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EXPOSURE ASSESSMENT OF MYCOTOXINS IN FOOD AND BIOLOGICAL SAMPLES THROUGH HIGH RESOLUTION MASS SPECTROMETRY-BASED STRATEGIES

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List of Abbreviations

ACN	Acetonitrile
AGC	Automatic Gain Control
AIF	All Ion Fragmentation
AOAC	Association of Official Analytical Chemists
BMDL	Benchmark Dose Lower Confidence Limit
bw	Body weight
C18	Octadecyl carbon chain(C18)-bonded silica
CBD	Cannabidiol
CEN	European Committee for Standardization
CONTAM Panel	Panel on Contaminants in the Food Chain
Crea	Creatinine
DLLME	Dispersive Liquid-Liquid Microextraction
DnS	Dilute and Shoot
dSPE	Dispersive Solid Phase Extraction
DTT	Dithiotreitol
EC	European Commission
EFSA	European Food and Safety Authority
ESI	Electrospray Ionization
FAO	Food and Agricultural Organization of the United Nations
FnS	Filter and shoot
FWHM	Full Width at Half Maximum
GC	Gas Chromatography
GCB	Graphitized Carbon Black

HBM	Human Biomonitoring
HPLC	High Performance Liquid Chromatography
HRMS	High Resolution Mass Spectrometry
IAC	Immunoaffinity Column
IARC	International Agency for Research on Cancer
IFCC	International Federation of Clinical Chemists
INC	Nut and Dried Fruit Council
ISO	International Organization for Standardization
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOAEL	Lowest Observed Adverse Effect Level
LOD	Limit of Detection
LOQ	Limit of Quantification
LRMS	Low Resolution Mass Spectrometry
m/z	Mass to charge ratio
MAPK	Mitogen-Activated Protein Kinases
MeOH	Methanol
MIP	Molecularly Imprinted Polymers
ML	Maximum Limit
MRL	Maximum Residue Limit
MS/MS	Tandem Mass Spectrometry
MSPE	Magnetic Solid Phase Extraction
NOAEL	No Observed Adverse Effect Level

PDI	Probable Daily Intake
ppm	Parts per million
PSA	Primary-Secondary Amine
PTDI	Provisional Tolerable Daily Intake
PTFE	Polytetrafluorethylene
Q-Orbitrap	Quadrupole-Orbitrap
QqQ	Triple quadrupole
Q-TOF	Quadrupole-Time of Flight
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RASFF	Rapid Alert System for Food and Feed
ROS	Reactive Oxygen Species
RPF	Relative Potency Factor
RSD_R	Inter-day Relative Standard Deviation
RSD_r	Intra-day Relative Standard Deviation
S/N	Signal to Noise ratio
SALLE	Salt-Assisted Liquid-Liquid Extraction
SAX	Strong Anionic Exchange
SPE	Solid Phase Extraction
SRM	Single Reaction Monitoring
SSE	Signal Suppression/Enhancement
TDI	Tolerable Daily Intake
TDS	Total Diet Study
TTC	Threshold of Toxicological Concern
UHPLC	Ultra-High Performance Liquid Chromatography

SUMMARY

Mycotoxins are toxic secondary metabolites produced by several fungi genera that are frequently found in food products. If ingested, mycotoxins can display a broad range of adverse effect. In order to reduce the health impact due to the dietary intake of mycotoxins, the Commission Regulation No 1881/2006, released by the European Commission, sets maximum limits (MLs) for certain mycotoxins in several food products. As a complementary tool, the Panel on Contaminants in the Food Chain (CONTAM Panel) have proposed tolerable daily intake values (TDIs) that establish the maximum quantity of a certain mycotoxin that can be daily ingested over a lifetime without an appreciable health risk. Traditionally, the exposure to mycotoxins have been estimated through the combination of consumption surveys and contamination data after analyzing food matrices, but current approaches are evolving towards more accurate and individualized strategies. Human biomonitoring represents an alternative tool that can provide reliable results through the direct measurement of mycotoxins and/or their metabolites in biological samples. Therefore, the present doctoral thesis focused on the development of analytical methodologies based on liquid chromatography coupled to either quadrupole-time of flight or quadrupole-Orbitrap high resolution mass spectrometry for the simultaneous determination of multiple mycotoxins in food and biological matrices for later exposure assessment.

Out of the 85 food samples analyzed, 60 tested positive for at least one mycotoxin (70%) but at low concentration levels (< 50 ppb). Co-occurrence of

mycotoxins was detected in 42 food samples, meaning the 70% of the total positive samples. The most common co-occurrence events included zearalenone alongside its derived forms and combinations of enniatins. Exposure assessment based on consumption and contamination data highlighted that daily intake of alternariol, alternariol monomethyl ether and zearalenone and its derived forms might not represent a health concern by themselves, but their contribution to the total exposure should be taken into consideration specially with susceptible cohorts like children.

Human biomonitoring consisted in the analysis of two different matrices: urine and hair as a novel matrix. Analysis of human urine revealed the presence of either citrinin or its metabolite dihydrocitrinone in 142 out of 300 samples (47%), whereas T-2 and HT-2 were detected in 63 (21%) and 90 (30%) samples, respectively. Exposure assessment based on the measurement of urinary citrinin biomarker revealed an exposure accounting for 8% to 40% of the tolerable daily intake, being children (< 18 years old) the most exposed population group. Although no correlation between the measured levels and the dietary intake can be established for T-2 yet, a high prevalence of the T-2 hydroxylated metabolites, especially 3-hydroxy-T-2 toxin, occurring in 299 out of 300 samples (99.7%) was observed.

Regarding hair samples, frequent contamination was observed with 43 out of 100 samples testing positive for at least one mycotoxin, being enniatins and aflatoxin B1 the most prevalent compounds. Concentration values ranged from 2.7 to 106 ng/g and corresponded to a cumulative exposure over five months

according to the length of the samples. Retrospective non-targeted screening in hair samples revealed the presence of 128 mycotoxins, including some relevant compounds such as patulin (85%). Therefore, the here-developed and validated methodology resulted to be useful for a broad range of mycotoxins and revealed hair as a potential biological matrix for monitoring their accumulation.

RESUMEN

Las micotoxinas son metabolitos secundarios tóxicos producidos por varios géneros de hongos y que se encuentran frecuentemente en productos alimenticios. La ingestión de micotoxinas puede provocar la aparición de un amplio abanico de efectos perjudiciales para la salud. Con el objetivo de reducir el impacto sanitario debido a la ingesta de productos contaminados por micotoxinas, la Comisión Europea estableció la Regulación No 1881/2006, donde se fijan límites máximos para ciertas micotoxinas en varias tipologías de productos alimenticios. Como herramienta complementaria, el Panel de Contaminantes en la Cadena Alimentaria (CONTAM Panel) han propuesto valores de ingesta diaria tolerable, que establecen la cantidad máxima de una micotoxina que puede ser ingerida diariamente sin que entrañe un riesgo apreciable para la salud. Tradicionalmente, la exposición a micotoxinas se ha estimado a través de la combinación de encuestas de consumo y datos de contaminación tras analizar matrices alimentarias, pero los enfoques actuales están evolucionando hacia estrategias más precisas e individualizadas. La biomonitorización en humanos representa una herramienta alternativa que puede proporcionar resultados fiables a través de la medida directa de una micotoxina y/o sus metabolitos contenidos en una muestra biológica. Así pues, la presente Tesis Doctoral se ha enfocado en el desarrollo y optimización de metodologías analíticas basadas en cromatografía líquida acoplada a espectrometría de masas de alta resolución con analizadores cuadrupolo-tiempo de vuelo y cuadrupolo-Orbitrap para la determinación simultánea de múltiples micotoxinas contenidas en matrices

alimentarias y biológicas para, finalmente, realizar una evaluación de la exposición.

De las 85 muestras alimentarias analizadas, 60 mostraron contaminación por al menos una micotoxina (70%), aunque a bajos niveles de concentración (< 50 ppb). Además, en 42 muestras (70% del total de muestras positivas) se detectó la presencia simultánea de varias micotoxinas, debido mayoritariamente a la combinación de zearalenona junto a sus formas derivadas o a la combinación de eniatinas. La evaluación de la exposición basada en datos de contaminación y consumo puso de manifiesto que la ingesta de alternariol, alternariol monometil éter y zearalenona junto a sus derivados no suponen un riesgo debido al consumo de las matrices analizadas, pero su contribución a la exposición total debe ser tomada en consideración especialmente en grupos de población susceptibles como niños.

La biomonitorización humana consistió en el análisis de dos matrices distintas: orina y pelo, esta última escasamente estudiada. Tras el análisis de orina humana, se detectó la presencia de, por un lado, citrinina o su metabolito dihidrocitrinona en 142 de 300 muestras (42%), mientras que las toxinas T-2 y HT-2 se detectaron en 63 (21%) y 90 (30%) muestras, respectivamente. La evaluación de la exposición a través del biomarcador de citrinina en orina reveló una exposición equivalente al 8-40% del valor de ingesta diario tolerable, siendo niños (< 18 años) el grupo de población más expuesto. Por otro lado, aunque actualmente no es posible realizar una correlación entre los niveles urinarios de T-2 y su ingesta, se detectó un alto número de muestras con metabolitos

hidroxilados de T-2, especialmente 3-hidroxi-T-2, que fue identificado en 299 de las 300 muestras analizadas.

La contaminación fue frecuente también en pelo, con al menos una micotoxina en 43 de las 100 muestras analizadas y siendo eniatinas y aflatoxina B1 los compuestos mayoritarios. Los valores de concentración oscilaron entre 2.7 y 106 ng/g, que se corresponden con una exposición acumulada durante 5 meses de acuerdo con la longitud de las muestras recogidas. El estudio retrospectivo no dirigido identificó la presencia tentativa de 128 micotoxinas, entre las que se encuentran algunas relevantes como patulina (85%). Así pues, la presente metodología desarrollada y validada en pelo resultó ser útil para un amplio abanico de micotoxinas y refuerza el posible de uso de esta matriz para monitorizar la acumulación de estos contaminantes.

SOMMARIO

Le micotossine sono metaboliti tossici secondari prodotti da diverse specie di funghi, principalmente appartenenti ai generi *Aspergillus*, *Penicillium*, *Alternaria* e *Fusarium*. Questi funghi sono in grado di colonizzare le colture agricole e produrre metaboliti secondari durante le pratiche di raccolta, lavorazione e conservazione, e di conseguenza essere presenti nei prodotti alimentari. La presenza di metaboliti tossici negli alimenti e nei mangimi e l'ingestione degli stessi rappresenta un problema di interesse globale per la salute umana a causa degli effetti tossici delle micotossine. Al fine di ridurre l'impatto sulla salute, il regolamento della Commissione n. 1881/2006, pubblicato dalla Commissione europea, stabilisce i limiti massimi (LMR) per alcune micotossine in diversi prodotti alimentari. Come strumento complementare, il gruppo di esperti scientifici sui contaminanti nella catena alimentare (gruppo CONTAM) ha proposto valori di assunzione giornaliera tollerabile (TDI) che stabiliscono la quantità massima di una determinata micotossina che può essere ingerita quotidianamente nel corso della vita senza un rischio apprezzabile per la salute. Tradizionalmente, l'esposizione alle micotossine è stata stimata attraverso la combinazione di indagini di consumo e dati di contaminazione dopo aver analizzato le matrici alimentari, ma gli approcci attuali si stanno evolvendo verso strategie più accurate e individualizzate. Il biomonitoraggio umano rappresenta uno strumento alternativo che potrebbe fornire risultati affidabili attraverso la misurazione diretta delle micotossine e/o dei loro metaboliti in campioni biologici.

Pertanto, la presente tesi di dottorato si è concentrata sullo sviluppo di metodologie analitiche basate sulla cromatografia liquida accoppiata alla spettrometria di massa ad alta risoluzione quadrupolo-tempo di volo o quadrupolo-Orbitrap per la determinazione simultanea di più micotossine in matrici alimentari e biologiche ed una successiva valutazione dell'esposizione.

