins in European beers was therefore developed taking advantage of triple quadrupole, which was most satisfactory for quantification purposes, followed by LTQ-Orbitrap® that allowed unambiguous identification and the screening capability.

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Applicability of hybrid linear ion trap-high resolution mass spectrometry and quadrupole-linear ion trap-mass spectrometry for mycotoxin analysis in baby food

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ABSTRACT

Recent developments in mass spectrometers have created a paradoxical situation; different mass spectrometers are available, each of them with their specific strengths and drawbacks. Hybrid instruments try to unify several advantages in one instrument. In this study two of wide-used hybrid instruments were compared: hybrid quadrupole-linear ion trap-mass spectrometry (QTRAP®) and the hybrid linear ion trap-high resolution mass spectrometry (LTQ-Orbitrap®). Both instruments were applied to detect the presence of 18 selected mycotoxins in baby food. Analytical parameters were validated according to 2002/657/CE. Limits of quantification (LOQs) obtained by QTRAP® instrument ranged from 0.45 to 45 $\mu g\,kg^{-1}$ while lower limits of quantification (LLOQs) values were obtained by LTQ-Orbitrap®: 7–70 $\mu g\,kg^{-1}$. The correlation coefficients (r) in both cases were upper than 0.989. These values highlighted that both instruments were complementary for the analysis of mycotoxin in baby food; while QTRAP® reached best sensitivity and selectivity, LTQ-Orbitrap® allowed the identification of non-target and unknowns compounds.

1. Introduction

Mycotoxins are regarded as the most serious of natural toxins that can contaminate cereals or derivates [1–3]. Due to the co-occurrence of different toxins in food matrices and their possible synergistic effect in humans, it is absolutely necessary to perform multi-analyte detection methods [4,5]. Moreover, the level of contamination can vary considerably worldwide according to geographical area, region and year and it can range from a few $ng\,g^{-1}$ to several $\mu g\,g^{-1}$ [6]. The different chemical groups of mycotoxins, the complexity of matrices and the low detection limits required increasing the importance of the choice of analytical strategy in this field.

Liquid chromatography-tandem mass spectrometry coupled with triple quadrupole has been widely accepted as the main tool in the identification, structural characterization and quantitative analysis of mycotoxins owing to its superior sensitivity, specificity and efficiency [3,7–9]. However, this mass analyzer is a targeted method that only monitors a relatively large number of analytes defined in advance; in such targeted analyses, signals from all other compounds are ignored [10,11]. As the number of substances to be screened and confirmed is high and not limited, one technique could never be capable sufficient to detect all mycotoxins and related compounds (as metabolites) in one run.

Fortunately, the establishment of directives based on mycotoxins analysis [12–15], validation criteria [16–18] and development of mass spectrometry have growth in parallel way; the use of hybrid instruments could overcome several drawbacks and reach the requirements and robustness data required.

In this work, two widely-used hybrid instruments, QTRAP® and LTQ-Orbitrap®, have been investigated to achieve both accurate and reliable target mycotoxins monitoring in wheat-based baby foods, as well as to find non-target and unknown mycotoxins.

On the one hand, triple quadrupole-linear ion trap-mass spectrometry or QTRAP® was born in the last decade; this instrument is a hybrid linear ion trap triple quadrupole in which the last quadrupole is replaced by a linear ion trap (LIT). The ion trap is capable of 3 levels of fragmentation (MS^3) as well as high sensitivity scan, besides the instrument is able to operate like a triple quadrupole or hybrid running, such as information dependent acquisition (IDA) method [19]. Most often, QTRAP® instrument has been exclusively used as triple quadrupole for mycotoxins analysis [20,21]. The analytical methods developed in these works had basically confirmatory purposes, fulfilling Commission Decision

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2002/657/EC. The methods had several advantages: both of them were rapid, accurate and selective working in triple quadrupole mode, but the applicability of hybrid mode was not studied.

Focus on the analysed matrix, the methods have been commonly applied for the establishment of monitoring programs for mycotoxins analysis in different types of cereals [2,3,19–21]. In the particular case of baby foods, they have been exclusively studied for concrete groups of mycotoxins using triple quadrupole mass spectrometers. For example, the literature shows methods for aflatoxins and ochratoxin A (OTA) [7], as well as fumonisins [8]. Thereby, a multi-mycotoxin method for baby food analysis has not been developed until this moment, and neither the applicability QTRAP® working in hybrid mode has been studied.

On the other hand, hybrid linear ion trap-high resolution mass spectrometry or LTQ-ORBITRAP® has recently appeared combining Orbitrap analyzer with an external accumulation device such as a linear ion trap, making possible multiple levels of fragmentation (MS^n) for the elucidation of analyte structure. The use of the LTQ Orbitrap allows high-quality accurate mass and acquisition of MS^n spectra [22,23]. Focus on mycotoxin analysis by Orbitrap® technology, it has not been commonly used for routine analysis. It could be due to this technology is recent, even so it has been just applied to cereals and beer [23–25]. However, this technology has been never applied to baby food analysis and it has not been evaluated against other hybrid instrument. Previous work carried out a first approach for determining 31 mycotoxins in grain comparing triple quadrupole with Orbitrap instrument [10]. The authors concluded that one of the major advantages of the high resolution full scan method is the possibility of screening unknown compounds, however the best sensitivity was obtained with triple quadrupole instrument.

This paper highlights the advantages, limitations and applicability of these two instruments and their validation to be applied for mycotoxins analysis in baby food. Since our knowledge, it is the first time that these two hybrid instruments (in the hybrid mode detection) are compared in the field on mycotoxins analysis in this food matrix.

2. Materials and methods

2.1. Reagents and materials

Acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Solid-phase used for matrix solid-phase dispersion (MSPD) extraction was Sepra C18-E (50 μm, 65 Å) endcapped silica-based C_{18} from Phenomenex (Torrance, USA). Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, USA). All solvents were passed through a 0.45 μm cellulose filter purchased from Scharlau (Barcelona, Spain). Analytical grade reagent formic acid (purity > 98%), and ammonium formate were obtained from Panreac Quimica S.A.U. (Barcelona, Spain).

The standards of aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), OTA, sterigmatocystin (STER), α-zearalenol (ZOL), zearalenone (ZEN), nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), diacetoxyscirpenol (DAS), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), beauvericin (BEA) were purchased from Sigma Aldrich (Madrid, Spain). T-2 toxin (T-2) and HT-2 toxin (HT-2) stock solutions (in acetonitrile) were purchased from Biopure referenzsubstanzen GmbH (Tulln, Austria). Fumonisin B_3 (FB_3) was supplied by the PROMEC unit (Programme on Mycotoxins and Experimental Carcinogenesis, Tygerberg, South Africa).

The stock solutions of aflatoxins (AFs) and OTA at 500 μg mL^{-1} were prepared in acetonitrile and STER, ZOL, ZEN, NIV, DON, 3-ADON, FB_1, FB_2, BEA were prepared at the same concentration in methanol. Stock solutions of DAS, FB_3, T-2 and HT-2 at 100 μg mL^{-1} were prepared in acetonitrile. All these standard solutions were kept in safety conditions at −20 °C.

All other working standard solutions were prepared immediately before use by diluting the stock solution with methanol/water (50/50, v/v).

2.2. Samples

Baby food samples (wheat-based) were purchased from different stores from Valencia (Spain) and Cork (Ireland) and kept at −20 °C in a dark and dry place. A wide range of brands and retailers, including pharmacies, supermarkets and smaller shops, were covered in order to ensure that the survey was representative of the baby food industry. The entire commercial samples were homogenized, and 200 g of subsample was collected in a plastic bag and stored under the same conditions until analysis [15]. A total of 25 samples of wheat-based baby foods were bought and analysed.

2.3. Extraction procedure

Sample preparation was optimized in a previous study [3]. A MSPD extraction method was applied to wheat-based baby foods. Samples (200 g) were prepared using an Oster® food processor (Professional Series Blender model BPST02-B00), mixing the sample thoroughly. Homogenized and representative portions of 1 g were weighed and placed into a glass mortar (50 mL) and were gently blended with 1 g of C_{18} for 5 min using a pestle, to obtain a homogeneous mixture. The homogeneous mixture was introduced into a 100 mm × 9 mm i.d. glass column, and eluted dropwise with 15 mL of elution solvent which was a mixture of acetonitrile/methanol (50/50, v/v) at 1 mM ammonium formate by applying a slight vacuum. Then, the extract was transferred to a 25 mL conical tube and evaporated to dryness at 35 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was reconstituted to a final volume of 1 mL with methanol/water (50/50, v/v) and filtered through a 13 mm/0.22 μm nylon filter purchased from Membrane Solutions (Texas, USA) before their injection into the liquid chromatography tandem mass spectrometry (LC–MS/MS) system.

For the preparation of fortified samples, 1 g of "blank" samples (sample in which it was corroborated before the analysis that no analytes were present) were spiked with 0.1 mL of a working mixture of mycotoxins at the appropriate concentration. Then, spiked samples were left to stand 3 h at room temperature before the extraction to allow the evaporation of the solvent and to establish equilibration between the mycotoxins and baby food sample. Ten replicates were prepared for each spiking level.

2.4. General chromatographic conditions and HPLC instrumentation

Separation of analytes was performed with a reversed-phase analytical column (Gemini C_{18}, 150 mm, 2 mm i.d, 5 μm; Phenomenex) maintained at 35 °C. As mobile phase, 5 mM ammonium formate and 0.1% formic acid in water (A) and 5 mM ammonium formate in methanol (B) were used. The gradient was as follows: at the start 5% of solvent B and after the percentage of solvent B was linearly increased to 95% in 10 min. The percentage of solvent B was kept for 5 min. Finally, the column was equilibrated to initial conditions for 10 min. The flow rate was 200 μl min^{-1} and the injection volume was 10 μl.

The 3200 QTRAP® mass spectrometer was coupled to Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA),

while LTQ-Orbitrap® was connected to Accela LC system (Thermo Scientific, Hemel Hempstead, UK).

2.5. Mass spectrometry conditions

The 3200 QTRAP® mass spectrometer (Applied Biosystems, ABSciex, Foster City, CA, USA) was equipped with a Turbo V™ Ion Spray (ESI) interface. The QTRAP® analyzer combines a fully functional triple-quadrupole and ion trap mass spectrometer within the same instrument. The analyses were performed using Turbo V™ Ion Spray in positive mode. The operation conditions for the analysis in positive ionization mode were the followings: ion spray voltage 5500 V, probe temperature 450 °C, curtain gas 20 (arbitrary units) and GS1 and GS2, 50 and 55 psi, respectively. Nitrogen served as nebulizer and collision gas. Selected reaction monitoring (SRM) experiments were carried out to obtain the maximum sensitivity for the detection of target molecules. The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) were performed by flow injection analysis for each compound and the values are summarized in Table 1, Supplementary data; entrance potential (EP) and collision cell exit potential (CXP) were set 10 and 4 V, respectively for all analytes. The mass spectrometer was operated in SRM mode and with a unit resolution for Q1 and Q3. For LC–MS/MS analysis, scheduled SRM (sSRM) was used at 50 s of SRM detection window and 1 s of target scan time, in this form was obtained more than 12 data points for all selected mycotoxins. Scheduled SRM is defined as a SRM with the amount of time for detection that surrounds the retention time for each transition.

In order to compare the performance distinctive of two operational modes of the QTRAP® triple quadrupole and triple quadrupole linear ion trap, IDA method was developed. Several experiments were carried out; the first experiment was a SRM which included the most abundant transition of the target compounds. The intensity threshold was set at 700 counts per second (cps); when intensity of the ions was arrived at the minimum, 3 enhanced product ion (EPI) scans (dependent scans) were unleashed at different collision energies (20, 35 and 50 eV). The monitoring of the sSRM ratio and the EPI scan (as an extra-information tool) were used. Analyst® version 1.5.2 software (Applied Biosystems/ABSciex) was used to control and also for data collection and analysis.

LTQ-Orbitrap® XL (Thermo Scientific) is a hybrid LIT–FT mass spectrometer. The linear ion trap (LTQ), part of the hybrid MS system, was equipped with heated electrospray interface (H-ESI), operating in positive ionization mode. Full-scan accurate mass spectra (mass range from 90 to 900 Da) were obtained at high resolution 100,000 full width at half height maximum (FWHM) and processed using Xcalibur v.2.0 and MassFrontier 7.0, both software from Thermo Scientific. The electrospray source conditions were: source voltage 4 kV, heated capillary temperature 275 °C, capillary voltage 30 V and sheat gas and auxiliar gas, 35 and 30 (arbitrary units), respectively. The mass spectrometer was operated in a data-dependent-acquisition (DDA) mode in which both MS and MS^n spectra were acquired specifying parent mass of target compounds. In this mode, the acquisition software probed the MS spectra in real-time on a full scan allowing accurate mass. The accurate mass is capable to find true unknowns since the method does not require any pre-selection of masses. The instrument is initially set to operate in full-scan mode until a parent ion appears to preset the instrument, which switches into the MS^n. The mass resolution was set at 100,000 FWHM for both screening and quantitative analysis. The products ions were generated in the LTQ trap at an optimized collision energy setting of selected mycotoxins. The scan type settings are presented in Table 2, Supplementary data. No exclusion list was used. The total cycle time depends upon the resolution; at a resolution of 100,000 FWHM the total cycle time is about 1 s. The results were used to create a (full-scan) accurate mass, both MS and MS^n, database to enable identification of compound in future screening analysis. The Orbitrap instrument was calibrated using a solution containing caffeine, MRFA, and Ultramark 1621, according to the manufacturer's instructions.