Degli 85 campioni alimentari analizzati, 60 sono risultati positivi ad almeno una micotossina (70%) ma a bassi livelli di concentrazione (< 50 µg/kg). La co-presenza di micotossine è stata rilevata in 42 campioni, corrispondenti al 70% del totale dei campioni positivi. Gli eventi di co-presenza più comuni includevano lo zearalenone insieme alle sue forme derivate e alle combinazioni di enniatine. La valutazione dell'esposizione basata sui dati di consumo e contaminazione ha evidenziato che l'assunzione giornaliera di alternariolo, alternariolo monometil etere e zearalenone e le sue forme derivate potrebbero non rappresentare un problema per la salute di per sé, ma il loro contributo all'esposizione totale dovrebbe essere preso in considerazione soprattutto per i gruppi suscettibili della popolazione, di fatti i bambini risultano maggiormente esposti a livelli più alti di potenziali sostanze tossiche.

Il biomonitoraggio umano ha riguardato l'analisi di due diverse matrici: urina e capelli. L'analisi delle urine umane ha rivelato la presenza di citrinina o del suo metabolita diidrocitrinone in 142 campioni su 300 (47%), mentre T-2 e HT-2 sono stati rilevati in 63 (21%) e 90 (30%) campioni, rispettivamente. La valutazione dell'esposizione basata sulla misurazione del biomarcatore urinario della citrinina ha rivelato un'esposizione che rappresenta dall'8% al 40% della dose giornaliera

tollerabile. Sebbene non sia ancora possibile stabilire una correlazione tra i livelli misurati e l'assunzione con la dieta per il T-2, è stata osservata un'alta prevalenza dei metaboliti idrossilati del T-2, in particolare la tossina 3-idrossi-T-2, presente in 299 campioni su 300 (99,7%).

Per quanto riguarda i campioni di capelli, è stata osservata una contaminazione frequente: 43 campioni su 100 sono risultati positivi ad almeno una micotossina, le enniatine e l'aflatossina B1 sono stati i composti più prevalenti. I valori di concentrazione variavano da 2,7 a 106 ng/g e corrispondevano a un'esposizione cumulativa valutata su cinque mesi in base alla lunghezza dei capelli. Lo screening retrospettivo non mirato nei campioni di capelli ha rivelato la presenza di 128 micotossine, compresi alcuni composti rilevanti come la patulina (85%). Pertanto, la metodologia sviluppata e validata è risultata utile per l'analisi di un'ampia gamma di micotossine ed ha rivelato che i capelli sono una potenziale matrice biologica da utilizzare per il monitoraggio d'accumulo da micotossine.

1. INTRODUCTION

1. Introduction

1.1. Mycotoxins

Mycotoxins are toxic compounds resultant from the secondary metabolism of several filamentous fungi, mainly *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria* species. These are low-molecular weight molecules (< 1,000 Da) with variable structural features, so their chemical behavior in terms of solubility, stability, reactivity and toxicity highly differs from each other. Currently, more than 400 mycotoxins have been reported and identified.

Mycotoxins can naturally occur in cereals, tree nuts, spices, cocoa, coffee, fruits and vegetables that are susceptible to fungal contamination. Therefore, mycotoxigenic fungi can colonize crops at some point from pre-to-post-harvest stages (storage, distribution and processing) and its growth can be promoted by environmental conditions such as pH, temperature, water activity or light. During the final stages of the exponential growth phase and the beginning of the stationary phase, these fungi can also produce mycotoxins. Although the optimal conditions for fungal growth are not always indicative of mycotoxin production, the appearance of mycotoxins is tightly linked to temperature (~25-30 ºC) and water activity (~0.95-0.99) (Moretti and Susca, 2017).

The Food and Agricultural Organization of the United Nations (FAO) released a statement affirming that a 25% of crops worldwide are contaminated with mycotoxins. Although this sentence has been widely cited, it must be considered alongside the context it was released in, when detection techniques

lacked sensitivity and thus reflecting an underestimation of mycotoxins. Currently, this figure has been estimated to be 65-90% given the new articles published over the last decade.

It is possible that mycotoxins occurring in crops and raw materials are also present within the final products from the food industry, considering that they can withstand the whole transformation process to a greater or lesser extent. Processing of raw materials includes a series of unit operations that can influence the overall content of mycotoxins, but it does not necessarily imply a reduction. Primary processing, meaning those operations intended for transforming raw materials into food commodities (sorting, washing, milling, dehulling…), can redistribute the total burden of mycotoxins towards different fractions of the material, mainly by-products. Secondary processing consists in adding value to food commodities through other chemical, physical or biological operations such as extrusion cooking, pressure cooking, baking, fermentation and roasting or frying, among others. The impact of these secondary operations is mycotoxin-dependent considering the differences in stability, and their concentration can even be increased by specific operations, such as the fermentation of dough in bread making (Mousavi Khaneghah et al., 2018). Nevertheless, the complete removal of mycotoxins throughout the whole food processing is rarely achieved (Karlovsky et al., 2016). Besides being present in susceptible crops, raw materials and derived-products, mycotoxins have also been reported in animal products as a consequence of the carry-over of mycotoxins contained in feeds (Montanha et al., 2018; Tolosa et al., 2020).

Consumption of food products contaminated by mycotoxins can represents a health concern for humans according to their reported toxicity. The acute or chronic diseases caused by mycotoxins are generally known as mycotoxicosis, whose symptomatology and target organ depend on the chemical features of each mycotoxin. Different toxic effects have been attributed to mycotoxins, such as immunosuppression, neurotoxicity or intestinal disorders, that have been related to chronic dietary exposure. However, the most concerning aspect of their toxicity relies on their carcinogenic potential. The International Agency for Research on Cancer (IARC) started a Monograph program for collecting and evaluating the carcinogenic effects of mycotoxins in humans. According to their carcinogenic potential, some mycotoxins were included in a classification as carcinogenic to humans (group 1), probably carcinogenic to humans (group 2A), possibly carcinogenic to humans (group 2B) and not classifiable as to its carcinogenicity to humans (group 3). Table 1 shows the number of mycotoxins included in each of the mentioned groups.

Table 1. IARC Monograph classification of mycotoxins

IARC group	Number of mycotoxins
1. Carcinogenic to humans	5
2. Probably carcinogenic to humans	0
3. Possibly carcinogenic to humans	5
4. Not classifiable as to its carcinogenicity to human	12

Nevertheless, the impact of a certain mycotoxin is not only determined by its toxicity but also by how spread it is. The ubiquitous character of some mycotoxins is a major reason for investigation, even if they are not included in IARC classification or their toxicity in humans is still under investigation.

1.1.1. Aflatoxins

Aflatoxins (AFB1, AFB2, AFG1, AFG2) are the most widely investigated mycotoxins regarding their occurrence and toxicity since they were classified in IARC group 1 as carcinogenic to humans. In fact, AFB1 is the most potent natural carcinogen in mammals. Chemically, the structural core of AFs is based on a di- or tetra-hydro-difuran ring linked to a coumarin with five or six carbons (Figure 1).

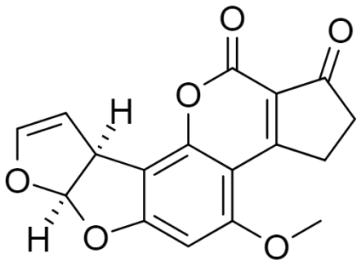

Figure 1. Aflatoxin B1

These mycotoxins are mainly produced by *Aspergillus flavus* and *A. parasiticus*, which are especially prevalent in tropical and subtropical areas that can provide high relative humidity (90%) and temperature (optimum temperature 32 ºC). These species are common pathogens of a broad range of crops including cereals (maize, barley, sorghum, rice and oat), tree nuts (pistachios, walnuts, almonds and peanuts) and spices. AFs have been reported in food products

derived from those crops, reflecting a considerable stability to the routinary processes within the food industry. Despite it is not a naturally-occurring but a metabolic product, AFM1 stands as another relevant compound from the AF family that can also be present in food products, especially in milk (Alshannaq and Yu, 2017).

The current toxicological knowledge is mainly focused in AFB1. This mycotoxin is metabolized by the p450 enzymes into its epoxidized forms, that are suggested to display mutagenic properties by forming adducts with DNA and altering gene expression and that can also bind to RNA and proteins. Throughout the mentioned mechanisms, acute exposure to AFB1 can lead to hepatic hemorrhage necrosis and edema, whereas effects derived from chronic exposure include immunosuppression, mutagenicity and carcinogenicity (EFSA CONTAM Panel, 2020a) Therefore, the IARC has included AFs in group 1 whereas AFM1 has been placed into group 2B.

1.1.2. Ochratoxin A

Ochratoxin A (OTA) is a pentaketide originated from the dihydrocoumarins family linked to a β-phenylalanine (Figure 2).

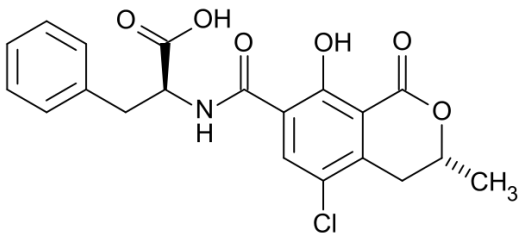

Figure 2. Ochratoxin A

Several species from different fungi genera are able to produce OTA, primarily *Aspergillus ochraceus* and *Penicillium verrucosum*, although other species such as *A. carbonarius* and *A. niger* can also be relevant according to the environmental conditions in a geographical area. Occurrence of OTA in low-temperated zones is usually related to *Penicillium* whereas *Aspergillus* is more common in hot and wet areas. Contamination with OTA have been detected in many different food commodities, including cereals (maize, barley, wheat and rice), beer, grapes/wine, coffee, tree nuts and spices (El Khoury and Atoui, 2010).

Toxicity of OTA is based on the increase of NADPH and the activity of p450 enzymes promoting the caspase activating pathway and inducing apoptotic processes. Additionally, OTA provokes oxidative stress in mitochondria and reticulum endoplasmic. All these mechanisms are responsible for the toxic effects of OTA, including neurotoxicity, teratogenicity, immunotoxicity and especially, nephrotoxicity, since kidney is its target organ (Tao et al., 2018). In this line, the appearance of renal tumors in mice and rats after exposure to OTA led to its classification within the group 2B in the IARC classification.

1.1.3. Patulin

Patulin (PAT) is mycotoxin structurally based on an unsaturated heterocyclic lactone (Figure 3). Several species belonging to *Aspergillus*, *Byssochlamys*, *Euenicillium*, *Penicillium* and *Paecylomyces* are able to synthesize PAT, but *P. expansum* is considered to be the major PAT-producing fungus. It has to be highlighted that PAT can be produced even in refrigeration conditions (Saleh and Goktepe, 2019). Although all these species can colonize fruits, vegetables, cereals

and feeds, the presence of PAT is especially remarkable in apples and apple-derived products, including juices and jam.

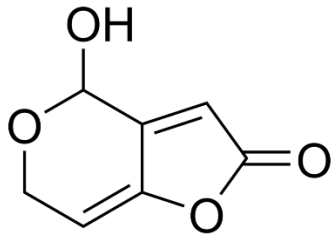

Figure 3. Patulin

From a toxicological perspective, PAT is a harmful mycotoxin that exerts its bioactivity by binding to sulfhydryl groups contained in many enzymes, including ATPase, RNA polymerase or lysosomal enzymes, among others. Chronic exposure to PAT has been related neurotoxicity, immune disorders and gastrointestinal effects by modifying the intestinal flora composition (Pal et al., 2017). This mycotoxin has been included in group 3 of the IARC classification since there are not enough evidence supporting its carcinogenic effects.

1.1.4. Citrinin

Citrinin (CIT) is a quinone with a planar structure that contains conjugated bonds (Figure 4).

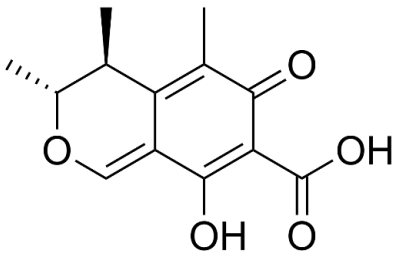

Figure 4. Citrinin

Many different fungal species can produce CIT, comprehended within *Aspergillus*, *Penicillium* and *Monascus* genera. Among them, *M. purpureus* stands a major CIT-producing fungus that is used for the obtention of red yeast rice, a widely consumed product in many parts of Asia. Although CIT has been reported in several grains (wheat, barley and rye), fruits and fruit juices, vegetables, beans, herbs and spices, red yeast rice represents the most prone food product to contamination with CIT (López Sáncheza et al., 2017).

Multiple mechanisms are apparently involved in CIT toxicity: increasing of reactive oxygen species (ROS), inhibition of DNA and RNA synthesis, activation of caspase-cascade system and inactivation of the HSP90 multichaperone complex. Consequently, genotoxic, hepatotoxic and teratogenic activity has been attributed to CIT (de Oliveira Filho et al., 2017). Nevertheless, due to the limited data on its carcinogenicity, CIT has been placed in group 3 of the IARC classification.

1.1.5. Fumonisins

Fumonisins (FBs) include a group of mycotoxins with a core structure based on two tri-carboxylic acid side chains esterified to an amino-mono-or-polyol chain. The most relevant FBs are FB1 (three hydroxyl groups), FB2 (two hydroxyl groups), FB3 (two hydroxyl groups) and FB4 (one hydroxyl group).

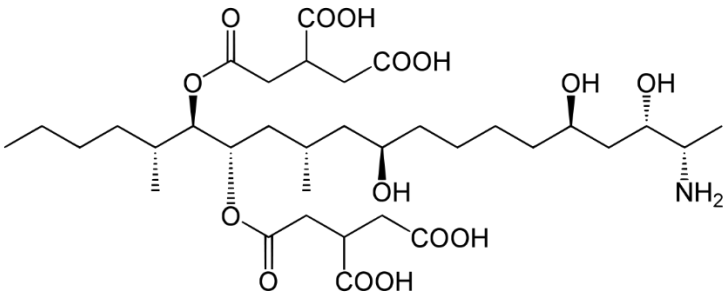

Figure 5. Fumonisin B1

Mycotoxins belonging to FBs family are produced by *Fusarium* species, especially *F. proliferatum* and *F. verticillioides*. Although these fungi are ubiquitously distributed worldwide, but FBs are mainly found in corn, soy, peas, raisins, wine and beer (Braun and Wink, 2018).

The toxicity of FBs is due to their structural resemblance to sphingosine and sphinganine, provoking a competitive inhibition of ceramide synthase and therefore interfering in *de novo* ceramide biosynthesis and in CoA-dependent acylation of sphinganine (Smith, 2018). Chronic exposure to FBs, primarily FB1 and FB2, has been related to esophageal and liver cancer, so they have been introduced within the IARC classification 2B.

1.1.6. Deoxynivalenol

Deoxynivalenol (DON) belongs to the trichothecene type B family, which are a group of compounds presenting a tricyclic core structure with a double bond in C9-C10, an epoxy group between C12-C13 and a ketone group in C8 (Figure 6). DON is regarded as the most important type B trichothecene.

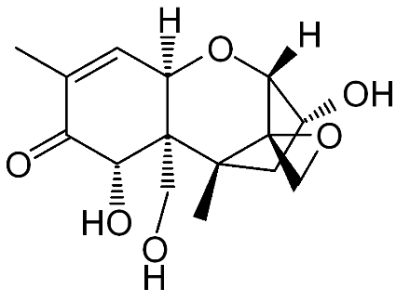

Figure 6. Deoxynivalenol

DON is produced by *Fusarium* species, primarily *Fusarium culmorum* and *Fusarium graminearum*, which can colonize a broad range of cereals especially wheat, corn, barley, oat or rice, among others, and its presence has also been repeatedly reported in derived products such as breakfast cereals, bread or salty snacks (Mishra et al., 2020). This fact remarks a high thermal and pH-dependent stability of DON.

The toxicity attributed to DON is due to its to promote the trans-activation of pro-inflammatory cytokines and several mitogen-activated kinases (MAPKs) disrupting the activity of the ribosomes (Pierron et al., 2016). These mechanisms reflect disorders on the immune system and severe damages in the intestinal barrier (EFSA CONTAM Panel, 2017c). According to the available information about its carcinogenic potential, DON has been classified into IARC group 3.

1.1.7. T-2 toxin

T-2 toxin (T-2) is considered as the major mycotoxin within the trichothecenes type A family, which present the same core structure that type B with the particularity that the ketone group in position C8 is substituted by a different functional group (Figure 7).

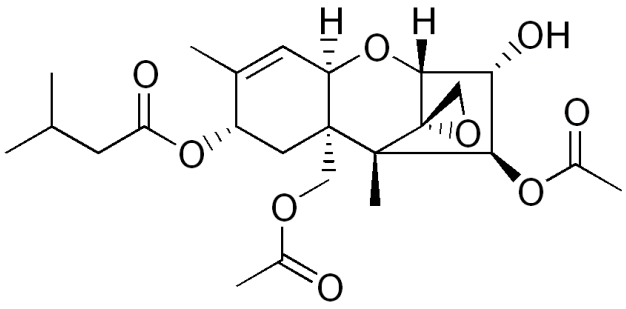

Figure 7. T-2 toxin

As a trichothecene, T-2 is produced by *Fusarium* species including *Fusarium soprotrichioides*, *Fusarium poae* and *Fusarium acuinatum* that usually appear in cold climate regions or under wet conditions. These fungi can be present in corn, wheat and oats so T-2 has been mainly reported in those cereals and derived-products often co-occurring with HT-2, another type A trichothecene (van der Fels-Klerx and Stratakou, 2010).

T-2 exerts its toxicity through several mechanisms: immunotoxicity has been reported through the upregulation of pro-inflammatory genes and the mRNA expression of several interleukins, whereas the production of ROS inducing apoptotic processes seems to be related with reproductive and hepatotoxicity (Wu et al., 2020). Similarly to DON, T-2 is included in group 3 of the IARC classification.

1.1.8. Zearalenone

Zearalenone (ZEN) is a macrocyclic β-resorcyclic acid lactone (Figure 8) that can be metabolized by the host plant into its derived forms including α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL).

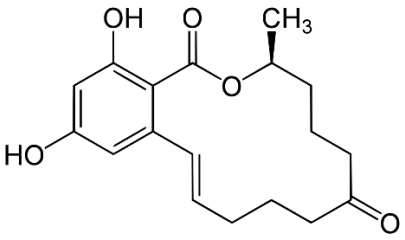

Figure 8. Zearalenone

ZEN is produced mainly produced by *Fusarium graminearum*, *Fusarium culmorum*, *Fusarium cerealis* and *Fusarium semitectum* that grows at the same conditions of trichothecenes-producing *Fusarium* species, so it is very common to find those mycotoxins co-occurring. ZEN usually occurs in corn and corn-derived products although it can also be found in other typology of cereals including barley, oat, wheat, rice and sorghum (Mahato et al., 2021).

The toxicity of ZEN and its derived forms is based on its structural resemblance to several estrogenic molecules, so they can bind the corresponding receptors creating an hormonal imbalance that provokes reproductive disorders (Kowalska et al., 2016). As a consequence of its estrogenic activity, ZEN was related to breast cancer in humans by few publications, but since causal relationship was not reported, ZEN is still included within group 3 of IARC classification.

1.1.9. Emerging Fusarium toxins

The so-called "emerging *Fusarium* toxins" are a group of compounds mainly represented by the family of enniatins (ENNA, ENNA1, ENNB and ENNB1) and beauvericin (BEA). The name was given more than ten years ago considering the lack information about their role as mycotoxins, but evidence about their prevalence and toxicity have been rapidly collected during the following years

(Gruber-Dorninger et al., 2017). Structurally, ENNs (Figure 9) and BEA (Figure 10) are closely related presenting a cyclic hexadepsipeptide-based organization.

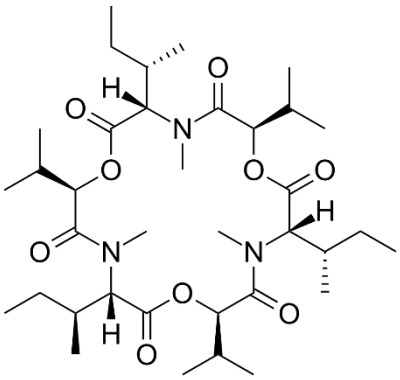

Figure 9. Enniatin A

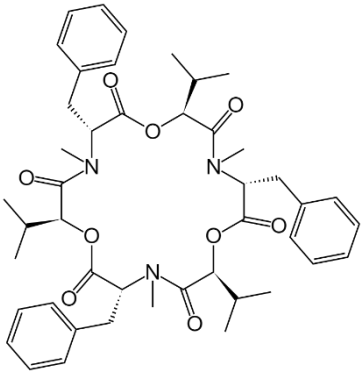

Figure 10. Beauvericin

Among the several *Fusarium* species that can produce these toxins, *Fusarium avenaceum* represents a major fungus able to synthesize up to six different ENNs and BEA. Emerging *Fusarium* toxins are extensively distributed worldwide although they preferably occur in grains, mainly wheat- and corn-derived products (Gautier et al., 2020).

The reported toxicity is related to their ionophoric character. These mycotoxins can insert into the cell membrane and form cation-selective pores, therefore altering the cell homeostasis (Fraeyman et al., 2017). Therefore, ENNs and BEA have been reported to exert immunomodulating effects, whereas genotoxicity of BEA and ENNB1 has also been confirmed (Maranghi et al., 2018). Nevertheless, the toxicity is still under investigation and, consequently, the IARC has not included these mycotoxins in their classification.

1.1.10. Alternaria toxins

Alternaria toxins comprehend a broad range of mycotoxins but alternariol (AOH) and alternariol monomethyl ether (AME) represents the more concerning compounds for humans. Both toxins share a common structure based on a dibenzopyrone core (Figures 11 and 12).

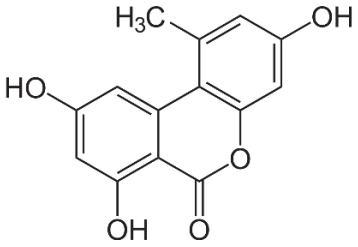

Figure 11. Alternariol

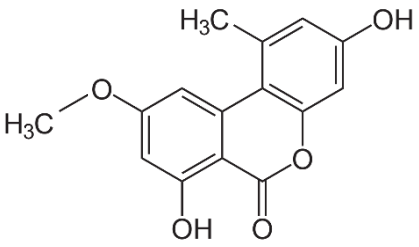

Figure 12. Alternariol monomethyl ether

Several fungi can produce these mycotoxins including *Alternaria alternata* *Alternaria arborescens* and *Alternaria tenuissima*, among others. This fungi can simultaneously produce AOH and AME, being extensively reported in cereals, mainly corn, wheat, rice and sorghum but also in tomato, sunflower seeds, fruits and fruits juices (Chen et al., 2021).

Similar mechanism of toxicity has been reported for both mycotoxins, based on their ability to alter the function of topoisomerases and promote ROS production. Therefore, genotoxic and mutagenic effects has been observed in esophageal cells (Tiessen et al., 2017), but since no clear evidence of its carcinogenicity has been reported, AOH and AME have not been listed within the IARC classification.

1.2. Regulatory framework

The presence of mycotoxins in food products represents a global concern for human health, so authorities, in cooperation with scientific committees, have developed a regulatory system that oversees the impact of mycotoxins in society throughout several mechanisms. The Joint FAO/WHO Expert Committee on Food Additives (JECFA), at a worldwide level, and the European Food Safety Authority (EFSA) throughout the Panel on Contaminants in the Food Chain (CONTAM Panel), that represents its European analog, are examples of scientific institutions in charge of the food and feed surveillance and toxicological research on mycotoxins, among other food contaminants. These institutions release routinary reports that can be derived to the competent authorities, such as the European Commission (EC), to legislate according to the recommendations reflected in the reports.

One of those mechanisms is based on the application of maximum limits (MLs) for certain mycotoxins occurring in certain processed or unprocessed foodstuffs. These levels are resultant from an evaluation based on toxicological data, susceptibility of each product to accumulation of mycotoxins and target population of a specific product. The current law establishing MLs is Commission Regulation (EC) No 1881/2006; nonetheless, this is not a static regulation since it has been subsequently amended as new evidence on toxicity and occurrence have been collected over the years. Table 2 shows the current MLs for each mycotoxin and foodstuffs.