2.6. Validation method for target analysis

2.6.1. QTRAP®

The criteria applied to study the identity of mycotoxins were according to the EU requirements [16]: (i) precursor ion and two transitions were monitored, (ii) the measured retention time of the suspected peak had to correspond to the measured retention time of the standard and finally (iii) the area ratio between the two monitored SRM traces had to be equal in the sample and in the standard or matrix-matched [22]. Moreover in this work, the EPI scan (as an extra-information tool) was carried out for positives samples.

The matrix-matched calibration curves were used for effective quantitative determinations. The linearity in the response was calculated using standard solutions and matrix-matched solutions were prepared by spiking wheat-based baby food presentations in triplicate at six concentrations levels into the analytical range: from the limit of quantification (LOQ) to 100 times this LOQ. The matrix effect (ME) was calculated for each mycotoxin in baby food, as the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A); the ratio ($B/A \times 100$) is defined as the matrix effect (ME%). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%.

Recoveries ($n = 10$) were carried out by spiking wheat-based baby food at LOQ concentration level and 100 times LOQ. The precision of the method (% RSD), was estimated by the repeated analysis ($n = 10$) of a spiked wheat-based baby food at LOQ and 100 times LOQ during the same day (intra-day) and on different five days (inter-day). In order to compare the sensitivity of SRM Scheduled™ modes, the limits of detection (LODs) were calculated using spiked baby food. The LODs were determined as the lowest mycotoxin concentration whose qualified transition (q) presented a signal-to-noise ratio ($S/N \geq 3$. The quantification limits (LOQ) were determined as the minimum detectable amount of analyte with a $S/N \geq 10$ for the quantified transition (Q) (Table 1).

2.6.2. LTQ-ORBITRAP® XL

Validation of the method was performed following directive and guide on that subject [16,17]. The following parameters were studied: confirmation of identity, specificity/selectivity, linearity, limit of detection (LOD), lower limit of quantification (LLOQ), precision as repeatability and within-lab reproducibility, process efficiency and recovery.

Confirmation of identity was based on the following criteria: (i) the measured accurate mass of $[M+H]^+$ or $[M+NH_4]^+$ must fit the theoretical accurate mass with a mass tolerance set at ±5 ppm. (ii) Fragment ions obtained by ion trap must be present and their relative abundances with respect to $[M+H]^+$ or $[M+NH_4]$ ion must coincide with those of a calibration standard within ±15%. (iii) The retention time window was set to ±2% from that of a calibration standard.

Linearity was evaluated using standard solutions and matrix-matched calibrations by analyzing in triplicate six concentrations levels between LLOQ and 100 times LLOQ. Matrix effects were studied as previous section. Other analytical parameters, such as limits of detection (LODs) and lower limits of quantification (LLOQs), were determined empirically by analyzing a series of decreasing

Table 1
Maximum levels (ML) for selected mycotoxins in baby food according to EC1881/2006, EC1126/2007 and EC165/2010 Commission Regulations and limit of detection (LOD), limit of quantification (LOQ) and lowest limit of quantification (LLOQ) expressed as $\mu g\,kg^{-1}$, obtained by different mass analyzers.

Mycotoxins	ML Baby food[a]	QTRAP *Scheduled* SRM		QTRAP® IDA method (EPI mode)		ORBITRAP® Full scan DDA	
		LOD	LOQ	LOD	LOQ	LOD	LLOQ
NIV		12	45	60	150	40	70
DON	200	5	15	12	30	15	30
3-ADON		4	12	10	30	15	35
DAS		1.5	4	4	12	10	25
HT-2		1.5	3.5	3	12	7	18
T-2		0.8	2.5	2.5	8	5	12
FB1	200 ($FB_1 + FB_2$)	10	30	30	60	32	55
FB2		12	36	30	65	30	60
FB3		10	30	30	60	32	65
ZEN	20	2	8	7	20	8	18
ZOL		2	6	5	20	10	25
BEA		1	3	3	8	5	12
AFB1	0.1	0.2	0.45	0.5	2	3	7
AFB2		0.25	0.75	0.8	3	4	8
AFG1		0.25	0.75	0.8	3	5	8
AFG2		0.25	0.75	0.8	3	4	8
STER		0.5	1.5	0.8	3	5	9
OTA	0.5	0.15	0.45	0.5	2.25	3	7

[a] Maximum level (ML) [12–14].

concentrations of the wheat baby food in multiple replicates ($n = 3$), using three different calibration lines. The LOD was the concentration at which the analyte response could be identified with relative standard deviation (% RSD) and mean relative error (MRE) (MRE% defined as [measured concentration − nominal concentration/nominal concentration × 100]) > 20% and ≤30%. The LLOQ was defined as the lowest concentration that could be quantified with RSD% and the absolute value of MRE% ≤ 20% [23]. LODs and LLOQs are listed in Table 1. The recovery experiments were carried out by spiking the sample in ten replicates at two concentration levels, LLOQ level and 100 times LLOQ level. In the same way, precision of the method was determinate in fortified wheat powered baby food at LLOQ level and 100 times LLOQ level and calculated as RSD of measurements in ten replicates during the same day and five non-consecutive days.

3. Results and discussion

3.1. Validation study

The methods were validated according to the criteria specified in Commission Decision 2002/657/EC for quantitative confirmation method [16]. The specificity of the methods was demonstrated by the analysis of "blank" baby food samples (samples without analytes) and fortified samples (samples on a mixture of mycotoxins at known concentration was spiked).

Characteristic values of performance, including limits of detection (LODs) and quantification (LOQs), recoveries; intra-day and inter-day precision were obtained in fortified samples at appropriate concentration level by ten replicates. The LODs and LOQs in $\mu g\,kg^{-1}$ for the methods are summarized in Table 1. The analytical parameters of the methods are summarized in Table 2 for QTRAP® and Table 3 for LTQ-Orbitrap®. These limits were between 3 and 20 fold better by QTRAP® instrument when sSRM method was used: this implies that for each compound one specific product ion was selected for quantifying and a second product ion was used for confirmation. The principle to the *scheduled* SRM is to monitor these transitions increasing the time that is available for acquiring one data point. It was therefore observed an enhancement of the selectivity and consequently improvement on LODs and LOQs.

These limits are an important point in this work, since maximum levels (MLs) established for mycotoxins in baby foods are more restrictive than other foodstuff [12–15]. In this way, QTRAP® system reached these MLs for all selected mytcotoxins when mass analyzer worked in sSRM mode with the exception of AFB_1. When the instrument were working in hybrid mode (IDA method) reached LODs and LOQs, which were higher than in sSRM mode, and this mode did not fulfil established MLs for OTA and aflatoxins. LTQ-Orbitrap® system did not fulfil MLs for aflatoxins and OTA.

For this reason, some authors have preferred to limit to a particular group of mycotoxins. For example, a sensible analytical method has been recently developed for AFs and OTA in baby food using ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS). Remarkable LOQs were reached lower than $25\,ng\,kg^{-1}$ and excellent accuracy was obtained [7]. On the other hand, a method for fumonisins was successfully developed reaching low LOQs: $2\,\mu g\,kg^{-1}$ for FB_1 and FB_2, and $5\,\mu g\,kg^{-1}$ for FB_3 [8]. Usually, a multi-mycotoxin method is a compromise of several parameters. The structural variability of mycotoxins is the main problem for their simultaneously extraction and detection.

In our research, this compromise was observed in different ways. LOQs were fulfilled for selected mycotoxins except AFB_1 when QTRAP® worked as a triple quadrupole and AFB_1 and OTA when QTRAP® worked in hybrid mode. As the number of compounds is increased, more complex is the analytical method. However, the LOQs reached in this study were according to recent multi-mycotoxins methods which were applied to cereals [2,3,19–21].

Focus on Orbitrap®, there is not available bibliography about baby food analysis. One study compared the Orbitrap® technology with triple quadrupole instrument [10]. This work carried out the validation for triple quadrupole instrument, but it was not validated the method for Orbitrap®. Even so, the LODs for Orbitrap® ranged from 4 to 2000 $\mu g\,kg^{-1}$. The authors concluded that the high resolution full scan method could be used for screening unknown compounds, while the best sensitivity and quantification were obtained by triple quadrupole.

Step by step, new analytical methods have appeared in this way. These methods are completely focused on Orbitrap® technology; beer was the starting point and two multi-mycotoxin methods were developed. The first method used Exactive Orbitrap®; in this case the LLOQs ranged from 0.5 to 65 μg/L [24]. The second one was developed using the LTQ-Orbitrap®; LLOQs ranged from 12 to 155 μg [23]. The difference between these instruments is the presence of LIT, which could be used to confirm the compounds by

Table 2
QTRAP® validation parameters: matrix effect, low and high recovery levels (values (%)) and relative standard deviations (RSD, %) given in brackets calculated at two concentration levels ($\mu g\,kg^{-1}$).

Mycotoxin	Matrix effects[a] (%)	Intra-day[d]		Inter-day[e]	
		Low level[b]	High level[c]	Low level[b]	High level[c]
NIV	65	81 (9)	75 (10)	80 (12)	78 (12)
DON	75	83 (7)	87 (7)	84 (8)	82 (8)
3-ADON	60	75 (8)	71 (5)	72 (7)	75 (4)
DAS	70	74 (7)	79 (3)	73 (6)	74 (7)
HT-2	83	79 (8)	73 (5)	74 (7)	74 (7)
T-2	85	71 (6)	72 (4)	73 (5)	73 (11)
FB1	95	95 (10)	92 (14)	94 (16)	93 (15)
FB2	98	91 (12)	93 (15)	95 (15)	92 (14)
FB3	96	95 (10)	95 (15)	94 (12)	93 (16)
ZEN	80	76 (5)	73 (4)	75 (5)	74 (9)
ZOL	77	78 (7)	75 (9)	76 (6)	73 (6)
BEA	68	71 (8)	69 (5)	73 (5)	74 (9)
AFB1	49	73 (4)	70 (8)	77 (7)	78 (11)
AFB2	52	77 (4)	73 (9)	74 (6)	76 (12)
AFG1	56	72 (5)	69 (7)	75 (4)	73 (9)
AFG2	55	78 (5)	75 (7)	73 (7)	72 (10)
STER	69	71 (5)	72 (6)	72 (4)	71 (6)
OTA	81	78 (6)	72 (4)	72 (5)	75 (9)

[a] ME %: slope matrix matched sample/slope standard in solvent × 100.
[b] Low level: LOQ level ($\mu g\,kg^{-1}$).
[c] High level: 100 times LOQ level ($\mu g\,kg^{-1}$).
[d] Number of replicates: 10.
[e] Different days: 5.

fragmentation study. In our study the LLOQs were ranged from 7 to 70 $\mu g\,kg^{-1}$ being LLOQs according to recent works [23–25].

The calibration curves for each compound by both methods were established using matrix-matched from LOQ to 100 times LOQ for QTRAP® and LLOQ to 100 times LLOQ for LTQ-Orbitrap® system. Linear regression analysis was performed by plotting peak area ratios versus analyte concentrations using a least-square linear regression mode. The linearity was acceptable for all analytes in the whole range of tested concentrations, as proved the correlation coefficients (r) upper than 0.991 values for all curves in the case of the QTRAP® mass spectrometer and upper than 0.989 for the LTQ-Orbitrap® system.

Matrix effects calculated in percentages, as it has previously been described above, were similar on both instruments (Tables 2 and 3), although ME (%) were slightly higher on LTQ-Orbitrap® system than on QTRAP® instrument. In the first one, the most striking fact was the enhancement observed in fumonisins, while in the second one was curious the suppression resulted in the detection of aflatoxins. These matrix effects should be compensated by using appropriate calibration method. In this study, on both methods, external matrix-matched calibration showed to be effective in compensation of matrix effects.

The intra-day and inter-day precisions of the methods were evaluated on spiked wheat-based baby food at two different concentration levels (LOQ and 100 times LOQ). The RSD values for

Table 3
LTQ-ORBITRAP XL validation parameters: matrix effect, low and high recovery levels (values (%)) and relative standard deviations (RSD, %) given in brackets calculated at two concentration levels ($\mu g\,kg^{-1}$).

Mycotoxin	Matrix effect[a] (%)	Intra-day[d]		Inter-day[e]	
		Low level[b]	High level[c]	Low level[b]	High level[c]
NIV	60	77 (10)	81 (11)	78 (10)	78 (12)
DON	63	83 (11)	78 (9)	81 (10)	81 (9)
3-ADON	59	84 (11)	80 (14)	81 (9)	82 (9)
DAS	62	74 (9)	72 (8)	73 (11)	78 (9)
HT-2	63	71 (8)	72 (9)	71 (7)	76 (11)
T-2	69	68 (10)	69 (10)	71 (8)	68 (11)
FB1	112	79 (11)	82 (11)	77 (19)	81 (12)
FB2	110	89 (15)	86 (14)	83 (18)	85 (15)
FB3	123	82 (12)	86 (12)	72 (11)	77 (12)
ZEN	79	75 (11)	77 (13)	73 (14)	71 (12)
ZOL	88	75 (8)	76 (8)	71 (15)	75 (12)
BEA	64	69 (15)	66 (18)	70 (13)	70 (19)
AFB1	60	72 (7)	78 (7)	69 (6)	77 (9)
AFB2	54	67 (10)	70 (11)	73 (13)	71 (9)
AFG1	51	71 (8)	73 (7)	74 (12)	72 (9)
AFG2	56	70 (8)	73 (9)	69 (11)	71 (10)
STER	66	69 (11)	73 (6)	72 (12)	71 (9)
OTA	77	71 (6)	70 (12)	78 (9)	73 (8)

[a] ME %: slope matrix matched sample/slope standard in solvent × 100.
[b] Low level: LLOQ level ($\mu g\,kg^{-1}$).
[c] High level: 100 times LLOQ level ($\mu g\,kg^{-1}$).
[d] Number of replicates: 10.
[e] Different days: 5.

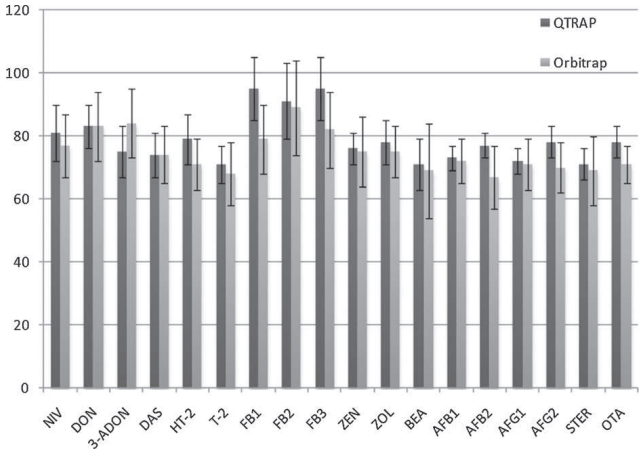

Fig. 1. Recoveries (%) and RSDs (*y*-error bars) at the LLOQ concentration level (LTQ-Orbitrap) of selected mycotoxins in baby food obtained by QTRAP and LTQ-ORBITRAP instruments.

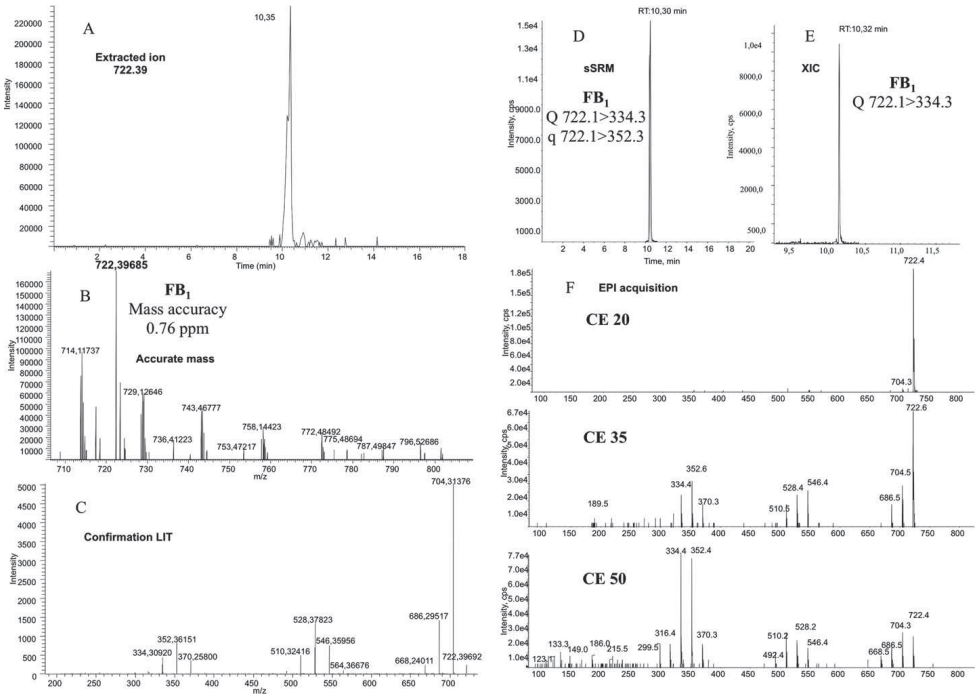

Fig. 2. Analysis of FB1 mycotoxin in a spiked wheat-based baby food at 75 μg kg-1 by LTQ-Orbitrap® (A–C) and by QTRAP® (D–F). Extracted ion 722.39 (A), accurate mass FB1 (B) and confirmation by ion trap (C). Schedule SRM transitions (D), XIC from the TIC (E) and EPI spectrum at different collision energy voltage (F).

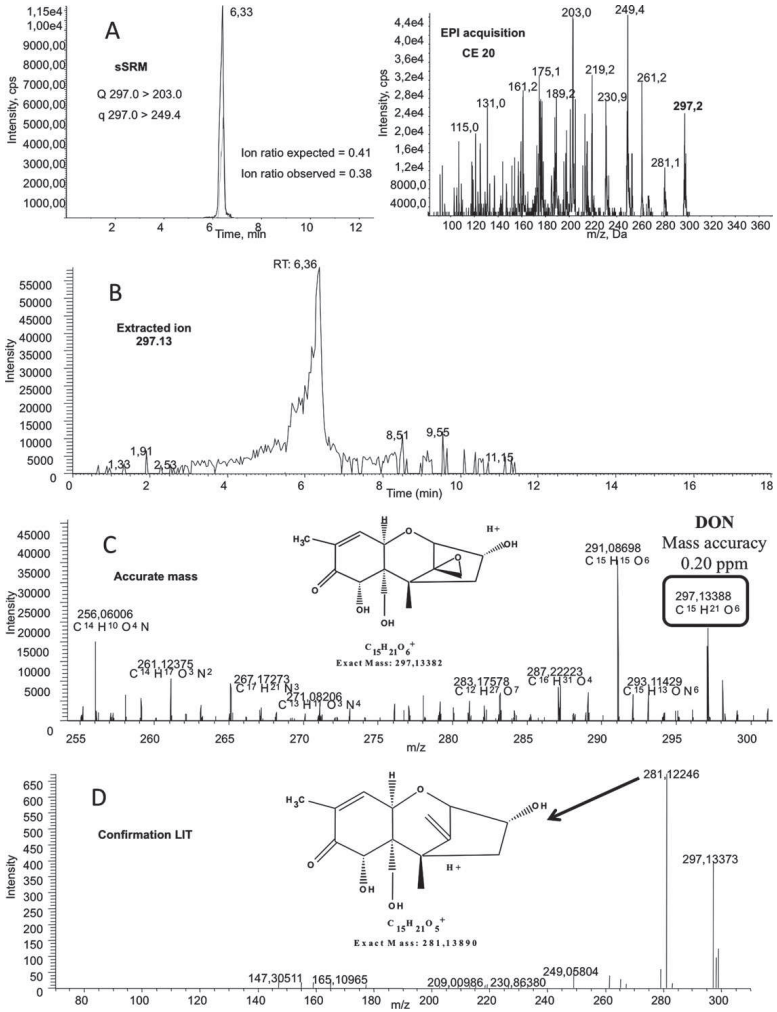

Fig. 3. Positive sample: DON. Figure (A) shows the sSRM chromatogram and EPI acquisition mode (20 eV) obtained in QTRAP®. Figures (B–D) show detection and confirmation using LTQ-Orbitrap®. Figure extracted ion 297.13 (B), accurate mass DON (C) and confirmation by linear ion trap (D).

intra-day analyses were in the range of 3–15% and the RSD for inter-day values ranged from 4 to 16%, showing good reproducibility for QTRAP® instrument (Table 2). In the same way, using the LTQ-Orbitrap® mass spectrometer, RSD values for intra-day analyses were in the range of 6–18% and the RSD for inter-day values ranged between 6 and 19% (Table 3). Although the RSD values obtained by LTQ-Orbitrap® technology were slightly higher than for QTRAP®, they were considered satisfactory.

The mean recovery values at LOQ and 100 times LOQ spiked levels ranged between 71–95% and 69–95%, respectively by QTRAP® (Table 2) and between 67 and 89% for LLOQ concentration level and 66–86% for 100 times LLOQ concentration level using LTQ-Orbitrap® (Table 3). Fig. 1 shows the recoveries and RSDs obtained at LLOQs concentration level (Table 1) of selected mycotoxins in baby food obtained by QTRAP® and LTQ-Orbitrap® instruments, showing acceptable and very similar values for both instruments.

In light of these results, soft differences were observed between the compared analyzers. When the linearity was studied, QTRAP® provided a slightly better linear response than LTQ-Orbitrap®. In the same way, LTQ-Orbitrap® evidenced higher matrix effects;

this fact can be explained since it is generally assumed that the specificity afforded by sSRM mode discriminates between target mycotoxins and matrix components. Results showed that accuracy was better in the QTRAP® system. The recoveries obtained were similar in both systems, which is normal due to the recoveries mainly depends on the extraction procedure and not on the determination systems. However, the differences in the RSDs between both analyzers gave an idea of the quantification accuracy.

To demonstrate the differences and similarities in the mass spectra, Fig. 2 depicts the analysis of FB_1 mycotoxin in a spiked wheat-based baby food at 75 $\mu g\,kg^{-1}$ by QTRAP® and by LTQ-Orbitrap®. On the one hand, the LTQ-Orbitrap® is able to acquire a full-scan (A), as well as fragmented ions under data-depending acquisition (C), which can be acquired in a single Orbitrap mass spectrum [22,26,27]. However, QTRAP® product ion mass spectra are generated using Q1 as a resolving RF/DC transmission quadrupole to select the precursor ion of interest. This precursor ion is then accelerated into the pressurized collision cell inducing fragmentation and the resulting fragment and residual precursor ions are transmitted into the Q3 linear ion trap (LIT) where they are mass selectively scanned out toward the detector while the Q3 LIT is performing the mass scan ions can be accumulated in Q0 further enhancing instrument duty cycle. This scan is referred as an EPI scan (F) [28].

In this study was observed an interesting difference between instruments in terms of qualitatively different products ions obtained and relative abundances of these fragments. This can be explained considering the different mechanisms of ion isolation and fragmentation, previously explained. Although the fragment ions obtained were the same in both instruments, their abundances were not the same. In fact, in the LTQ-Orbitrap® spectra it was observed that the main fragment ion for FB_1 was m/z 704.3136 that corresponds to the lost of water molecule. It can be assumed that the fragmentation mechanism in the LTQ-Orbitrap® is softer than in the QTRAP®, whose main fragments were m/z 334.4 and m/z 352.4.

3.2. Application to baby food samples

Once the proposed methods were optimized and validated, the two mass analyzers were applied for monitoring 18 mycotoxins in a total of 25 commercial baby-food samples. All the samples were analysed by HPLC-QTRAP®-MS and HPLC-LTQ-Orbitrap®-MS. Samples in which mycotoxins were detected (positive samples), an extra confirmation tool was carried out by IDA method.

After the analysis of all the samples, only one sample was positive for DON. Fig. 3 shows the chromatogram in both instruments. Fig. 3A shows the chromatogram in sSRM, showing two selected transitions and the ion ratio expected and observed according to EU guidelines [16]. This analyzer allowed obtaining a spectrum of second generation, EPI method, increasing the number of identification points (IPs) and the identification was unambiguous (Fig. 3A). The calculated concentration was $60.1 \pm 3.8\ \mu g\,kg^{-1}$ ($n = 5$).

This sample was also analysed by the LTQ-Orbitrap® and DON was detected. Fig. 3B shows an extract ion of DON, showing adequate retention time and calculated concentration was $57.8 \pm 5.3\ \mu g\,kg^{-1}$ ($n = 5$), insignificant difference with QTRAP® quantification was observed. The error mass was 0.2 ppm (Fig. 3C) confirming the presence of this mycotoxin in this sample. Besides, the ion-trap showed DON pathway for the sample completely equal to the standard obtained (Fig. 3D). DON could be fragmented by the ion-trap generating the deepoxy-deoxynivalenol metabolite known as DOM-1, meat the loss of epoxy group.

In order to enlarge the capability of the method, a simple strategy, described in previous work [23], is followed for the identification of target and non-target analytes in the samples. At this regard it should be pointed out that sample preparation itself implies some selection of recovered analytes.

In this context, all the samples were analysed on looking for emergent micotoxins, such as enniatins A, B, A_1, B_1 and fusaproliferin as it has been done in a previous work [23]. MassFrontier 7.0 was used as complementary software in order to identify these compounds and unknowns in the 25 samples. No one non-target mycotoxin was found in any sample.

4. Conclusions

Two hybrid instruments were checked to analyse mycotoxins from baby foods. On the one hand, QTRAP® working in sSRM mode allowed a reliable quantification of 18 mycotoxins from wheat-based baby food. Besides, QTRAP® working in full mass rang and using IDA method that permitted to develop EPI mode, could improve identification and confirmation, decreasing slightly LOQ levels respect sSRM mode.

On the other hand, LTQ-Orbitrap® has the ability to perform quantitative target and non-target analysis using full-scan FTMS in the instrument and it allows simultaneously target analysis in LIT. The ultra-high resolution mass was therefore used to identify target and non-target mycotoxins and LIT was valuable for analyte confirmation. Thereby, all the samples were analysed by HPLC-LTQ-Orbitrap® in order to find the presence of other non-targets mycotoxins as enniatins and fusaproliferin.

No one of non-target mycotoxin was found in the samples.