Table 2. Maximum limits (MLs) for mycotoxins in foodstuffs

Mycotoxin	Foodstuffs	ML (ng/g)
AFB1	Tree nuts, dried fruits, cereals, spices (chillies, chilli powder, cayenne, paprika, nutmeg, ginger, turmeric), cereal-based baby food, dietary foods for medical purposes	0.1-8.0
AFM1	Raw, heat-treated and milk for further processing	0.05
Sum of AFs	Tree nuts, dried fruits, cereals and derived products, spices (chillies, chilli powder, cayenne, paprika, nutmeg, ginger, turmeric, white and black pepper), cereal-based baby food, dietary foods for medical purposes	4.0-15.0
OTA	Cereals and derived products, dried vine fruit, coffee, wine, grape juice, cereal-based baby food, dietary foods for medical purposes, spices (chillies, chilli powder, cayenne, paprika, nutmeg, ginger, turmeric, white and black pepper), liquorice	0.5-80
PAT	Fruit juices, drinks and solid products derived from apple, cereal-based baby foods	10-50
DON	Cereals and derived products including cereal-based baby foods	200-1,250

Mycotoxin	Foodstuffs	ML (ng/g)
ZEN	Cereals and derived products including maize-based baby foods and refined maize oil	20-400
Sum of FBs	Unprocessed maize, maize-derived products including baby foods	200-4,000
CIT	Food supplements based on rice fermented with red yeast *M. purpureus*	100
Ergot scletoria and ergot alkaloids	Unprocessed cereals except maize and rice	500

In order to ensure a reliable evaluation of the MLs, Commission Regulation (EC) No 401/2006, partially modified by Commission Regulation (EU) No 178/2010, sets a guideline for analysis of mycotoxins in food matrices. These criteria aimed to achieve a representative sampling and a precise quantification depending on the targeted mycotoxin.

Whenever any non-compliance of these MLs in foodstuffs or feeds is detected, a whole system triggers in order to notify the incidence, track the affected products and take actions depending on the seriousness of the matter. The Rapid Alert System for Food and Feed (RASFF) allows a quick flow of information among EU and several other countries on any food or feed

contaminant that can pose a risk to public health. The process starts with the detection of an incidence by a border control, official analysis of marketed products, companies-own checks, consumer complaints or after the report of a food poising. Then, the incidence is notified and included within the RASFF database providing the following information: type of mycotoxin (or contaminant), type of product, origin of the contaminated products, distribution status of the product across the market and action taken.

The annual report released by the RASFF classifies all the notifications collected during the whole year in terms of food products, type of hazards, etc. Therefore, these reports provide crucial information on food safety and encourage the research on those hazards that pose the highest risk to public health. The last report from the RASFF included notifications collected during 2020, reflecting a total of 424 notifications due to the presence of mycotoxins, in most cases due to the detection of AFB1 in tree nuts. Mycotoxins represent the third most relevant hazard during 2020 right after pathogenic microorganisms and pesticide residues (EC, 2021). However, if the same number of mycotoxin analysis were carried out as those of microorganisms or pesticides, they would very possibly occupy the first position. The worldwide relevance of mycotoxins in a food safety context is evidenced by the number of notifications over the last ten years (Figure 13), meaning that they still represent a major hazard that requires attention from the scientific community and regulatory authorities.

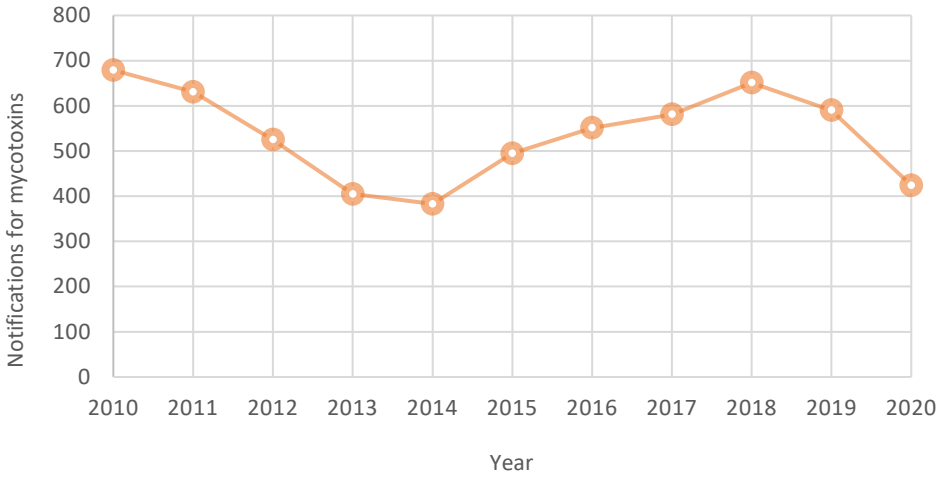

Figure 13. Number of notifications for mycotoxins collected in the RASFF database over the last ten years

This system ensures a proper control of mycotoxins that occur in food products above their corresponding MLs, but it does not consider alternative situations when the presence of regulated mycotoxins complies the current legislation or when non-regulated mycotoxins are present in a food product. This fact translates into a potential exposure to mycotoxins throughout the consumption of contaminated food products. Hence, another tool has been established in order to assess the exposure to mycotoxins through dietary intake. The tolerable daily intake (TDI) values establish the maximum quantity of a certain mycotoxin that can be daily ingested over a lifetime without an appreciable health risk. These values comprehend a toxicological evaluation of the chronic effects of mycotoxins after *in vivo* experiments, determining the no observed adverse effect level (NOAEL) or the lowest observed adverse effect level (LOAEL) and dividing it by an uncertainty factor.

According to the type of mycotoxin and its attributed toxicity, several TDIs have been suggested by scientific committees that serve as guidance value for exposure assessment studies (Table 3). In some cases, a group-TDI is established if derived forms from a certain parent mycotoxin exhibit similar toxicity. Additionally, if these forms exert toxicity but not at the same degree as the parent toxin, a relative potency factor (RPF) is assigned to each form. The RPF is based on a comparison between the parent and the derived toxin. In other cases, when the assessed mycotoxins exert clear genotoxic and carcinogenic effects, a different approach based on the margin of exposure (MOE) is used. This margin is delimited by the benchmark dose lower confidence limit for 1% and 10% extra risk compared to the background(BMDL1 and BDML10, respectively), that account for the lower limit of 95% confidence interval referring to an incidence of liver cancer of 1% and 10%, respectively, in a control group). Lastly, when no specific toxicological data is available, the threshold of toxicological concern (TTC) replaces the TDI until further evidence is collected.

Table 3. Regulated mycotoxins for exposure assessment

Mycotoxin	Value	Reference
AFs	BMDL10 = 0.34 µg/kg bw day BMDL1 = 0.078 µg/kg bw day	(EFSA CONTAM Panel, 2007)
OTA	BMDL10 = 14.5 µg/kg bw day	(EFSA CONTAM Panel, 2020b)
CIT	TTC = 0.2 µg/kg bw day	(EFSA CONTAM Panel, 2012)
ZEN + derived forms	TDI = 0.25 µg/kg bw day (RPF system)	(EFSA CONTAM Panel, 2016)
DON + derived forms	TDI = 1 µg/kg bw day	(EFSA CONTAM Panel, 2017b)
FB1 + FB2 + FB3+ FB4	TDI = 1 µg/kg bw day	(EFSA CONTAM Panel, 2018)
T-2 + derived forms	TDI = 0.02 µg/kg bw day (RPF system)	(EFSA CONTAM Panel, 2017a)
PAT	TDI = 0.4 µg/kg bw day	(WHO, 2000)

1.3. Analysis of mycotoxins

Food safety is still regarded as a matter of concern that requires from routinary supervising in order to ensure the proper compliance of the current legislation. Analysis of food commodities becomes even more crucial considering the globalization of markets, where raw materials are collected, processed or distributed worldwide. Therefore, there is a necessity of sensitive and reliable analytical methodologies for the quantification of mycotoxins in food products. In this line, several scientific organizations, such as the Association of Official Analytical Chemists (AOAC), the European Committee for Standardization (CEN) or the International Organization for Standardization (ISO) have push towards the harmonization of mycotoxin analysis in food matrices. Currently, 72 official analytical methodologies have been validated after inter-laboratory studies for a reliable and sensitive quantification of specific mycotoxins in specific food products that could occur above the MLs set in legislation (Bessaire et al., 2019). However, all these methodologies are focused on single mycotoxins or single families (AFs, FBs or T-2+HT-2), which are useful for assessing the compliance of the legislation but fail to provide an actual overview of the impact of mycotoxins in food matrices.

The current trend in mycotoxin analysis, in both food and biological matrices, is the development of multi-mycotoxin methodologies able to provide much more information about the mycotoxins profile of a certain sample. This is especially useful not only for surveillance purposes but also when facing human exposure studies, since the co-occurrence of mycotoxin in food and biological

samples is a well-documented fact (Arce-López et al., 2020; Lee and Ryu, 2017). Hence, methodologies must be flexible enough to include as many as possible analytes without compromising the sensitivity.

Current methodologies for mycotoxin determination require from a proper sample preparation using compatible solvents for the extraction of mycotoxins, usually followed by a purification step to obtain a cleaner extract prior to quantification.

1.3.1. Sampling

Sampling represents a crucial stage that aims to collect representative samples for providing reliable outcomes that fulfil the initial purpose of the study. Therefore, a proper sampling must be an objective-driven process. A proper collection considers where, when and how to take the samples in order to have representative measurements. Several sampling methods have been developed and reflected in Commission Regulation (EC) No 401/2006 (EC, 2006) for specific food products. On the other side, when working with biological samples, other variables (age, gender, socioeconomic status, etc.) should be considered in order to extract consistent findings from a specific population. Referring to human studies, a minimum sample size of 72-120 per subgroup of population have been proposed for ensuring the statistical consistency of results (Vogel et al., 2019).

1.3.2. Extraction

The extraction process consists in the release of mycotoxins embedded into the food or biological matrix using appropriate solvents. According to the broad

polarity range of mycotoxins, choosing the right solvent will strongly contribute to the success of extraction. Acetonitrile, water, methanol and ethyl acetate are the most common solvents when dealing with liquid matrices. Ideally, the solvent should be immiscible with the matrix, but salts can also be added in order to generate a salting-out effect for inducing the separation between two miscible phases (*e.g.*, aqueous matrix extracted with acetonitrile). For solid matrices, mixtures of acetonitrile-water or methanol-water at different ratios are the most frequently used solutions. The pH of the solvent/mixture can also be modified for improving the performance in certain cases. Another factor that strongly influence the efficiency of the extraction is the time in which the solvent/mixture is in contact with the matrix. Therefore, the use of shaking, sonication or homogenization are commonly introduced within the extraction process in order to facilitate the solvent-matrix interaction.

Depending on the typology of matrix or targeted mycotoxins, samples could also require from a pretreatment stage prior to extraction. This pretreatment is frequently applied over samples solid samples (*e.g.*, tree nuts or cereals) aiming for reducing the particle size and increasing the exchange surface between matrix and solvent, as in the case of milling or grinding. For liquid samples, a dilution step may be incorporated for achieving a cleaner extract. On the other hand, enzymatic treatments are also introduced in extractive procedures for mycotoxins occurring in biological samples. The human metabolism of mycotoxins includes conjugation reactions, mainly with glucuronides, so enzymatic digestions with glucuronidases are routinely applied

over plasma, blood, serum or urine samples in order to extract and, lately, quantify the parent mycotoxin.

Currently, sample preparation is evolving towards the use of eco-friendly solvents, miniaturization implying a lesser spend of sample/solvent and quick and easy workflow for obtaining rapid results. Therefore, following those principles, conventional methodologies have been substituted for other alternatives related to microextraction and solid-phase extraction.