In conclusion, QTRAP® instrument is more suitable for quantitative purposes and it allows extra information by IDA methods for unambiguous identification. It allows an increase of identification points. Nevertheless, LTQ-Orbitrap® has other advantage: in addition to quantification of mycotoxins from baby food, the ultra-high resolution mass could identify non-target and unknowns mycotoxins. This potential comes from the ultra-high resolution mass allowing an exact mass accuracy. Moreover, this instrument allows a retrospective data analysis, which means that from Full-Scan it could be studied. Thereby, the extracted ion chromatogram of a specific analyte could be processed after the chromatogram has been acquired.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.12.039.

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4.12 Rapid mycotoxins analysis in human urine. A pilot study.

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Rapid mycotoxin analysis in human urine: A pilot study

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ABSTRACT

A simple and rapid method effective for quantitative determination of deoxynivalenol (DON), T-2 toxin (T-2), HT-2 toxin (HT-2), zearalenone (ZEN), ochratoxin A (OTA), aflatoxins (AFs) B_1, B_2, G_1 and G_2 and fumonisins FB_1 and FB_2 in urine was developed. The urine was diluted with phosphate buffer solution (PBS) and thoroughly mixed. For clean-up and extraction, the mixture was loaded on a MYCO 6in1™ IAC. Hybrid triple quadrupole-linear ion trap mass spectrometer (QTrap®) was used for the detection. Extra tools for confirmation of selected mycotoxins in positive samples, Information Dependent Acquisition (IDA) experiments, were also developed. The use of immunoaffinity columns followed by the LC–MS/MS analysis showed acceptable average recoveries between 83% and 116% and reached acceptable precision values (relative standard deviation (RSD) ⩽ 14%). In a pilot study with 27 volunteers, OTA, DON and AFG_2 were detected. However, this study needs to be extended in order to understand the relation between the mycotoxins intake and mycotoxin levels in human urine.

1. Introduction

Certain genera of toxigenic fungus might produce mycotoxins (Tournas and Uppal Memon, 2009; Richard et al., 2007). These mycotoxins arrive to humans by different ways; the most frequent one is from food. For this reason, efforts have been normally made by researchers and by national authorities to assess mycotoxin incidence in food determining the contamination levels of raw food material or derivate products (Rubert et al., 2011; Zinedine et al., 2010; Villa and Markaki, 2009; Vishwanath et al., 2009; Tam et al., 2006). However, variations in food processing, food intake, contamination levels, intestinal absorption, toxin distribution and excretion lead to individual variations in toxin levels (Bullerman and Bianchini, 2007; Speijers and Speijers, 2004). For this reason, the study of the metabolism and evaluations of mycotoxins in biological fluids have been given increasing attention since the results may offer valuable indications on the real risk for consumers.

There are two possible situations: the metabolism of the selected mycotoxin is well-established with extensive literature or, on the other hand, there are almost no studies of the metabolisms.

For example, the metabolism of OTA, DON and fumonisins has been widely studied. Gilbert et al. (2001) showed the correlation between urinary OTA concentration and dietary intake appears to be stronger than the corresponding relationship between plasma OTA level and intake. In fact, several authors have detected OTA in human urinary samples (Muñoz et al., 2010; Manique et al., 2008; Pena et al., 2006).

In the same way, the metabolism of DON has gained importance in the last years, and there are several studies that relate DON intake to urinary DON level. Turner et al. (2009, 2011), suggested that un-conjugated DON can persist and it can be excreted in urine. For fumonisins, based on the absence of measurable metabolites, FB_1 has been suggested as a biomarker (Xu et al., 2010; Shephard et al., 2007). However, only when the exposure level is very high, FB_1 could be detected in urine (Ahn et al., 2010; Silva et al., 2010).

Nowadays, studies on the metabolism of several mycotoxins, such aflatoxins, are relatively scarce. Moreover, the uncertainness exists, since it has been related individual difference in the enzymatic system. However, some authors have developed different procedures to determine these mycotoxins in urine in order to relate their presence to a high exposure. In this way, Thieu and Pettersson (2009) found ZEN, DON and AFB_1 and AFB_2 in urine from pigs. In the same way, but in humans, Polychronaki et al. (2008) suggested that the occurrence of AFB_2 and AFG_2 in several urine samples from Guinean children could come from the conversion of both AFB_1 and AFG_1 in the liver.

T-2 toxin is rapidly absorbed after it ingestion, and the fraction eliminated as unmodified compound in urine is negligible. There are significant differences between species in the metabolic pathways of T-2 toxin, however, it is well-known that it main metabolite is HT-2. No studies have been conducted with humans to assess the presence of T-2 or its metabolites in biological fluids (Cano-Sancho et al., 2010).

In this work the immunoaffinity columns (IAC) MYCO 6in1™ followed by LC–QTRAP–MS/MS was applied to the analysis of 11 mycotoxins in human urine samples. The objective was to evaluate the occurrence of 11 un-metabolized mycotoxins in the Valencian

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population, in order to evaluate human exposure and it risk. This study is a preliminary study since it needs to be extended in the future to understand the relation between the mycotoxins intake and mycotoxin levels in urine, both metabolized and un-metabolized.

2. Materials and methods

2.1. Chemicals and reagents

HPLC grade acetonitrile and methanol were supplied by Merck (Darmstadt, Germany). Deionized water (>18 MΩ cm^{-1} resistivity) was purified using Milli-Q® SP Reagent water system plus from Millipore Corp. (Bedford, USA). Analytical grade reagent formic acid (purity >98%) and ammonium formate were purchased from Panreac Quimica S.A.U. (Barcelona, Spain).

The individual stock solutions of AFB_1, AFB_2, AFG_1, AFG_2, OTA at concentration of 500 μg ml^{-1} were prepared in acetonitrile and DON, ZEN, FB_1, FB_2 were prepared at the same concentration in methanol; all these standards were purchased from Sigma–Aldrich (Madrid, Spain). T-2 and HT-2 toxin stock solutions in acetonitrile at concentration of 100 μg ml^{-1} were purchased from Biopure referenzsubstanzen GmBH (Tulln, Austria). All the individual working solutions were kept in safety conditions at −20 °C.

Immunoaffinity columns MYCO 6in1™ were from Vicam (Watertown, USA). Phosphate buffer solution (PBS) was prepared from PBS buffer tablets purchased from Sigma–Aldrich (Madrid, Spain).

2.2. Sampling

In this pilot study, 27 healthy volunteers were asked to provide urine samples. Samples were collected from a group of 17 male and 10 female (The ages of all the volunteers ranged between 21 and 77 years old) during September and November 2010 in Valencia (Spain). No ingestion or administration was given to the subjects, for what, this research project did not involve any human risk for the participating subjects. However, the collection of human urine was according to Declaration of Helsinki and it was based only in the evaluation of selected mycotoxins at concentration in the urine. For this reason, all participants signed an informed consent before sampling.

Since it is the most concentrated, first morning urines were collected in sterile urine beakers from each individual and stored at −20 °C until analysis.

The samples with undetectable levels of mycotoxins were used for spiking and recovery studies.

2.3. Extraction and clean-up

The extraction procedure of selected mycotoxins from urine was done according to Silva et al. (2010) that reported an extraction method for FB_1 and FB_2 in human urinary samples. In this study some modifications were done. An aliquot of human urine sample was filtered through a Whatman No. 541 filter paper. Afterwards, 10 ml were diluted with 10 ml of PBS (pH 7.4) and thoroughly mixed for 3 min with a vortex mixer. For clean-up, the mixture was loaded on a MYCO 6in1™ immunoaffinity column attached onto a vacuum manifold with a flow rate of about 1 drop s^{-1}. The column was washed with 10 ml of PBS, after the cartridges were dried for 30 min. Eleven selected mycotoxins were then slowly eluted with 5 ml of a mixture of methanol/acetonitrile (50/50) (v/v). After, the extract was transferred to a 15 ml conical tube and evaporated to dryness at 50 °C with a gentle stream of nitrogen using a multi-sample Turbovap LV Evaporator (Zymark, Hoptkinton, USA). The residue was then reconstituted to a final volume of 1 ml with a mixture of methanol/water (50/50) (v/v) and filtered through a 13-mm/0.45-μm nylon filter purchased from Membrane Solutions (Texas, USA) before their injection into the LC–MS/MS system.

2.4. Equipment

LC–tandem MS analyses were carried out in a system consisting of a Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) coupled to a 3200 QTrap® mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a TurboV™ ionspray (ESI) interface. Separation of analytes was performed using a Gemini C_{18} (Phenomenex, 150 mm × 2 mm, 3 μm of particle size) LC-column preceded by a guard column with the same packing material (3 μm of particle size). The flow rate was set to 0.2 ml min^{-1} and the oven temperature was 35 °C, being eluent A water slightly acidified with 0.1% of formic acid and 5 mM ammonium formate, and eluent B methanol with 5 mM ammonium formate. The elution gradient started with 75% of eluent B, increasing to 100% B in 10 min. During a further 3 min the column was cleaned and readjusted to the initial conditions and equilibrated for 7 min. The volume injection was 20 μl.

The operation conditions for the analysis in positive mode were the followings: Ion spray voltage 5500 V, curtain gas 12 (arbitrary units), GS1 and GS2, 50 and 60 psi, respectively, probe temperature (TEM) 500 °C. Nitrogen served as nebulizer and collision gas. The optimization of MS parameters as declustering potential (DP), collision energy (CE) and collision cell entrance potential (CEP) were performed by flow injection analysis for each compound; entrance potential (EP) and collision cell exit potential (CXP) were set at 10 and 4 V, respectively, for all analytes. For LC–MS/MS analysis, scheduled single reaction monitoring (sSRM) was used with 45 s of SRM detection window and 2 s of target scan time. All the optimized parameters are presented in Table 1.

Analyst version 1.5.1 software (Applied Biosystems/AB Sciex) was used to control all components of the system and also for data collection and analysis. In order to compare the performance distinctive of two operational modes of the QTrap® (triple quadrupole and triple quadrupole linear ion trap) IDA method was developed. Several experiments were carried out; the first experiment was a selected reaction monitoring (SRM) which included the most abundant transition of the target compounds. The intensity threshold was set at 500 counts per second (cps), when intensity of the ions was arrived at the minimum 3 enhanced product ion (EPI) scans (dependent scans) were unleashed at different collision energies (20, 35 and 50 eV). The monitoring of the SRM ratio and the enhanced product ion (EPI) scan (as an extra-information tool) was used for confirmation of the positives findings.

Table 1
SRM optimized parameters for selected mycotoxins and their genera producers.

Genera producer	Mycotoxin	Retention time	Precursor ion (m/z)	Product ion	DP (V)	CE (eV)	CEP
Fusarium	DON	2.1	297	175^{Q}	36	81	18
			$[M+H]^+$	114^{q}		51	
	HT-2	4.9	442	215^{Q}	31	19	18
			$[M+NH_4]^+$	105^{q}		57	
	T-2	6.9	484	215^{Q}	36	23	20
			$[M+NH_4]^+$	185^{q}		27	
	FB_1	4.8	722	334^{Q}	101	51	26
			$[M+H]^+$	352^{q}		45	
	FB_2	7.9	706	336^{Q}	131	49	18
			$[M+H]^+$	318^{q}		51	
	ZEN	8.1	319	301^{Q}	46	13	20
			$[M+H]^+$	187^{q}		25	
Aspergillus	AFB_1	3.0	313	241^{Q}	76	43	22
			$[M+H]^+$	128^{q}		87	
	AFB_2	2.8	315	259^{Q}	60	40	32
			$[M+H]^+$	288^{q}		40	
	AFG_1	2.6	329	200^{Q}	81	53	22
			$[M+H]^+$	243^{q}		35	
	AFG_2	2.5	331	217^{Q}	50	43	20
			$[M+H]^+$	189^{q}		43	
Aspergillus and *Penicillium*	OTA	7.9	404	239^{Q}	60	40	14
			$[M+H]^+$	102^{q}		100	

Q, quantification transition; q, qualification transition.

Table 2
Linearity, matrix effect, limits of quantification (LOQs), recovery values (%) and relative standard deviations (%) given in brackets calculated at two concentration levels (ng ml-1).

Compound	Linearity range (ng ml^{-1})	Correlation coefficient (r^2)	Matrix effect (%)[a]	LOD (ng ml^{-1})	LOQ (ng ml^{-1})	Intraday[b]		Interday[c]	
						Low level (LOQ)	High level (100 × LOQ)	Low level (LOQ)	High level (100 × LOQ)
DON	35–3500	0.9982	92	10	35	108 (8)	106 (3)	101 (9)	108 (5)
HT-2	10–1000	0.9993	91	3	10	97 (9)	96 (7)	96 (9)	96 (8)
T-2	6–600	0.9995	89	2	6	92 (4)	89 (6)	91 (6)	90 (5)
FB_1	15–1500	0.9953	103	5	15	92 (6)	93 (5)	91 (7)	91 (5)
FB_2	15–1500	0.9992	101	4	15	89 (9)	90 (5)	85 (10)	91 (8)
ZEN	10–1000	0.9993	105	3	10	88 (10)	89 (8)	83 (14)	85 (9)
AFB_1	1.5–150	0.9994	98	0.5	1.5	109 (12)	108 (9)	103 (12)	102 (7)
AFB_2	1.2–120	0.9978	94	0.4	1.2	112 (11)	116 (9)	105 (12)	113 (8)
AFG_1	1.2–120	0.9995	101	0.4	1.2	102 (13)	115 (7)	98 (10)	115 (7)
AFG_2	2–200	0.9990	95	0.8	2	89 (12)	91 (9)	86 (13)	90 (6)
OTA	1.5–150	0.9997	95	0.5	1.5	94 (4)	89 (3)	90 (5)	83 (9)

[a] ME%: slope matrix matched urine/slope standard in solvent × 100.
[b] Number of replicates: 10.
[c] Different days: 5.

2.5. Validation method

Linearity was evaluated using standard solutions by analyzing in triplicate six concentrations levels. The matrix effect (ME) for each analyte in urine was calculated, defined as the percentage of the matrix-matched calibration slope (B) divided by the slope of the standard calibration in solvent (A). Thus, the ratio (B/A × 100) is defined as the absolute matrix effect (ME%). A value of 100% indicates that there is no absolute matrix effect. There is signal enhancement if the value is >100% and signal suppression if the value is <100%.

The recovery experiments were carried out by spiking the blank urine sample in ten replicates at two concentration levels LOQ and 100 times LOQ (showed Table 2). In this way, intraday and interday parameters of the method were determined at LOQ and 100 times LOQ concentration levels by repeat the analysis of urine samples in ten replicates the same day and five non-consecutive days.

Other analytical parameters, such as limits of detection (LODs) and limits of quantification (LOQs) were defined as the concentration which the signal-to-noise (S/N) obtained was closely to 3 and 10, respectively. These limits were calculated by Analyst version 1.5.1 software (Applied Biosystems/AB Sciex) and both parameters were determined by the analysis of decreasing concentration of the spiked urine.

3. Results and discussion

3.1. Extraction and clean-up

In this study, sample preparation of extracts was performed according to Silva et al. (2010). However, in this work the number of mycotoxins analyzed was extended and, in order to improve the recoveries for them, some parameters were optimized. In a first step, it was tested the initial volume of PBS required: 10 ml of human urine was diluted with 5, 10, 15 and 20 ml of PBS, and thoroughly mixed during 3 min with a vortex mixer. The best dilution was obtained with 10 ml of urine and equal volume of PBS, since recoveries were higher than 75% and relative standard deviation (RSD, %) lower than 16%. For the clean-up, the mixture urine/PBS, was loaded on a MYCO 6in1™ immunoaffinity column attached onto a vacuum manifold with a flow rate of about 1 drop s^{-1}.

Another critical parameter using IAC is the wash-step; in this study the column was washed with 5, 10, 15 and 20 ml of PBS. 10 ml of PBS let absent interference and effective reproducibility (RSD < 14%).

The last step was to optimize the elution solvent. With this purpose, the 11 mycotoxins were slowly eluted with different solvents or mixtures of them. 3 ml of these possibilities were checked: (i) methanol, (ii) acetonitrile, (iii) methanol/acetonitrile (50/50) (v/v) and (iv) methanol/water (50/50) (v/v). The mixture of methanol/acetonitrile (50/50) (v/v) was more effective than others, due to recoveries obtained were higher than 80%. Moreover, the volume of this mixture was studied in order to improve the recoveries: 3, 4, 5 and 6 ml of elution solvent were checked. After this study, 5 ml of the mixture was selected, reaching recoveries that ranged between 83% and 116%.

3.2. Method performance

The validation process was carried out using a blank urine sample with no detectable mycotoxin.

The calibration curves showed a linear trend in the range of LOQ and 100 × LOQ with a coefficient of determination (r^2) higher than 0.9953 (Table 2).

The intraday and interday precision of the method were evaluated on spiked urine extract at two different concentration levels (LOQ and 100 times LOQ). The RSD values for intraday analyses were in the range 3–13% and the RSD for interday values were between 5% and 14%, showing good repeatability and reproducibility for IAC–HPLC–MS/MS. The mean recovery values at LOQ and 100 × LOQ spiked levels were 88–116% for the intraday test and 83–115% for the interday test (Table 2).

There are several strategies to solve matrix effects; one of them is external calibration using matrix-matched and the other one using internal standards (IS). First, in order to evaluate the presence of matrix effects, the signal suppression–enhancement (SSE) for each analyte in urine was calculated, according to the ratio (B/A × 100) explained previously in the Section 2.5. The simple sample preparation by IAC avoided matrix effect for selected mycotoxins, since these effects were not significant or negligible (Table 2). The developed method fulfilled the parameters established by EU (2002).

3.3. Qualitative analysis: IDA method

Fig. 1 depicts the IDA experiment performed for determining FB_2 in urine. Fig. 1A shows the Total Ion Chromatogram (TIC) of all SRM transitions recorded. To isolate each compound separately, their SRM transition can be extracted (extracted ion chromatogram, XIC) from the TIC (Fig. 1B). Finally, Fig. 1C presents the EPI spectrum obtained for FB_2, where the residual precursor ion and two main fragments are present. However, SRM acquisition mode was preferred for routine analyses, and IDA mode was applied only as an extra-tool to confirm positive urine samples with mycotoxins.

3.4. Application of the proposed method. A pilot study

Mycotoxins were determined in urine from 27 volunteers. FBs, ZEN, T-2, HT-2, AFB_1, AFB_2, and AFG_1 were not detected in any of the samples analyzed.

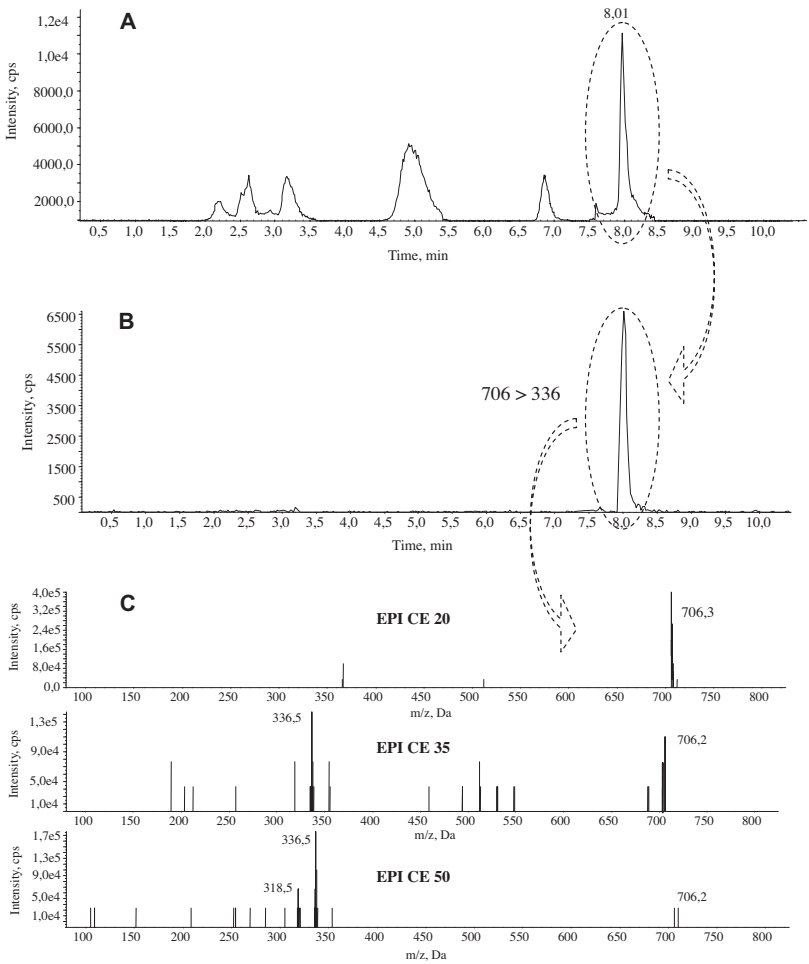

Fig. 1. IDA experiment of FB_2 in urine sample. (A) TIC of total SRM transitions, (B) XIC from the TIC and (C) EPI spectrum FB_2 at LOQ concentration level at different collision energy voltage.

The presence of OTA, DON and AFG_2 was detected at trace level: these mycotoxins were found at concentration values between LOD level and LOQ level for each mycotoxin.

OTA was found in three (11.1%) urine samples at lower concentration than the limit of quantification. This percentage was lower than results related in the literature. As an example, Fazekas et al. (2005) determined 61% positive for OTA of the studied urine samples. Pena et al. (2006) confirmed the presence of OTA in 70% of urine samples at concentrations above the LOQ and in the same way, Akdemir et al. (2010) found OTA in 83% of the urine samples. Although these studies reached LOQ level lower than this study, it is important to keep in mind that this work is a multi-mycotoxin monitoring and this fact can compromise the sensitivity. Moreover, the detector used in all these examples was a fluorescence detector which can reach best sensibility for some compounds. Other variables as the population, the diet, the extraction, etc., could explain the different results obtained.

In our study, surprisingly, one sample was positive for AFG_2 (confirmed by IDA experiment). About the metabolism of this mycotoxin, Polychronaki et al. (2008) speculated the conversion of AFG_1 to AFG_2 in the liver since this mycotoxin was observed at high frequencies in Guinean children (36%).

Finally, DON was confirmed in nine of 27 analyzed urine samples (33.3%). These results can be considered acceptable since Turner et al. (2008) detected DON in 296 urine samples from a total of 300 samples in a large-study conducted in UK. Owing to the fact that DON was the mycotoxin with high prevalence in this study, Fig. 2 depicts a chromatogram of a urine

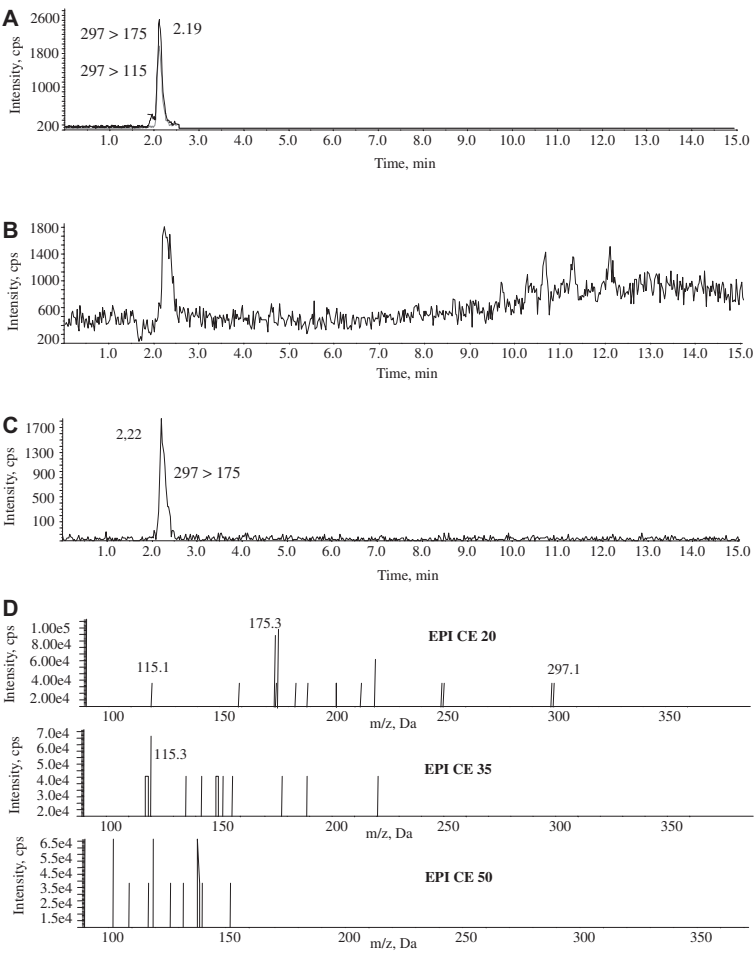

Fig. 2. DON positive sample. (A) Schedule SRM DON transitions, (B) TIC of DON, (C) XIC from the TIC and (D) EPI spectrum DON at LOQ concentration level at different collision energy voltage.

sample that contained DON at concentration slightly above the LOQ level.

4. Conclusions

The validated method was confirmed to be an accurate, precise, and sensitive methodology for the detection of 11 mycotoxins in human urine samples. In conclusion, IAC provided acceptable recoveries and good clean-up for these samples. The specificity of this procedure eliminated the matrix effects and other interferences.

The application of the method in 27 volunteers did not detect mycotoxins in human urine at LOQ concentration levels, but it showed trace levels of several free mycotoxins like OTA, DON and AFG_2.

In conclusion, we can suggest that the studied population has low exposure to mycotoxins. However, further work is currently underway to relate the food mycotoxin contamination and the presence of these mycotoxins in human urine.

5. Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgment

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5. Discussió

5. DISCUSSIÓ

5.1 MULTI MYCOTOXINS EXTRACTION PROCEDURES

The development of analytical methods for mycotoxins analysis in food is a laborious work for investment of time in sample pre-treatment besides the extraction procedure.

In fact, mycotoxins are present at very low levels in foods, as well as it great structural variability. Fortunately, combining of selectivity of LC-MS and the optimal extracts obtained from mycotoxin extractions it allows a rapid, easy, cheap and effective analytical method. In this form, mycotoxins from different genus and structures can be extracted and detected simultaneously. The extractions evaluated were:

- Matrix solid-phase dispersion (MSPD)
- Solid-phase extraction (SPE)
- Immunoaffinity columns (IACs)
- Solid liquid extraction (SLE)
- QuEChERS

5.1.1 EVALUATION MSPD

MSPD has been commonly applied as extraction procedure to organic contaminants [1], but it has not been usually used for mycotoxin extraction. Most often, MSPD has been applied to solid matrices owing to the facility to disrupt the sample. This extraction has been also applied to viscous and liquid matrices [1]. In our research, MSPD has been evaluated for solid and viscous matrices. Solid matrices have low water content; therefore, it makes the homogenization of sample

simple. By contrast, the equilibrium between liquid sample and solid-phase is tedious, as well as the disruption is not effective.