1.3.2.1. Solid phase extraction

The solid phase extraction (SPE) technique consists in the extraction and concentration of mycotoxins contained within a liquid matrix based on their affinity for a solid stationary phase. These materials are composed by small particles packed in columns or cartridges that are able to selectively retain mycotoxins to a greater or lesser extent. The routinely operation starts at conditioning the column using a solvent that prepare the stationary phase to actively interact with mycotoxins. Then, the liquid matrix is passes through the packed column and mycotoxins remain linked to the stationary phase. The column is washed with a solvent that can remove impurities without affecting the interaction mycotoxin-stationary phase and, lastly, another solvent is used for eluting the previously retained mycotoxins. The choice of the stationary phase and solvents used for washing and eluting is based on the mycotoxins of interest. Traditional materials for SPE are based on silica, carbon and polymeric sorbents and several companies have developed their own columns/cartridges based, as

reviewed by Fontanals et al. (2019), but other alternatives are available according to more specific mycotoxin-stationary phase interactions.

Stationary phases based on immunoaffinity (immunoaffinity columns, IACs) are commonly used for the extraction of specific mycotoxins that are recognized by monoclonal antibodies immobilized within the stationary phase. These antibodies are finally denaturated in order to disrupt the bond between the toxin and its antibody. Due to the high specificity of this interaction, procedures based on IACs can provide clean and concentrated extracts that facilitate further analysis, with the main drawback that these are not universal but single mycotoxin- or family- driven methodologies with a high cost. IACs are commonly used for the analysis of CIT, AFs, OTA, T-2 or ZEN in both food and biological samples as reviewed by Delaunay et al. (2020).

Another alternative methodology is the use of molecularly imprinted polymers (MIPs). These molecules are synthesized by co-polymerization of functional monomers into the mycotoxin of interest, serving as a template, and a cross-linker, that constitutes the scaffold. Then, the mycotoxin is removed from the structure in order to free the functional monomers and to actively work once packed into cartridges or columns. Therefore, these methodologies also provide high specificity and have been recently applied for the detection of AFs, OTA or ZEN in food matrices, as reviewed by Janik et al. (2021), and offer a great potential for further development.

1.3.2.2. Magnetic solid phase extraction

Magnetic solid phase extraction (MSPE) represents an alternative for SPE methods since it does not require from any physical support (column or cartridge). A magnetic sorbent is directly added to a liquid matrix in order to adsorb mycotoxins of interest. Then, a magnetic field (*e.g.*, external magnet) is applied for separation of sorbent and removal of the liquid matrix. Once the matrix has been removed, a compatible solvent is added to desorb mycotoxins from the magnetic sorbent. Finally, the magnetic particles are removed from the extract using a magnetic field. Common sorbents consist in magnetic nanoparticles with surface modification by activated carbon, octadecyl, carbon nanotubes, graphene or graphene oxide (Manousi et al., 2020). Recent investigations have chosen MSPE for the quantification of AFs, FBs, ZEN or emerging *Fusarium* toxins in food and biological samples (Arroyo-Manzanares et al., 2020).

1.3.2.3. QuEChERS

This methodology was firstly introduced by Anastassiades et al. (2003), who were searching for a simple methodology that allowed a multi-residue extraction from food samples. After confirming its suitability, this procedure was name after the portmanteau *Quick*, *Easy*, *Cheap*, *Effective*, *Rugged and Safe*: QuEChERS.

This procedure presents an easy workflow based on the addition of acetonitrile for the extraction of analytes followed by the addition of a magnesium sulfate or sodium sulfate in order to remove the humidity from the matrix and

achieve the migration of mycotoxins towards the organic phase. An aliquot is then collected and subjected to dispersive clean-up with octadecyl carbon chain(C18)-bonded silica (C18) and primary-secondary amine (PSA) prior to analysis.

Over the years, QuEChERS methodology has been extensively used for the extraction of mycotoxins from a broad typology of matrices. Additionally, many modifications have been introduced to the original QuEChERS in order to adapt the protocol to a specific matrix or specifics mycotoxins: substitution of acetonitrile with an acetonitrile-water system, different ratios acetonitrile-water, quantity of magnesium/sodium sulfate or selection and quantity of sorbents for clean-up.

1.3.2.4. Salt-assisted liquid-liquid extraction

The salt-assisted liquid-liquid extraction (SALLE) emerged as a miniaturized method for the extraction of mycotoxins from aqueous matrices. It consists in the addition of a great amount of salt (*e.g.*, NaCl) alongside the organic solvent so water molecules preferentially solvate the ions from salt and, therefore, inducing a quick separation between both aqueous and organic layers. The low volume of solvent used provides highly concentrated extracts, so subsequent stages of evaporation/reconcentration are not always required. Additionally, the low quantity of sample perfectly fit within a biomonitoring context, considering that the availability of biological samples can be limited. A recent example of its application is the studied conducted by Carballo et al. (2021), who validated a SALLE procedure for the quantification of DON, OTA, ZEN and their corresponding metabolites in urine samples.

1.3.2.5. Dispersive liquid-liquid microextraction

The dispersive liquid-liquid microextraction (DLLME) technique was firstly proposed by Rezaee et al. (2006). It is based on a ternary system: to an aqueous matrix, a mixture of two organic solvents is rapidly injected creating a cloudy solution of microdroplets that increase the exchange surface, so the equilibrium state is quickly reached.

Out of the two organic solvents, one act as dispersers and the other as the extractant, where mycotoxins will finally be extracted. Therefore, choosing the right system is crucial for achieving a successful extraction. The disperser solvent must be miscible with the extractant and the aqueous matrix, so the most common choices are acetonitrile, acetone or methanol. On the other side, the extractant must be miscible with the disperser but denser and immiscible with the matrix, in order to provide a proper migration of mycotoxin between the separated phases. Frequent solvents are chloroform, carbon tetrachloride or dichloromethane. The low quantity of extractant solvent provides a high concentration factor. This technique has been incorporated into recently developed methodologies for the quantification of multiple mycotoxins in food and biological samples.

1.3.2.6. Direct methods

The evolution of analytical methodologies points towards simple and generic multi-class, multi-analyte features that allow a high sample throughput at a minimum expense. Among these techniques, dilute-and-shoot (DnS) is the

most common approach. Despite DnS does not necessarily imply the extraction of mycotoxins, it has been proven to work as a standalone methodology for sample preparation.

Original DnS consists in the dilution of a liquid matrix into a suitable solvent, reducing the presence of matrix interferents, and direct introduction into a detection platform. Nevertheless, this approach has evolved over the years trying to fit the necessities of specific matrices, so several variations have been introduced into the basic DnS: liquid matrices may also incorporate a step of centrifugation or deproteinization whereas solid matrices require from a previous extraction with an appropriate solvent.

An extreme variation of DnS is the so-called filter-and-shoot (FnS), which consists in just introducing the sample into the detection platform without any previous treatment. Although this would be the ideal sample treatment, only "simple" liquid matrices allow its application. Due to its limitations, only few studies on urine have successfully applied this methodology for the quantification of mycotoxins (Heyndrickx et al., 2015).

1.3.3. Clean-up

Once mycotoxins have been released from the original matrix, the extracts usually undergo purification or clean-up in order to remove matrix interferents that could be co-extracted alongside mycotoxins. The design of the clean-up stage highly depends on the nature of the matrix and the subsequent analytical technique. In many cases, two different extraction methodologies are successively applied in order to achieve a better purification of the extracts. However, the

dispersive solid phase extraction (dSPE) is the most frequent choice. A sorbent or mixture of sorbents are directly added to the extract followed by a shaking or vortexing step, in order to retain the co-extracted interferents. Additionally, dSPE offers an easier and quicker operation and less expense of reagents compared to SPE-based clean-up methods.

Traditional dSPE sorbents in mycotoxin studies are C18, able to remove non-polar or neutral compounds especially from aqueous extracts, and PSA, that can retain organic acids, polar pigments and sugars from organic extracts. However, these materials can also adsorb mycotoxins presents in the extract when used in excess.

1.3.4. Confirmation techniques

Analytical techniques for detection/quantification require from a high selectivity and sensitivity considering that mycotoxins usually occur at ultra-trace levels. Many different methodologies are currently applied in the mycotoxin field but chromatographic separation followed by mass spectrometry (MS) technology continues to be the gold standard due to the possibility of simultaneously assess multiple mycotoxins with high selectivity and sensitivity, its relatively easy workflow and data treatment.

1.3.5. Chromatographic techniques

The main objective of chromatographic methodologies consists in the separation of the targeted analytes that will further undergo MS analysis, based on the different physicochemical properties of analytes.

1.3.5.1. Gas chromatography

Gas chromatography (GC) is a methodology that induce separation among analytes depending on their volatility and affinity for the stationary phase. Although mycotoxins are not volatile compounds, their hydroxyl groups must be derivatize with chemical agents (*e.g.*,trimethylsilyl ether) for being subjected to GC. Many studies on trichothecenes have been carried out using GC as chromatographic technique, as reviewed by López-Ruiz et al. (2019).

1.3.5.2. Liquid chromatography

Liquid chromatography (LC) represents the most widely used variant attending to its versatility, that allows the development of not only multi-analyte but also multi-class methodologies.

In the mycotoxin field, reversed-phase LC is the choice. This technique consists in the use of a hydrophobic stationary phase (*e.g.*, C18) and a mobile phase (*e.g.* acetonitrile-water or methanol-water in organic acid medium). The detectors currently used are MS for multi-mycotoxins, fluorescence for AFs and OTA and UV spectrometry for PAT.

LC-based methodologies have been of common use as shown by the considerable amount of studies published only during the last year, as reviewed by Iqbal (2021).

1.3.6. Mass spectrometry techniques

MS consists in the ionization of the analytes, contained within a sample, that are subsequently subjected to separation based on their mass to charge ratio (*m/z*) in a vacuum system. Combined with chromatographic techniques, the use of MS can provide quantitative measurements of the assayed analytes with a high selectivity at a concentration order of ppb. Several analyzers have been devised and introduced into MS platforms for the proper distinction among ions, but, based on the typology of analyzer, current MS technologies can be classified into low-resolution and high-resolution MS (LRMS and HRMS, respectively). The main differences between the two approaches consists in the mass measurement of an ion: LRMS can measure until two decimal figures whereas HRMS can reach four of five, which has crucial implications on the identification and quantification procedure. According to the Commission Decision 2002/657/EC (EC, 2002) regarding the performance of analytical methods, LRMS must include an additional fragmentation step of the ion in order to generate two characteristic product ions that will be lately monitored for having a reliable identification and quantification. This type of approach that implies fragmentation of a precursor ion is called tandem mass spectrometry (MS/MS). On the contrary, HRMS does not require from that additional step since a precursor ion can be properly identified through its exact mass measurement, although fragmentation can also be applied in another different context that will be further discussed. This section will focus on HRMS since all the studies conducted during this thesis relied on this methodology.

1.3.6.1. Low resolution mass spectrometry

LRMS methodologies imply the use of a mass detector that integrates two or more analyzers in tandem. In the mycotoxin field, triple quadrupole (QqQ) represents the most common option: the first quadrupole acts as a filter for selecting a precursor ion with a specific *m/z*; the selected precursor ion goes through the second quadrupole, the collision chamber, where a gas is injected for inducing the fragmentation; the third quadrupole acts as a mass detector of the product ions.

The use of these analyzers allows a single reaction monitoring (SRM), increasing the signal to noise ratio (S/N) by reducing the amount of chemical noise and resulting in a higher sensitivity. Therefore, the monitoring of specific transitions translates into highly selective and sensitive multi-analyte methodologies. As an example, Rodríguez-Carrasco et al. (2014) developed a GC-MS/MS methodology for quantifying fifteen mycotoxins in urine samples at concentrations down to 0.25 ng/mL.

1.3.6.2. High resolution mass spectrometry

HRMS methodologies provide a more efficient and easier workflow since the ion of interest can be directly monitored without the necessity of fragmentation. Two analyzers are mainly used in HRMS platforms, quadrupole-Orbitrap (Q-Orbitrap) and quadrupole-time-of-flight (Q-TOF).

The Q-Orbitrap technology is based on a previous filtering of the ions through a quadrupole that later enter Orbitrap compartment, which is provided

with one electrode at both ends and another electrode at a central position. Once inside, ions start to oscillate around the central electrode with their own specific frequencies that are conveniently converted into *m/z* data. If fragmentation events are also set, the precursor ions are derived into a dissociation chamber and product ions return to the Orbitrap for calculating their *m/z*.