The optimisation of MSPD method needs to evaluate several parameters as solid-phase and solvent extraction [2, 3]. MSPD extraction method has been mainly used normal and reversed-phases. However, the reversed-phases, such as C_8 and C_{18} have been predominantly applied to organic contaminants [1, 4]. The technique involves homogenisation of a small amount of sample tissue with bulk bonded silica based sorbent using a pestle and a mortar. The mechanical shearing forces produced by the grinding process disrupt the structure of the tissue, dispersing the sample over the surface of the support sorbent by hydrophilic and hydrophobic interactions. The process produces a mixture semi-dry and free flowing, and a homogenous blend of sample and sorbent is the result.

Studies about solid-phases for MSPD showed that polar solid-phases as normal phases (amino, florisil, phenyl and silica) were not able to extract all mycotoxins. This fact could be produce since the presences of polar groups in the structure of mycotoxins can interaction with these polar solid phases not allowing their elution at the pass of the medium polar solvents.

On the other hand, reversed-phases, such as C_8 and C_{18} demonstrated to be the best solid support owing to the strong hydrophobic character. Both studies in which MSPD was applied demonstrated that C_{18} was the best sorbent for multi-mycotoxin extraction procedure [2, 3].

The extraction solvent has to be also evaluated, checking a great variety of solvents with different polarities such as methanol, dichloromethane, acetonitrile, ethyl acetate, and mixtures of them. The nature of elution solvent is an important step since of target mycotoxins should be efficiently desorbed while the remaining

matrix components should be retained in the column. Furthermore, solvent is characterized by it polarity and elution strength for a specific sorbent.

In a first work, target mycotoxins were aflatoxins and OTA [2]. In this case, acetonitrile was the best organic solvent, because of acceptable recoveries were obtained. However, when the number of mycotoxins was extended, the polarity of the extraction solvent had to be further than previous work. Therefore, the study of extraction solvent had to be reviewed again. Mixture of methanol and acetonitrile (50/50, v/v) demonstrated to be more effective than others, as well as, the use of modifiers in the mixture helped to improve the recoveries. In this study, ammonium formate was selected as a modifier [3].

To sum up, evaluation and optimisation of MSPD using C_{18} as a solid support and methanol-acetonitrile with ammonium formate as solvent extraction reached recoveries from 65-101% [2, 3, 5-7].

MSPD showed to be an effective extraction and purification in one step. This extraction was mainly applied to cereal and derivate products: malt, instant cereal beverage, baby food, cereal flours, coffee, barley and a tuber as tiger-nut. At the end, the maximum range of mycotoxins extracted with this technique was 21 [5].

5.1.2 EVALUATION OF SPE AND IAC

The high water content of several samples is a limiting factor to use MSPD. For this reason when liquid matrices were studied other alternatives were tested: SPE and IAC. These techniques consist in a separation process by which compounds that are dissolved or suspended in a liquid mixture are separated from other compounds in the mixture according to their physical and chemical properties using cartridges [8]. The basic principle of SPE can be considered as a simple

chromatographic process with the sorbent being the stationary phase. It is especially suitable for the analysis of aqueous samples. A typical SPE sequence starts with the conditioning of the column, by activating it with a solvent and/or water. The aqueous sample is then eluted and the analyte is trapped together with interfering compounds. Ideally most of the matrix interferences should be removed by a rinsing step, with the analyte staying on the column. Consequently the analyte is eluted with an organic solvent and further pre-concentration takes place by evaporation with N_2 stream. In this form, SPE columns separate target compounds, mycotoxins, from other compounds in the mixture according to their physical and chemical properties using different solid materials.

In this work, the use of SPE columns has been applied to the analysis of mycotoxins in beer [9]. SPE C_{18} bonded silica columns are most frequently used since they are very pressure resistant and give reproducible results, as well as they allow a wide range of extracted mycotoxins. C_{18} has allowed recoveries between 66-108% for 18 target mycotoxins.

On the other hand, IACs show low or negligible matrix effects and recoveries higher than other extraction procedures, due to the specificity of these columns. IACs contain antibodies that selectively bind the toxin of interest from the crude extract. During the clean-up step, interferences should be washed from the column while the toxin is immobilized on the column. In the last step, the toxin is eluted from the column for determination purposes. Using this highly specific technology, almost all clean-up related issues can be solved.

These columns have been usually used after sample pre-treatment in order to obtain cleanest extracts. However, a novelty application was the use of those columns to analyses biological samples: in our research, IACs, MYCO 6in1™ multi-analyte columns were used to extract eleven mycotoxin from human urine.

At the end, eleven target mycotoxins were adequately extracted and the recoveries ranged from 83-108% [10]. The specificity of these columns limit the number of mycotoxins, by contrast, matrix effects are negligible and recoveries very satisfactory.

5.1.3 EFFICIENCY AND EFFICACY OF SELECTED EXTRACTION PROCEDURES

The last step was to compare conventional and recent extraction procedures. MSPD, QuEChERS, SLE and SPE clean-up method are commonly used for mycotoxins analysis, but, and as it has been explained four methods have showed advantages and drawbacks depending of the analysed matrix and target mycotoxins. SLE with or without clean-up step, MSPD and QuEChERS were compared to evaluate its capacity to extract simultaneously 32 mycotoxins in barley. Regarding recoveries data obtained for each extraction procedure, SPE clean-up method presented lower recoveries than other techniques, which ranged from <50% to 80%. In our research, HLB Oasis cartridges showed an unsuccessful efficiency for target mycotoxins for what C_{18} cartridge was used looking for a wide number of mycotoxins; however it was also rejected due to low recoveries obtained.

Evaluating deeply the recoveries obtained by MSPD, QuEChERS and SLE it was observed that MSPD was able to extract all selected mycotoxins and recoveries ranged from 67 to 87%, however penitrem A, D3G and some ergot alkaloids showed low recoveries and high RSDs (%). On the other hand, SLE showed the highest recoveries and all target mycotoxins were completely extracted, however penitrem A and fumonisins showed unacceptable recoveries. At the end, modified QuEChERS did not present the highest recoveries, but this method was

able to extract adequately all target mycotoxins in barley, and recoveries ranged from 64 to 93% without any exception.

Summarizing, modified QuEChERS was selected in order to take advantage of its potential for simultaneous extraction of target mycotoxins. Moreover, QuEChERS gave low time consuming during the extraction procedure, as well as it was easier and cheaper than MSPD, SLE and SPE clean-up method [11].

During this Thesis, the majority of matrices were solids, such as cereals and derivate products. Focus on this type of matrix was observed that MSPD and QuEChERS could satisfactorily extract a wide range of mycotoxins. Both extractions demonstrated to be an effective alternative against classic extraction procedures.

However, high water content on several products could complicate these extractions. For this reason, when liquid matrices were studied, classical extraction as SPE and IAC were selected in order to obtain reliable data.

5.2 SEPARATION AND DETECTION METHODS

5.2.1 LIQUID CHROMATOGRAPHY TRIPLE QUADRUPOLE (LC-MS/MS)

Nowadays, LC-MS is the wide-used technique to identify simultaneously micotoxins. During the last decade a great number of analytical methods have been developed using this technique. In all cases, triple quadrupole analysers have been commonly coupled [12-14].

For this reason, initial point to start was the use of LC-QqQ-MS/MS [2, 3, 15, 16]. First of all, aflatoxins and OTA were optimized by LC-QqQ-MS/MS

owing to its toxicity [2]. The mobile phases used were water and acetonitrile with modifier ammonium acetate, which allowed an improvement of signal and an adequate separation of target mycotoxins. However, these mobile phases were optimised in order to extend the number of mycotoxins detected [3]. The problem was that acetonitrile did not allow the ionisation and separation of wide number of mycotoxins. When the acetonitrile was substituted by methanol, an improvement of the response was observed. Moreover, the use of formic acid and ammonium formate as modifiers allowed the detection and separation of fumonisins and type A trichothecenes [3]. Theses mobile phases have been commonly related in the literature [12-14].

The triple quadrupole detector has demonstrated to be the most reliable tool for mycotoxin analysis in foodstuff, since it can quantify and qualify unambiguously [2, 3, 15, 16]. In all related cases, the levels of quantification (LOQs) were lower than maximum levels established by EC 1881/2006 [17]. Moreover, the developed analytical methods were according to Commission Decision 2002/657/EC [18].

This technology permits a simple and cheap routine analysis of mycotoxins from different foods.

5.2.2 LIQUID CHROMATOGRAPHY TRIPLE QUADRUPOLE LINEAR ION TRAP (LC-QTRAP®)

Comparing triple quadrupole detector and QTRAP® were observed equal amounts of fragment ion data. However, the qualitative information acquired was different. On the one hand, LC-QqQ-MS/MS working in SRM mode could obtain a limited number of identification points, while QTRAP® working in hybrid mode could give a variety of new scan types and the possibility to combine them in order to improve the applications.

Experimentally, it was compared the performance of the two operating modes of the QTRAP®: SRM acquisition mode and information depending acquisition (IDA) method were developed along this Thesis. The IDA method consist in 3 EPI scans (dependent scans) released at different collision energies (20, 35 and 50 eV). The inclusion of this IDA experiment provided an unequivocal identification of the mycotoxins in the matrix. It has been widely explained along the experimental section [5, 6, 10]. However, SRM acquisition mode provided sensitivity and effective quantification, as QqQ detector.

In our research, QTRAP® worked as a triple quadrupole and a hybrid instrument, in all works; this instrument demonstrated to be a powerful tool for routine mycotoxin analysis providing extra qualitative information by IDA methods.

5.2.3 LIQUID CHROMATOGRAPHY HIGH RESOLUTION MASS SPECTROMETRY (LC-HRMS)

In this Thesis, the last part of work was carried out using high resolution mass spectrometry (HRMS), which has been scarcely used for mycotoxins analysis. However, nowadays LC-HRMS is being gradually included for routine mycotoxin analysis.

Orbitrap® technology has predominantly been the election technology, allowing full scan for compound identification, high-throughput and high-performance screening for qualitative and quantitative analysis of mycotoxins. This technology has been coupled to LIT obtaining a hybrid mass analyser (LTQ-Orbitrap®) or it has been used itself (Exactive®).

Initially, in our studies LTQ-Orbitrap® was used for the validation of an analytical method in beer, as well as for the screening of non-target mycotoxins.

The lower limits of quantification (LLOQs) or lowest calibration levels (LCLs) were slightly higher than those obtained using triple quadrupole system [19]. However, the LTQ-Orbitrap® is able to acquire a full-scan, as well as fragmented ions under data-depending acquisition, which can be acquired in a single Orbitrap® mass spectrum. However, the full scan acquisition allowed unambiguous identification of target mycotoxins by accurate mass, besides LIT confirmed by pathway study. In this form, the positive samples are reliably confirmed [9]. Even though this extra qualitative information decreased slightly LCLs or LLOQs.

On the other hand, Exactive® or Orbitrap® MS by itself has been commonly used due to facility of use, accurate mass, high-throughput screening and compound identification. Usually, the applicability for routine analysis has been demonstrated with the development of analytical method in selected matrix. However, a different way to demonstrate its application was to compare the efficiency and efficacy of different extraction procedures, which have been carefully studied using Orbitrap® technology. This issue has been normally carried out using triple quadrupole analysers. However, in this work the use of Orbitrap® MS technology demonstrated to be an effective and powerful tool for the validation of analytical method [11].

In this Thesis LC-HRMS demonstrate to be an alternative to LC-MS/MS. This advanced technique was able to quantify and qualify at very low levels target mycotoxins. Moreover, HRMS offers extra information owing to non-target and unknown myocotoxins can be screened.

5.2.4 APPLICABILITY OF HYBRID INSTRUMENTS

At the end of this Thesis, one work using QTRAP® and LTQ-Orbitrap® highlighted the advantages, limitations and applications of them. The validation was carried out for mycotoxins analysis in baby food [7].

To demonstrate the differences and similarities in the mass spectra acquisition, both instruments were evaluated. In this study it was observed an interesting difference between instruments in terms of qualitative products ions obtained and relative abundances of these fragments. This can be explained considering the different mechanisms of ion isolation and fragmentation, previously explained. Although the fragment ions obtained were the same in both instruments, their abundances were not the same [7].

At the end of this comparison, QTRAP® instrument showed to be more suitable for quantitative purposes and it allows extra information by IDA methods for unambiguous identification, allowing an increase of identification points. Nevertheless, LTQ-Orbitrap® could quantify target mycotoxins and identify non-target and unknowns mycotoxins. This potential comes from the ultra-high resolution mass, which allows an exact mass accuracy. Moreover, this instrument allows a retrospective data analysis, which means that from Full-Scan it could be studied. Thereby, the extracted ion chromatogram of a specific analyte could be processed after the chromatogram has been acquired.