The Q-TOF analyzers have a certain resemblance with QqQ, with two main differences: the second quadrupole is not mandatory for confirmation, so it can either work as a collision chamber or just let all the ion pass, whereas the last quadrupole is substituted by a TOF tube. Once the ions reach the tube, an electric field accelerates the ions orthogonally and then they free fall into the detector. The time until ions reach the detector is translated to *m/z*.

The use of HRMS represents a step-forward for mycotoxin analysis due to several advantages compared to LRMS. Both methodologies provide similar selectivity, accuracy and sensitivity when working through targeted approaches. Nonetheless, HRMS spectrometers can combine two different operation modes for catching the whole "picture" contained within a sample: *full scan* and *all-ion fragmentation*. This means that all the precursor ions are recorded and fragmented whereas all their corresponding product ions are simultaneously recorded too. This powerful feature allows the developing of non-targeted strategies that do not require from any analytical standard in order to determine the presence of a certain compound. Usually, this approach is supported by databases that can match the spectral data to known compounds with a determined confidence.

This HRMS feature has become especially relevant for assessing human exposure to mycotoxins throughout the occurrence of their metabolites in biological samples. In fact, non-targeted approaches are becoming a rising trend for establishing tentative metabolic profiles of mycotoxins such as ENs, DON, ZEN or OTA in biological samples from different species, as recently reviewed by Arroyo-Manzanares et al. (2021).

1.4. Exposure assessment

The White Paper on Food Safety establishes that food safety policies must be built around risk analysis, which can be conducted through a three-step process: risk assessment, risk management and risk communication. While management and communication tasks fall upon authorities, scientific research oversees the risk assessment. The main goal of risk assessment consists in establishing the probability in which a specific hazard can exert adverse effects, so food safety-driven research must respond to any of these specific parts of the risk assessment:

1. Hazard identification
2. Hazard characterization
3. Exposure assessment
4. Risk characterization

Current toxicological knowledge on mycotoxins, obtained through hazard identification and characterization, have allowed to establish reference values (TDIs) in order to characterize the risk of developing adverse effects based on the amount of mycotoxin that humans have been exposed to. Hence, exposure

assessment stands as the tool that estimates the human dietary intake of mycotoxins, that break into the food chain through contaminated food products.

Several methodologies have been developed to assess the exposure to mycotoxins, but current approaches are evolving into the obtention of individualized outcomes that provides more realistic estimations.

1.4.1. Total diet studies

Total diet studies (TDS) represent the traditional way of estimating the exposure to mycotoxins. These studies are based on the combination of two different datasets: occurrence of mycotoxins in food products and food consumption surveys. TDS include the multi-mycotoxin analysis of all representative food products included in the diet, whereas the intake is later calculated using consumption surveys. Usually, these studies take place at national levels, thus using national consumption surveys such as ENALIA and ENALIA 2 in Spain or the Italian National Consumption Survey INRAN-SCAI that establish consumption values per food categories. Nevertheless, considering the dimensions of these studies, TDS are usually simplified to the analysis of mycotoxins in a certain food product. Recent examples of TDS on mycotoxin are those conducted by Vin et al. (2020) in France or Ingenbleek et al. (2019) in Sub-Saharan Africa.

These traditional approaches present several drawbacks and its scope is considerably limited. Each dataset is somehow biased: consumption data assume homogeneous contamination throughout the whole sampling belonging to the

same food category whereas consumption data assume homogeneous dietary patterns, even if data is segmented by gender or age ranges.

1.4.2. Meta-analysis

Meta-analysis have emerged as a novel approach for exposure assessment studies that collect contamination data reported by different studies and, using statistical tools, integrate all those results into a single outcome with a determined confidence. Then, these values are combined with consumption data for obtaining values of exposure. Although meta-analysis implies a more computational workflow, it follows the same principle as TDS, so same limitations are also expected. Recent meta-analysis have estimated the exposure to DON through cereal-based products consumption in Spain (Narváez et al., 2022) or to AFM1 in Middle East (Rahmani et al., 2018). Considering that this is a computational tool with a quick, easy workflow and statistical consistency, it could represent a complementary approach for future mycotoxin studies.

1.4.3. Human biomonitoring studies

Human biomonitoring (HBM) studies represent the most promising approach for assessing the exposure to mycotoxins. Once ingested, mycotoxins undergo several metabolic pathways for achieving the complete elimination from the organism, so either mycotoxins or their corresponding metabolic products can be traceable in different biological matrices. As opposed to the uncertainties of TDS, HBM stands as a step-forward methodology that provides individual measurements of exposure throughout the analysis of single samples.

In terms of the matrix of choice, HBM on mycotoxins have usually relied on blood (plasma or serum) and urine. Although urine is the preferred matrix due to its easy and non-invasive collection, their applicability is determined by the scope of the investigation: urine is suitable for assessing short-term exposure (1-3 days) whereas blood can provide information about short-to-medium term exposure (up to 3 months).

The use of HBM is based on biomarkers of exposure, which can be mycotoxins themselves, metabolites or other compounds whose presence in biological samples can quantitatively relate to the ingested dose. Potential biomarkers of exposure must be subjected to a strict validation process, so very few are currently available, as shown in Table 3.

Table 3. Validated biomarkers of exposure to mycotoxins

Mycotoxin	Biomarker	Matrix	Reference
AFB1	AFM1	Urine	Zhu et al. (1987)
	AF-N7-Guanine	Urine	Groopman et al. (1993)
FB1	FB1	Urine	van der Westhuizen et al. (2011)
OTA	OTA	Urine	Gilbert et al. (2001)
		Plasma	Breitholtz et al. (1991)
DON	DON	Urine	Turner et al. (2008)
ZEN	ZEN + α-ZEL + β-ZEL	Plasma	Prelusky et al. (1989)

Mycotoxin	Biomarker	Matrix	Reference
CIT	CIT	Plasma	Blaszkewicz et al. (2013)
	CIT + Dyhydrocitrininone (DH-CIT)	Urine	Degen et al. (2018)

The determination of mycotoxins without validated biomarkers can also provide an overview of the exposure. In this line, an appropriate approach is to simultaneously determine the presence of the parent mycotoxin alongside its metabolites. Nevertheless, analytical standards for many of those metabolites are not commercially available. To overcome this challenge, HRMS methodologies are being applied to detect their tentative presence or even to develop semi-quantitative methodologies based on the analytical standard of their parent mycotoxin. In this line, the metabolic profile of ENNB previously predicted after *in vitro* assays conducted by Fæste et al. (2011) was later confirmed by Rodríguez-Carrasco et al. (2018) after developing a suspect screening strategy with HRMS-data from human urine samples.

The use of novel matrices for HBM of mycotoxins, such as hair, have been proposed to overcome the problems related to the surveillance window. In fact, accumulation of mycotoxins in human hair has already been demonstrated (Kintz et al., 2018; Sewram et al., 2003), following similar mechanisms as those proposed for abuse drugs. This stable chemical environment that hair can provide would enlarge the surveillance window. However, the potential of hair as a novel matrix remains unexplored.

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2. OBJECTIVES

2. Objectives

The general objective of the present research was to assess human exposure to multiple mycotoxins throughout the analysis of either food products or biological samples.

To achieve the mentioned goal, several specific objectives were subsequently set:

1. To compare, optimize and validate several methodologies based on ultra-high performance liquid chromatography coupled to high resolution mass spectrometry for the analysis of multiple mycotoxins in nutraceuticals, food products and biological samples.
2. To quantify the burden of multiple mycotoxins in marketed food products and biological samples.
3. To assess mycotoxin human exposure by a deterministic approach combining contamination and food consumption data, using tolerable daily intake values as reference.
4. To assess mycotoxin human exposure by direct measurement of urinary biomarkers, using tolerable daily intake values as reference.
5. To perform retrospective suspect screenings of mycotoxins and related compounds from HRMS data of previously analyzed food and biological samples.

3. RESULTS

3. Results

3.1. Ultra-high performance liquid chromatography coupled with quadrupole Orbitrap high-resolution mass spectrometry for multi-residue analysis of mycotoxins and pesticides in botanical nutraceuticals

1. Introduction

Nutrition is known to be an essential component of the health state, so having an unbalanced diet can lead to several disorders and diseases [1]. Due to current lifestyles, new and fast ways to maintain proper dietary habits are required. Nutraceuticals have emerged as an alternative to increase the input of nutrients, contributing to an improvement in health. These products are bioactive compounds naturally occurring in food or produced de novo in human metabolism, biologicals or botanicals, each intended to impart a physiological or medicinal effect after ingestion [2]. They can be delivered either in foods and beverages or in other non-conventional forms, such as capsules, tablets, powders or liquid extracts. In terms of marketing, nutraceuticals include a large number of different products packaged for specific groups by age, gender, physical conditions and activity level. The global market was valued at US$109 billion in 2015 and is projected to reach US$180 billion by 2020 [3].

Inside the variety of products classified as nutraceuticals, food supplements based on botanical ingredients represent the second largest segment, behind vitamins and minerals. Most recently, cannabidiol (CBD) dietary supplements

made of *Cannabis sativa* L. extracts have quickly become popular products. CBD is a phytocannabinoid present in the resin secreted from trichomes in female *C. sativa* plants and is mainly found in inflorescences. The bioactivity of this compound has been related to an enhancement of its antioxidant and neurological activity, among others, by the promotion of several metabolic pathways [4–6]. However, the European Union (EU) does not consider CBD supplements as a novel food [7] and lets member states set their own rules over its marketing, leading to a convoluted situation in terms of regulation. Despite several ambiguities in its legislation, the European market for CBD-based supplements was valued at US$318 million in 2018 and with a strong growth projection [8].

Due to the complex nature of *C. sativa* and other botanicals, potential contaminants can be coextracted during the different stages of the manufacturing process and placed into the final product. Among all the potential non-desirable compounds in herbal-based supplements, mycotoxins and pesticides are the most commonly reported [9,10]. Mycotoxins are secondary metabolites mainly produced by the fungi genera *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps* and *Alternaria*. These compounds can be present in food and feed commodities and display immunosuppressive, nephrotoxic or carcinogenic effects, among others [11]. According to their carcinogenic potential, some mycotoxins, like aflatoxins, have been included in the classification list of human carcinogens provided by the International IARC [12]. These mycotoxins are produced by the genera *Aspergillus*, which has been categorized as a major fungus occurring in *C. sativa* inflorescences alongside other mycotoxin producing fungi, like *Fusarium* spp., so

different mycotoxins could be also expected [13,14]. On the other hand, pesticides include a broad range of compounds routinely applied to protect crops from different pests. However, residues coming from these products can accumulate in plants intended for human consumption, leading to several health issues related to neurotoxicity, carcinogenicity and pulmonotoxicity, as well as developmental and reproductive disorders [15–18].

In terms of regulation, maximum residue limits (MRLs) for different types of contaminants have been set by the EU. Regulation (EC) No. 396/2005 [19] establishes limits for pesticides, whereas Regulation (EC) No. 1881/2006 [20] covers mycotoxins, attaching MLs in food and feeds. Nevertheless, nutraceutical products are not considered by the legislation yet but, due to a potential carryover during the manufacturing process, contamination could be expected not only in raw material, but also in other by-products. Moreover, several studies have reported the sole presence of pesticides [21,22], mycotoxins [23,24] and both types of contaminants [25–27] in diverse food supplements, remarking the necessity to evaluate the contamination profile of these products considering their rising consumption and popularity.

To overcome this point, the development of analytical procedures is needed. Concerning the extraction of contaminants, QuEChERS [21,23,24] and "dilute and shoot" procedures have been recently applied to food supplements delivered as gelatin capsules, traditional capsules, tablets, powder extracts or liquid presentations [25–27]. Analytical methods used in the detection and quantification of contamination include ELISA detection [28], GC coupled with

mass spectrometry (MS) [22] and ultra-high performance liquid chromatography (UHPLC) coupled with MS/MS [23,24] and high-resolution Orbitrap mass spectrometry (Q-Orbitrap HRMS) [25–27]. Due to its high resolving power, sensitivity and accurate mass measurement, high-resolution mass spectrometry stands as a suitable alternative for evaluating a large number of contaminants present in complex matrices at low concentrations. Therefore, the aim of the present study was to provide an analysis of pesticide residues and mycotoxins produced by major *C. sativa* fungi occurring in CBD-based food supplements, using UHPLC coupled with Orbitrap HRMS. To achieve this, a novel methodology was developed in order to identify and quantify 16 mycotoxins after evaluating different extraction procedures, followed by a post-target screening of 283 pesticides based on a comprehensive spectral library. To the best of the authors' knowledge, this is the first multi-class analysis of CBD-based supplements through the use of high-resolution mass spectrometry techniques.

2. Results and Discussion

2.1. Optimization of Extraction Procedure

The molecular complexity of this matrix demands an effective extraction in order to detect and quantify several mycotoxins in a reliable way. A QuEChERS methodology previously developed on this typology of sample [24] was selected as the starting point, whereas different volumes of extraction solvent and the type of sorbent for clean-up was tested.

2.1.1. Evaluation of the Volume of Extraction Solvent

The extraction procedure was first evaluated in triplicate by spiking the sample at 10 ng/g using the following volumes of extraction solvent per gram of sample: 2.5, 5, 7.5 and 10 mL.

The extraction performed with 2.5 mL showed recovery values below the minimum limit (70%) for the vast majority of the studied analytes as a consequence of solvent saturation (Figure 1a). Satisfactory recoveries (70–120%) were obtained after performing the extraction with 5 mL of solvent for the majority of compounds, with the exception of β-ZEL (155%) and ZAN (150%), which were significantly more efficient than the other volumes tested ($p < 0.05$). On the other hand, the extractions performed with 7.5 and 10 mL showed a gradual decrease in recoveries due to the larger dilution of the analytes. Therefore, 5 mL of AcN was selected as the optimal volume of extraction solvent for this type of CBD capsule.

2.1.2. Evaluation of the Type of Sorbent for Clean-Up

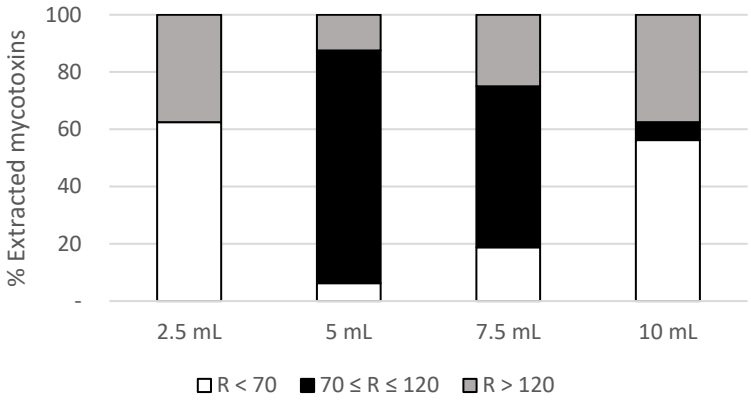
The molecular composition of the soft gel capsules mainly consists on fatty acids and proteins. Because of the complex nature of this matrix, an efficient clean-up is required in order to avoid interference with the analytes. To achieve this, clean-up with different sorbents (100 mg), including C18, as previously suggested [24], GCB, Z-Sep+ and PSA was performed.

PSA exhibited a good performance for the vast majority of analytes (Figure 1b) but was unable to recover other important mycotoxins, such as AFB1 and

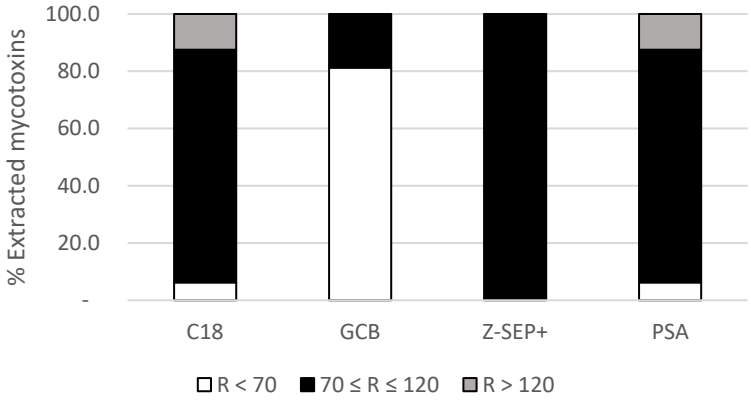
AFG1. The moderate affinity of PSA with polar compounds may explain low recoveries for aflatoxins, being consistent with other works based on oily matrices [29,30]. Similarly, extraction with C18 was efficient for most compounds and only some low-polarity mycotoxins showed recoveries out of the range set, like ZAN (150%) and β-ZEL (155%). Clean-up using GCB showed poor results, allowing us to detect only NEO (85%), HT-2 (89%) and T-2 (89%). This sorbent is able to retain planar molecules and mycotoxin adsorption has been previously reported [31], which might be the reason for the low recoveries obtained here. Finally, extraction performed with Z-Sep+ showed satisfactory recoveries (70–120%) for all the mycotoxins studied.

On the other hand, the influence of the matrix was minimal (80% ≤ SSE ≤ 120%) for all targeted analytes when using Z-Sep+ and PSA. Clean-up based on Z-Sep+ has been successfully applied to the extraction of analytes from lipid matrices [32,33]. Furthermore, Z-Sep+ is also able to form irreversible links with carboxylic groups present in proteins [34], standing as the most suitable sorbent for the here-analyzed matrix. Similarly, the use of PSA has been suggested to remove coextracted fatty acids and other ionic lipids [35]. On the contrary, a strong matrix effect was evidenced for half the analytes when using C18 and GCB. Signal suppression was detected after using C18, obtaining SSE ranging from 40% to 69%, whereas signal enhancement occurred after GCB clean-up, with SSE increasing from 128% to 167%. Since both sorbents have a preferential affinity for non-polar compounds, matrix interferents were not fully removed but coextracted. The presence of these coextracted species can change the ionization efficiency, leading to improper SSE and preventing a reliable quantification.

Although no significant differences were observed between the use of Z-Sep$^+$ and PSA (p > 0.05), Z-Sep$^+$ was chosen because of its better performance minimizing matrix interference.



(a)



(b)

Figure 1. Percentage of mycotoxins extracted with a recovery value (R) below 70% (white), between 70% and 120% (black) and above 120% (grey), corresponding to extractions performed with: (a) different volumes of solvent at a spiking level of 10 ng/g; (b) different sorbents for clean-up at a spiking level of 10 ng/g.

2.2. Analytical Method Validation

The optimized method was validated for the simultaneous extraction of 16 mycotoxins in CBD-based products. Results are shown in Table 1. Good linearity was observed for all analytes in the range assessed (0.20–100 ng/g), with regression coefficients (r2) above 0.990 and a deviation ≤20% for each level of the calibration curve. Comparison between calibration curves built in a blank matrix and in neat solvent showed a minimal interference in the matrix (±20%) for the studied analytes. Hence, external calibration curves were used for quantification purposes. Limits of quality (LOQs) obtained for all studied analytes were between 0.20 and 6.25 ng/g. Regarding trueness, recovery values corresponding to a fortification level of 20 ng/g ranged between 63 and 103% and between 63 and 113% for the lowest fortification level (10 ng/g). Referring to the additional spiking level (2 ng/g) for aflatoxins, recoveries ranged between 63% and 86%. Precision study revealed both inter- and intra-day relative standard deviation (RSD_R and RSD_r, respectively) values below 20% for all the mycotoxins analyzed. These results confirmed that the optimized procedure is suitable for a reliable quantification of the mycotoxins analyzed, fulfilling the criteria set by Commission Decision 2002/657/EC [36]. Table 2 reviews the available literature regarding mycotoxins in herbal-based supplements. As shown, the here-obtained LOQs were lower than the ones reported in previous studies using UHPLC-Q-Orbitrap HRMS. As established by Regulation (EC) No. 1881/2006 [20], MLs for aflatoxins in many food matrices must not reach levels which are below those LOQs (5 ng/g), whereas LOQs obtained in this study were between 5 and 25 times lower. Other analytical methods based on LRMS [37] required longer and

more complicated extraction procedures than the QuEChERS developed here. Even ELISA detection has been used for quantification of mycotoxins in medicinal herbs [28], but a very specific extraction had to be performed for different groups of analytes using several multi-functional columns. The QuEChERS procedure developed in this study, in combination with UHPLC-Q-Orbitrap mass spectrometry, was extremely simple and reliable, allowing for the quantification of all mycotoxins with high sensitivity.

Table 1. Method performance

Analyte	Linearity (r^2)	SSE (%)	Recovery (%)				Precision (%) [RSD_r, (RSD_R)]				LOQ (ng/g)
			2 ng/g[1]	10 ng/g	20 ng/g	50 ng/g	2 ng/g[1]	10 ng/g	20 ng/g	50 ng/g	
AFG2	0.9975	111	78	77	81	98	16 (19)	5 (6)	6 (6)	5 (4)	0.78
AFG1	0.9982	106	81	86	86	105	12 (9)	16 (19)	7 (6)	11 (10)	1.56
AFB1	0.9984	115	71	91	98	107	14 (13)	10 (8)	4 (4)	4 (3)	0.20
AFB2	0.9998	111	86	88	91	103	18 (15)	10 (8)	7 (5)	5 (4)	0.20
NEO	0.9988	112		88	93	104		18 (14)	16 (18)	17 (18)	0.78
HT-2	0.9984	108		113	101	92		12 (14)	16 (11)	12 (15)	6.25
T-2	0.9990	83		89	98	110		19 (13)	9 (7)	7 (10)	0.78
α-ZEL	0.9943	81		81	94	100		11 (11)	10 (14)	5 (16)	6.25
β-ZEL	0.9985	84		106	103	89		8 (18)	15 (16)	9 (11)	3.13
ZAN	0.9992	108		111	100	105		15 (13)	18 (11)	5 (13)	1.56
ZEN	0.9991	109		104	103	93		5 (16)	15 (14)	10 (19)	3.13

Analyte	Linearity (r^2)	SSE (%)	Recovery (%)				Precision (%) [RSD_r, (RSD_R)]				LOQ (ng/g)
			2 ng/g[1]	10 ng/g	20 ng/g	50 ng/g	2 ng/g[1]	10 ng/g	20 ng/g	50 ng/g	
ENN B	0.9998	102		63	63	65		18 (19)	18 (18)	6 (7)	6.25
ENN B1	0.9982	99		83	89	85		12 (11)	8 (6)	8 (8)	1.56
ENN A	0.9942	84		96	91	80		11 (9)	14 (17)	11 (12)	3.13
ENN A1	0.9972	87		92	101	90		12 (14)	9 (6)	7 (14)	1.56
BEA	0.9971	119		80	71	63		18 (17)	10 (18)	10 (19)	6.25

2.3. Application to Commercial CBD-Based Products

The validated UHPLC-Q-Orbitrap HRMS procedure was applied to ten commercially available samples in order to evaluate the occurrence of mycotoxins. Results are shown in Table 3. A considerable occurrence of mycotoxins was observed, since contamination with at least one analyte was found in 70% of the samples. Up to six different mycotoxins (T-2, ZAN, ZEN, ENNB1, ENNA, ENNA1) were quantified at a range from below LOQ to 11.6 ng/g, all produced by *Fusarium* genera, reported as a major *C. sativa* pathogen fungus [14]. Previous studies regarding mycotoxins in different herbal-based extracts have revealed the occurrence of similar mycotoxins independently of the matrix and the dosage form (Table 2). Despite the fact that the percentage of positive samples varied among the different studies (19%–99%), when the sensitivity of the analytical method increased, reaching lower LOQs, the number of positive samples dramatically increased. This indicated that mycotoxin contamination in herbal-based products at low levels is frequent.

In the here-analyzed samples, ZEN appeared to be the most common mycotoxin, with an incidence of 60% and concentration levels ranging from 4.2 to 11.6 ng/g (mean level = 6.9 ng/g). A high incidence of ZEN has also been previously reported in supplements made of different herbals from Czech and US retail markets (84%, n = 69) at a wide range of concentrations (5–824 ng/g, mean value = 75.7 ng/g) [24]. Moreover, ZEN was previously found in 96% of medicinal herbals from Spain (n = 84) as well, but in a tighter range (1–44.1 ng/g, mean value = 8.9 ng/g) [28].