5.2.5 EVALUATION OF DIFFERENT MASS ANALYSERS FOR MYCOTOXINS ANALYSIS

For an LC-MS system, it is necessary to verify the performance of LC and the MS separately. In this context, LC system, chromatographic column and mass

spectrometers were completely verified for each LC-MS system in order to obtain accurate data using different mass analyser.

The typical method characteristics or analytical parameters that need to be evaluated are: selectivity/specificity, accuracy, precision (repeatability and reproducibility), limit of detection (LOD) or detection limit, limit of quantification (LOQ) or quantification limit, and linearity and linear range. In this way, during this Thesis several mass spectrometers were used to analyse mycotoxins in foodstuff according to mentioned characteristics. ***Table*** 1 summarizes instrumental parameters.

These mass analysers have different advantages and drawbacks; therefore, the ideal application for each one is different. It is normal, since each mass analyser has a particular acquisition data owing to their capacities. Keeping in mind these premises; QqQ, QTRAP®, Orbitrap® (Exactive®) and LTQ-Orbitrap® were compared for legislated mycotoxins in order to find the best application of each of them. All of these instruments showed an excellent sensitivity, simplifying extraction procedure and clean-up steps.

Focus on the sensitivity, precision and linearity, four selected mass analysers fulfilled successfully Commission Decision 2002/657/EC. Delivering into details of sensitivity, QqQ and QTRAP® showed the lowest LOQs, whereas LTQ-Orbitrap® showed highest LLOQs. Focus on hybrid instruments were observed a decreasing of sensitivity when hybrid mode was used; it is the case of QTRAP® running in IDA method (EPI mode).

Special attention was paid to confirmation aspects. In all cases, repeatability of retention time was compared for required value ±2.5% and all of them were satisfactory. On the other hand, the number of identification points was studied for each mass analyser.

Table 1. Instrumental parameters show the most relevant instrumental parameters obtained along the developed works.

	QqQ	QTRAP®		Orbitrap® Full Scan	LTQ-Orbitrap® Full Scan DDA
		SRM	EPI mode		
LOQs/LCLs/LLOQs (µg kg^{-1})	0.5-100	0.1-100	2.25-150	1-100	8-155
Correlation coefficient	>0.99	>0.99	>0.99	>0.99	>0.99
Linear range	LOQ-100xLOQ	LOQ-100xLOQ	LOQ-100xLOQ	LCL-100xLCL	LLOQ-100xLLOQ
% RSD	<19%	<17%		<17%	<19%
IPs	4	4	4-8.5	2	2-14.5

For mycotoxins a minimum of 3 IPs are required, besides according to Commission Decision 2002/657/EC the relationship between a range of classes of mass fragment and earned identification points get different punctuation. ***Table 2*** summarizes the identification points earned.

For instance, to earn 3 identification points in mycotoxins analysis performed by QqQ, QTRAP®, Orbitrap® and LTQ-Orbitrap®, different acquisition modes have to be applied. Triple quadrupole and QTRAP® working on MRM or SRM can acquire one precursor ion plus two products ions, therefore, 4 IPs are reached. However, QTRAP® working on hybrid mode (IDA method) can increase the number of IPs until 4-8.5 IPs, as it have been shown along this Thesis [5-7, 10].

On the other hand, according to Commission Decision 2002/657/EC, HRMS is defined as the resolving power of 10.000 for the entire mass range at 10% valley. Nowadays, this value is roughly expressed as 20.000 FWHM (full width at half maximum). Moreover, Commission Decision 2002/657/EC does not specify a criterion for mass accuracy.

Table 2. Identification points earned.

MS technique	Identification points earned per ion
Low resolution mass spectrometry (LR)	1.0
LR-MS^n precursor ion	1.0
LR-MS^n transition products	1.5
HRMS	2.0
HR-MS^n precursor ion	2.0
HR-MS^n transition products	2.5

Thereby, the first impression is logical; the directive is not useful enough. Therefore, the necessity to include mass accuracy criterion within IPs system exist, in this form it could be guaranteed the uses for TOF, Q-TOF and Orbitrap® technology.

Focus on Orbitrap® technology, Commission Decision 2002/657/EC has created a paradoxical situation: ultra high mass resolving power settings of 100.000 or 50.000 FWHM and the mass error not exceeding 5 ppm cannot obtain required number of IPs (N=3). It is the case of Exactive instrument working in Full Scan mode, which can only reach 2 IPs. One solution would be the use of source collisional induced dissociation (CID) and high-energy collisional dissociation (HCD), which provide MS/MS data enough in order to solve this problem. However, the selectivity of detection shows unnecessary the transition products, as well as ultra high mass resolving power settings and the mass error lower than 5 ppm force to keep up to date the Commission Decision 2002/657/EC. On the other hand, LTQ-Orbitrap® combining Full Scan mode using Orbitrap® and Data Depending Acquisition (DDA) by LIT can reach between 4.5 to 14.5 IPs.

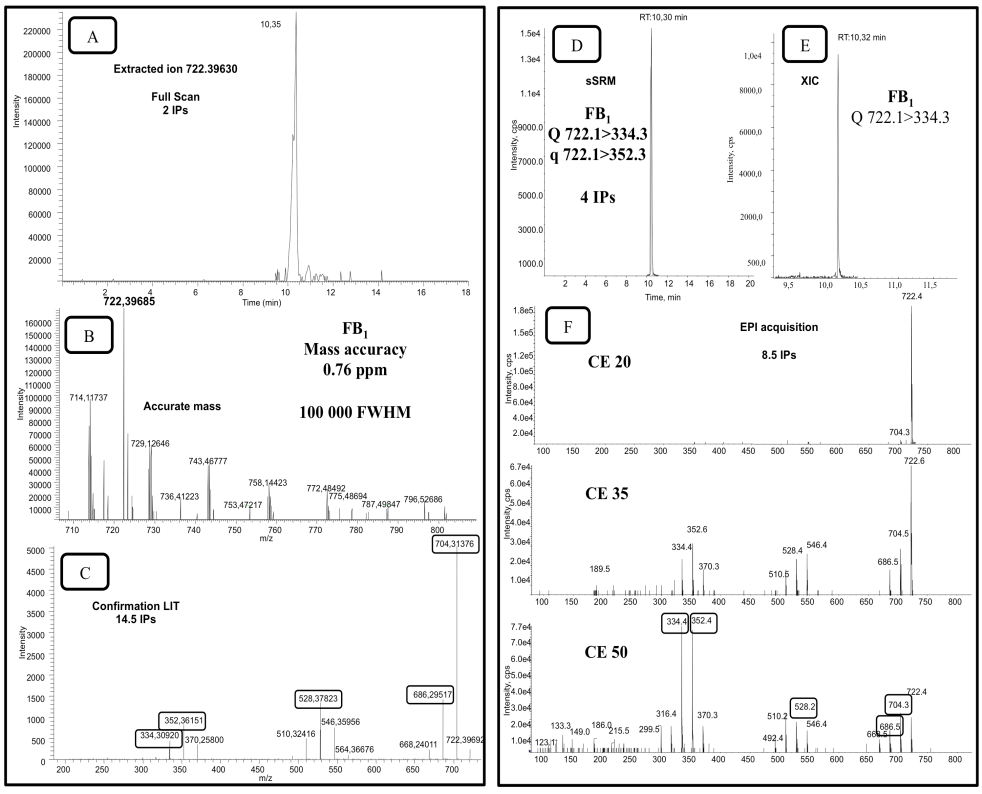
The ***figure 1***, shows all the acquisition modes, which were used along this Thesis. As an example, FB_1 was identified by SRM, EPI mode, Full Scan and Data Depending Acquisition (DDA). IPs were calculated for each acquisition mode.

Focus on FB_1 confirmation purpose example, when the low resolution mass spectrometry is compared, EPI acquisition mode showed to be up to SRM. In this particular case, it is owing to the third quadrupole of QTRAP®, which is replaced by a LIT giving an extra product ions respect to other QqQ analysers. Both cases reached the minimum of IPs established by Commission Decision 2002/657/EC, but extra information provided by QTRAP® can help to understand structural elucidation, as well as the confirmation is more unambiguous since 8.5 IPs can be counted.

By contrast, when HRMS was used for FB_1 confirmation, it was confirmed the necessity to keep up to date Commission Decision 2002/657/EC. A Full Scan did not permit to obtain IPs enough, although the instrument was working at ultra high mass resolving power settings of 100.000 FWHM and the mass error not exceeding 1 ppm. Obviously, when LTQ-Orbitrap® was used combining Full Scan and DDA the number of IPs is increasable, in this example 14.5 IPs were reached.

During this Thesis, QqQ and QTRAP® detectors were used for target mycotoxin analysis. They allowed to develop rapid and easy analytical methods for routine mycotoxin analysis in different food matrices. However, when the main aim of experimental work was to quantify target mycotoxins, as well as, to identify non-target mycotoxins, the election was Orbitrap® MS technology. This technology, LC-HRMS, was also applied in order to demonstrate its application for routine mycotoxins analysis.

Figure 1. Analysis of FB_1 mycotoxin in a spiked wheat-based baby food at 75 µg kg^{-1} by LTQ-Orbitrap® (A, B and C) and by QTRAP® (D, E and F). Extracted ion 722.39630 (A), accurate mass FB_1 (B) and confirmation by linear ion trap (C). SRM transitions (D), XIC from the TIC (E) and EPI spectrum at different collision energy voltage (F).



5.3 OCCURRENCE OF MYCOTOXINS IN FOODSTUFF

The number of analysed mycotoxins has been extended research by research. Initially, aflatoxins and OTA were studied in malt, instant cereal beverage and coffee. Consecutively, *Fusarium* toxins and other genus toxins were included to multi mycotoxins analysis. In this form, target mycotoxins were detected from selected matrices. The majority of matrices along this Thesis were cereals and their

derivate products, such as barley, baby food and different flours. Moreover, one regional product with great regional economic importance was studied. Tiger-nuts and its beverage, horchata, were selected for a survey.

The occurrence of mycotoxins can be seen in ***Table 3*** and ***4***. Focus on ***Table 3*** it can be observed the incidence of *Aspergillus* toxins mainly; aflatoxins, STER and OTA are summarized. In these studies, OTA was mainly identified in different samples. The incidence of OTA was reported along the monitoring of beverages, such as beer and tiger-nuts milk (horchata) and cereals. Most often, the incidence of OTA was linked to aflatoxins.On the other hand, the occurrences of alfatoxins were commonly detected in tiger-nuts and their beverages, as well as malt. Both cases the presence of these mycotoxins could be related on an inappropriate storage of the raw material.

In ***Table 4*** is showed the occurrence of *Fusarium* mycotoxins. Type B trichothecenes and BEA were predominantly detected for selected samples. DON and fumonisins were the legislated mycotoxins, commonly found in foodstuff. However, the incidence of emergent mycotoxins was gradually demonstrated. Focus on BEA and enniatins were normally found in cereal and derivate products, even though BEA was also detected in tiger-nuts.

These tables summarized the amount of mycotoxins, which were found along of this Thesis. In global, the levels of mycotoxins were lower than established by EU directives [17].

In these tables the total of samples analysed can be seen, number of positive by sample for each mycotoxin and mean concentration (mean conc.) for them, expressed as a $\mu g\ kg^{-1}$ for solid matrices and $\mu g\ L^{-1}$ for liquid matrices.

Table 3. Occurrence of alflatoxins, STER and OTA for selected matrices. Concentration expressed as µg kg^{-1}.

	AFB_1		AFB_2		AFG_1		AFG_2		STER		OTA	
	Positives	Mean conc	Positives	Mean conc	Positives	Mean conc	Positives	Mean conc	Positives	Mean conc	Positives	Mean conc
Malt (n=10)	2		2		4	1.3±0.3	4	1.5±0.4				
Coffee (n=5)											2	2.6±0.5
Orxata (n=190)[a]	12	1.3±0.5	2	1.35±0.3			1	1.2			2	2.2±0.2
Tiger-nuts (n=131)	28	1.5±0.5	5	1.6±0.2	2	1.4±0.1	17	2.2±0.7			14	8.6±9
Wheat flour (n=25)					2	0.62±0.1	1	1			3	3.2±0.3
Oat flour (n=3)			1	1.6								
Bakery preparation (n=8)							1	1.2				
Cereal liquid baby food (n=2)											1	0.35
Oat powered baby food (n=5)											1	0.5
Corn powdered baby food (n=6)							1	1.2				
Rice powdered baby food (n=4)									2	30±28		
Red ale beer (n=13)[a]											1	5.1
Ale beer (n=16)[a]											1	3.2
Pale lager beer (n=29)[a]											4	4.7±1.7

[a] µg L^{-1}

Tabla 4. Occurrence of *Fusarium* mycotoxins. Concentration expressed as µg kg^{-1}.

	NIV		DON		BEA		FB_1		FB_2		ZEN		ENA		ENA1		ENB		ENB1		T-2		HT-2	
	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.	Positives	Mean conc.
Tiger-nut (n=83)			5	45±15	11	65±54																		
Wheat flour (n=25)	9	97±6	5	141±132	6	261±228					1	39												
Corn flour (n=9)	1	92							2	439±168	1	71												
Rice flour (n=3)					3	417±137																		
Oat flour (n=3)			1	153	2	276±70																		
Bakery preparation (n=8)	1	76	2	106±104	3	351±312																		
Multi cereal baby food (n=8)	5	116±23			3	22±8	2	88±18			1	10												
Cereal liquid baby food (n=2)	1	70			1	12																		
Wheat powdered baby food (n=2)	2	130±28	1	60±4	2	13±8																		
Cereals and fruit purée baby food (n=3)	1	90			1	15																		
Corn powdered baby food (n=6)					2	16±8	1	90	1	75	1	15												
Rice powdered baby food (n=4)					4	45±40																		
Soy powdered baby food (n=2)					1	25																		
Oat powdered baby food (n=5)					1	9																		
Barley (n=15)			7	37±8									4	134±141	4	231±313	4	558±981	4	522±867	2	20±15	3	45±29
Stout beer (n=16)[a]							2	101±37	2	93±8	1	37.8												
Red ale beer (n=13)[a]							1	92	1	96														
Ale beer (n=16)[a]																								
Pale lager beer (n=29)[a]							7	91±15	7	78±6											3	7±4	3	17±2

[a] µg L^{-1}

5.4 DAILY INTAKE OF MYCOTOXINS

Human exposure to mycotoxins occurs primarily trough intake of contaminated food, although there is evidence that others ways such inhalation or dermal contact could be involved. Since the toxicity has only been evaluated for a few mycotoxins, the total impact of these naturally occurring contaminants on

human health cannot be assessed. Even the most well documented toxins, the tolerable daily intakes (TDI) established by international working groups remain temporary or provisional due to exposure. More data, both on levels in foods and on the intake of these foods in various population groups, are needed to perform reliable exposure analysis [20, 21].

Throughout this thesis, large number of food samples were analysed for aflatoxins, trichothecenes, OTA, fumonisins, ZEN, BEA and enniatins. The occurrence of these mycotoxins has been summarized in ***Table 3*** and ***4***; regarding these results, it is clearly demonstrated the regular presence of low levels of mycotoxins in several foods. However, and considering the potential negative health impact of the presence of the analysed toxins, the meaning of this contamination can best be evaluated by characterizing the risk in terms of estimated daily intake. With this aim, although there are insufficient exposure data to estimate a daily intake of mycotoxins for Spanish population, estimation of risk of consumers based upon the data obtained along this Thesis was carried out.

The exposure assessment was performed by estimate deterministic approach, which is an evaluation built by combining mean contamination values of analytes (***Table 5***) with food consumption data and divided by the body weight. In this study the dietary dairy intake, calculated as ng kg^{-1} body weight (b.w.) day^{-1} was evaluated and the resulting consumer dietary exposure was compared with temporary TDI (tTDI) of the respective mycotoxins, to evaluate the possible health risk associated with the intake.

The consumption data used for exposure assessment calculations usually is derived from dietary surveys conducted at the national level on a representative sample of individuals. Ideally, such concentrations are available in an exhaustive, consistent list of food categories, but, in practice, these conditions are rarely met.

In this study data on dietary consumption were mainly obtained from "Agriculture and Ministry". For foods that were not included in the database, it was assumed the intake of other similar product or the highest consumption levels representing the worst situation [22, 23].

Table 5. Estimate intake of mycotoxins in adults based on food consumption (standard body weight= 60 kg).

	Daily Intake (kg/day)	Daily Dietary Intake (µg/kg b.w./day)															
		AFB_1	AFB_2	AFG_1	AFG_2	OTA	NIV	DON	T-2	HT-2	ZEN	FB1	FB2	BEA	ENA	ENA1	ENB	ENB1
Malt	0.001[a]			$2x10^{-5}$	$2x10^{-5}$													
Coffee	0.001[a]					$4x10^{-5}$												
Orxata	$3x10^{-4}$	$7x10^{-6}$	$7x10^{-6}$		$6x10^{-6}$	$1x10^{-5}$												
Tiger-nuts	$3x10^{-3}$	$8x10^{-5}$	$8x10^{-5}$	$8x10^{-5}$	$1x10^{-4}$	$4x10^{-4}$		$2x10^{-3}$						$3x10^{-3}$				
Wheat flour	0.006[b]			$6x10^{-5}$	$1x10^{-4}$	$3x10^{-4}$	0.01	0.01			$4x10^{-3}$							
Oat flour	0.006[b]		$2x10^{-4}$					0.02						0.03				
Rice flour	0.006[b]													0.04				
Corn flour	0.006[b]						$9x10^{-3}$						$4x10^{-3}$					
Bakery preparation	0.006b				$1x10^{-4}$		$8x10^{-3}$	0.01						0.04				
Red ale beer	0.04[c]					$3x10^{-4}$						0.06	0.06					
Ale beer	0.04[c]					$2x10^{-3}$												
Pale lager beer	0.04[c]					$3x10^{-3}$			$5x10^{-3}$	0.01		0.06	0.05					
Stout beer	0.04[c]										0.02	0.07	0.06					
Barley	0.01							$6x10^{-3}$	$3x10^{-3}$	$8x10^{-3}$					0.02	0.04	0.09	0.09

[a] Value of intake of coffee
[b] Value of intake of rice
[c] Value of intake of beer

Foods have been classified according to range of age to which it is addressed. In ***Table 5*** are summarized the results obtained for food commodities

for adults while in ***Table 6*** are only summarized dietary daily intakes (DDIs) of food products intended for babies and infants. Acceptable Daily Intake (ADI) is defined by the United Nations' Joint Food and Agricultural Organization / World Health Organization Expert Committee on Food Additives (JECFA) as the amount of a contaminant, expressed on a body weight basis, that can be ingested daily-by a human-over a lifetime without appreciable health risk [24, 25]. For calculation of the daily intake per person, a standard body mass of 60 kg is used in the case of adults and a standard body mass of 8 kg.

Table 6. Estimate intake of mycotoxins in babies and infants based on food consumption (standard body weight= 8 kg).

	Daily Intake (kg/day)	Daily Dietary Intake (µg/kg b.w./day)								
		AFG_2	STER	OTA	NIV	DON	ZEN	FB_1	FB_2	BEA
Multi cereal baby food	**0.05**				**0.7**		**0.06**			**0.12**
Cereal liquid baby food	**0.05**			$2x10^{-3}$	**0.4**					**0.07**
Wheat powdered baby food	**0.05**				**0.7**	**0.4**				**0.08**
Cereals and fruit purée baby food	**0.05**				**0.5**					**0.1**
Corn powdered baby food	**0.05**	$7x10^{-3}$					**0.1**	**05**	**0.5**	**0.1**
Rice powdered baby food	**0.05**		**0.2**							**0.3**
Soy powdered baby food	**0.05**									**0.2**
Oat powdered baby food	**0.05**			$3x10^{-3}$						**0.05**

Daily intake according to manufacture

5.4.1 AFLATOXINS

Aflatoxins are potent toxins that cause liver disease and liver cancer in humans, animals and domestic pets. Unlike most other mycotoxins, there is no tolerable daily intake (TDI) for aflatoxin B1 since it is carcinogenic.

Owing to their risk, aflatoxins were analysed in all studied samples. Based on analytical data, major occurrence of aflatoxins were in horchata and chufa, however, these products are limited to a local consumption, and obviously, the calculation of their DDI was difficult but doing an estimation of intake, the DDI of aflatoxins from these food commodities was regarded as relatively small. It was also considered low the intake through flours.

This situation becomes more worrying in baby foods (***Table 4*** and ***6***). Concentration level of AFG_2 found in one corn powdered baby food sample was below of ML established; however, since international expert groups have not specified TDI for aflatoxins, the calculated DDI cannot be directly compared with tolerable level.

5.4.2 OCHRATOXIN A

The Joint Committee FAO/WHO of Experts on Food Additives (JECFA, 2001)) has established that provisional tolerable weekly intake of OTA is 0.1 µg kg^{-1} b.w. and this corresponds approximately to 0.014 µg kg^{-1} b.w. per day. The European Food Safety Authority (EFSA) has recently proposed the tolerable weekly intake of OTA is 0.12 µg kg-1 b.w., which correspond to a tolerable daily intake of 0.017 µg kg^{-1} b.w [26].

As shown in ***Table 5*** and ***Table 6***, the calculated human OTA intake found in this study are below the levels proposed as TDI, as well in adults as in infants.

5.4.3 TRICHOTHECENES

The Scientific Committee on Food (SCF) evaluated the toxicity of DON, NIV and T-2/HT-2 in a set of opinions [27]. In a group evaluation of these four analytes, the Committee concluded that combined or synergistic effects were not probable, although currently available data on this matter was limited. Hence, the Committee confirmed the single temporary TDI values for each toxins as follows: for DON 1 µg kg^{-1} b.w. per day, for NIV 0.7 µg kg^{-1} b.w. per day and for T-2 and HT-2 0.06 µg kg^{-1} b.w. per day.

Regarding the results for NIV, it is important to highlight that DDI calculate for two baby food samples were the t-TDI established; however, this intake was calculated supposing that the same baby food is the only solid food ingested. Normally, a mix of baby foods is provided to reach the necessities of the babies.

The calculated DDI of DON appears to be below the tTDI of 1 µg kg^{-1} b.w. per day proposed by SCF in both foods intended for adults and for babies.

Focusing on T-2 and HT-2, the DDI values calculated from foods intended for adults were below the tTDI established. These mycotoxins were not detected in any baby food sample.

5.4.4 FUMONISINS

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has recommended a provisional maximum tolerable daily intake (PMTDI) of 2 µg kg^{-1} b.w. per day for fumonisins B_1, B_2 and B_3, alone or in combination [25]. According to these results, all the positives samples for fumonisins represented a DDI below the tTDI established by JECFA.

5.4.5 ZEARALENONE

Zearalenone has previously been evaluated by the Joint Food and Agriculture Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) which established a provisional maximum tolerable daily intake (PMTDI) of 0.5 µg kg^{-1} b.w. per day in 2000, based on the estrogenic activity of zearalenone and its metabolites, in the most sensitive animal species, the pig. Also, in 2000, the Scientific Committee on Food (SCF) established a temporary TDI (t-TDI) of µg kg^{-1} b.w. per day. This TDI was designated as temporary and included an additional uncertainty factor because of some deficiencies in the database. The SCF recommended that additional studies were needed to determine the no-hormonal-effect level in pre-pubertal pigs, on the potential genotoxicity of zearalenone, on species differences in metabolism, and on blood levels of zearalenone in humans in order to help clarify the toxicokinetic behaviour.

In our study, the worse case is corn-based baby food that supposes a DDI of 0.1 µg kg^{-1} b.w. per day, closer than the tTDI established.

5.4.6 OTHER MYCOTOXINS

Changing climatic conditions together with ongoing innovation of agricultural practices have resulted in some changes in the spectrum of *Fusarium* species invading crops in the field. Consequently, the extent of mycotoxin contamination and the type of mycotoxins formed are changing continuously. In addition to the "traditional" (regulated) Fusarium mycotoxins, other so-called "emerging mycotoxins", such as enniatins and beauvericin, have been reported to occur in cereals and cereal derivates.

It is worthy of note and surprising the frequent presence of BEA in baby foods which has not been considered. However, as not tTDI for BEA is specified by any organisation, the calculated intakes can not be directly compared with tolerable level.

The intake of enniatins was only detected for the consumption of barley. As BEA, no tTDI was established for these mycotoxins.

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6. Conclusions

6. CONCLUSIONS

1. Matrix Solid-Phase Dispersion showed to be an effective extraction and purification in one step. This method was mainly applied to solid matrices, demonstrating to be an advantageous extraction for legislated and non-legislated mycotoxins.
2. However, the high water content of several samples is a limiting factor to use MSPD. In these cases, mycotoxins from liquid matrices were successfully extracted using SPE and IAC.
3. Comparing the efficiency and efficacy of conventional and alternative extraction procedures. It was observed that modified QuEChERS was superior to SLE, MSPD, and SPE method. Thereby, a wide range of mycotoxins could be extracted at low cost, reducing time consuming and increasing throughputs.
4. Liquid chromatography coupled to triple quadrupole has demonstrated to be the most reliable tool for routine mycotoxin analysis in foodstuff, since it can quantify and qualify unambiguously target mycotoxins.
5. QTRAP® allowed to obtain reliable quantification as triple quadrupole and extra qualitative information using the hybrid mode. Therefore, QTRAP® technology provided the ability to identify and to quantify mycotoxins in a single run increasing the number of IPs, demonstrating to be a powerful tools for routine analysis.
6. The applicability of the ultra high resolution mass spectrometry was demonstrated to be effective for routine mycotoxin analysis and screening of non-target mycotoxins.
7. Moreover, linear ion trap-high resolution mass spectrometry working on hybrid mode provided extra transition products, therefore, increasing IPs.

8. All the studies carried out about the presence and co-occurrence of selected mycotoxins demonstrated commonly incidence of these toxins in selected foods. However, the mean concentrations of mycotoxins were normally below of the Maximum Levels. In fact, only one wheat flour sample presented OTA concentration level higher than Maximum Level.
9. The main problem is non-legislated mycotoxins or non-established matrices, since the mean calculated concentrations are not interpretable, despite of the high presence of these mycotoxins in several foodstuff.
10. Estimated daily intake of mycotoxins from data obtained evidenced the low exposure to mycotoxins in Spanish inhabitants. However, the high Dietary Daily Intake of Nivalenol in baby food is striking fact, which needs to be supervised.
11. The evaluation of mycotoxins in biological fluids may offer valuable indications on the real risk for consumers. In a pilot study, the presence of several free mycotoxins as OTA, DON and AFG_2 in human urine was demonstrated, although at trace concentration levels.