Referring to T-2, results reported contamination in one sample at 2.0 ng/g, in contrast with the prevalent presence of T-2 in 78% (*n* = 69) of the same Czech and US samples, at concentrations rising from 69 to 1,870 ng/g (mean value = 162 ng/g) [24]. High levels of T-2 were also observed in milk thistle samples from Spain (363–453.9, mean value = 408.9 ng/g) in only two out of seven samples [38]. In the other hand, T-2 was quantified in 98% (*n* = 84) of the Spanish medicinal herbals, but in much lower concentrations (0.6–256 ng/g, mean value = 22.645 ng/g) [28].

Similarly, ZAN was quantified in one sample at 1.9 ng/g. This mycotoxin has been scarcely targeted in dietary supplement studies, but has been previously quantified at similar concentrations as those here-reported in two samples of Chinese medicinal herbals (*n* = 33) [39].

Results also showed ENN contamination. ENNB1, ENNA and ENNA1 were found in the same sample at 11.6, 4.2 and 5.8 ng/g, respectively, whereas ENNB1 was detected in two other samples below the LOQ (1.56 ng/g). These emerging *Fusarium* mycotoxins have been previously found in herbal products (84–91%, *n* = 69) widely ranging from 5 ng/g up to 10,900 ng/g (mean value = 354 ng/g) [24]. Similarly, ENNB1 was the most common toxin out of these emerging *Fusarium* mycotoxins, being consistent with the results here obtained.

All the mycotoxins found in the present study correspond to low- to non-polar compounds, which should be prevalently expected due to the nature of the matrix.

Co-occurrence of at least two mycotoxins was also observed in four out of ten samples. Results showed the presence of ZEN in combination with ENNs B1, A and A1, ZAN or T-2, which are common associations found by previous studies in herbal-based supplements [24,28]. It must be highlighted that synergic or additive effects have been observed as a consequence of these combinations in *in vitro* assays [40]. Based on what has been discussed and considering the uprising trend of *C. sativa*-based products, alongside the use of environment-friendly raw materials cultivated without pesticides, quality controls regarding mycotoxins should be set for these products in order to ensure safe consumption.

Table 2. Available methods for measurement of mycotoxins in herbal-based supplements

Samples procedence (no.)	Positives samples (%)	Major analytes detected	Concentration reported (ng/g)	Determination		
				Sensitivity (LOQ, ng/g)	Detection method	Reference
Medicinal or aromatic herbs (84)	99	ZEN	1.0-44.1	0.14	ELISA detection (EIA reader, SIRIO S)	[28]
		T-2	0.6-256.9	0.28		
		DON[3]	20.5-343.5	14.8		
		CIT[3]	14.9-354.8	16.5		
Traditional Chinese herbs (60)	83	ZEN	2.1-15.5	0.4	QQQ (Applied Biosystems) ESI+ MRM mode	[37]
		AFs[3]	0.2-19.5	0.1		
		MPA[3]	0.2-22.7	0.02		
Milk thistle (83)	19	AFB1	0.04-1.9	0.03	LC-FLD (Waters)	[41]
Green coffee bean (50)	36	OTA[3]	1–136.9	2.5	QQQ (AB SCIEX) ESI^+ and ESI^- MRM mode	[23]
		OTB[3]	1–20.2	2.5		
		FB1[3]	50–415	100		
		MPA	5–395	10		

Samples procedence (no.)	Positives samples (%)	Major analytes detected	Concentration reported (ng/g)	Determination		
				Sensitivity (LOQ, ng/g)	Detection method	Reference
Milk thistle (7)	29	T-2	363-453.9	30.5	QQQ (AB SCIEX) ESI+ MRM mode	[38]
		HT-2	826.9-943.7	43.8		
Herbals (69)	96	ZEN	5-824	10	QQQ (AB SCIEX) ESI^+ and ESI^- MRM mode	[24]
		T-2	69-1,870	10		
		HT-2	59-1,530	50		
		ENNB	5-9,260	5		
		ENNB1	5-10,900	5		
		ENNA	5-8,340	5		
		ENNA1	5-2,340	5		
Gingko biloba (8)	50	AFB1	5.0-54	5	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	[25]
		AFB2	4-300	10		
		T-2	18-20	30.5		

Samples procedence (no.)	Positives samples (%)	Major analytes detected	Concentration reported (ng/g)	Determination		
				Sensitivity (LOQ, ng/g)	Detection method	Reference
Green tea (10)	10	AFB1	5.4	5	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	[26]
Royal jelly (8)	0					
Soy (11)	27	AFB1	8.2-17.1	5	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	[27]
		AFG2	6.4	5		
Cannabis sativa (10)	70	ZEN	4.2-11.6	3.13	Q-Orbitrap (Exactive, Thermo FisherScientific) ESI+ and ESI- HRMS	Current study

ESI+ = positive ion mode; ESI− = negative ion mode; HRMS = high-resolution MS; LOQ = limit of quantification; MRM = multiple reaction monitoring; QQQ = triple quadrupole; AFs = aflatoxins; DON = deoxynivalenol; CIT = citrinin; FB1 = fumonisin B1; MPA = mycophenolic acid; OTA = ochratoxin A; OTB = ochratoxin B.

Table 3. Occurrence of studied mycotoxins in the analyzed samples

Sample	Mycotoxin (ng/g)					
	T-2	ZAN	ZEN	ENN B1	ENN A	ENN A1
1			11.6	11.6	4.2	5.8
4			6.5			
5				<LOQ		
7			8.1			
8		1.9	4.7			
9			4.2	<LOQ		
10	2.0		6.3			

2.4. Identification of Non-Target Compounds through Retrospective Analysis in Studied Samples

The post-target screening approach allowed us to detect pesticide residues in the analyzed samples using a spectral library. Results are shown in Figure 2. Up to 46 different pesticides were tentatively identified based on the pesticides mass spectral library. Ethoxyquin was putatively found in five samples, being the most prevalent pesticide. The main function of ethoxyquin is to avoid fungal contamination during the postharvest stage of the plant through its scald-preventive properties [42]. Surprisingly, the use of this pesticide is forbidden by the EC Decision 2011/143/EU. Piperonyl butoxide was found in four samples. This compound is not a pesticide by itself but can inhibit the resistance mechanisms of insects, being widely used in combination with other different pesticides [42].

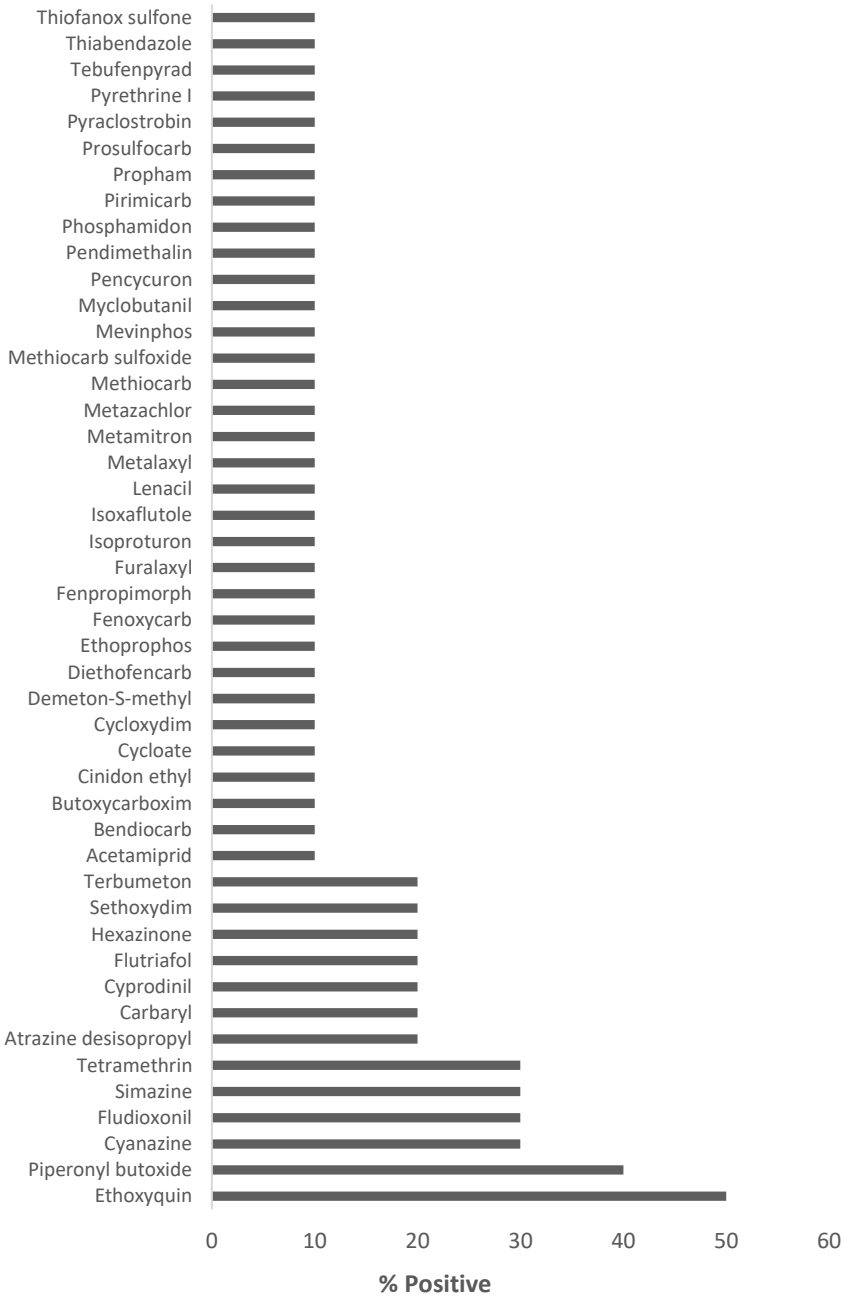


Figure 2. Occurrence of non-target pesticides in analyzed samples.

The tentative presence of cyanazine and simazine, both found in three different samples, must also be noted. The use of these pesticides was prohibited by EC Regulation No. 1107/2009 [43] and Commission Decision 2004/247/EC [44], respectively. Therefore, the occurrence of forbidden pesticides found in the here-analyzed samples highlights the necessity of monitoring potential contaminants in *C. sativa*-derived products acquired from online shops.

3. Materials and Methods

3.1. Chemicals and Reagents

Acetonitrile (ACN), methanol (MeOH), and water for LC mobile phase (HPLC grade) were acquired from Merck (Darmstadt, Germany). Formic acid and ammonium formate were obtained from Fluka (Milan, Italy). Sodium chloride (NaCl), magnesium sulfate (MgSO4), C18, graphitized carbon black (GCB), PSA and zirconium oxide (Z-Sep+) were obtained from Sigma Aldrich (Milan, Italy).

Mycotoxin standards and metabolites, namely aflatoxins (AFB1, AFB2, AFG1, and AFG2), HT-2 toxin (HT-2), T-2 toxin (T-2), neosolaniol (NEO), zearalenone (ZEN), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), beauvericin (BEA) and enniatins (ENNA, ENNA1, ENNB, and ENNB1) were purchased from Sigma Aldrich (Milan, Italy). Individual stock solutions of all analytes were prepared by diluting 1 mg of each mycotoxin in 1 mL of methanol. The working standard solution including all the mycotoxins was made by adequate diluting in MeOH:H2O (70:30 v/v) 0.1% formic acid to reach the required concentrations for performing the spike experiments: 20, 10 and 2 µg/mL. All solutions were kept in safe conditions at −20 °C.

3.2. Sampling

For the analysis of real samples, ten different CBD gelatin capsules were obtained from online shops based in different European countries. The capsules are made of gel mass, which contains gelatin, water, glycerin and other minor additives whereas the fill formulation consists of olive oil mixed with hemp oil containing CBD at certain concentrations. The weight of each capsule depended on the manufacturer; there were 0.25, 0.5 and 1 g capsules. Only soft gel capsules were studied since it was the prevalent presentation available for CBD supplements. On the other hand, one sample of CBD supplements delivered as soft gel capsules was acquired from a local store (Naples, Italy). After confirming the absence of contaminants, they were used for preparing fortified samples for recovery assays and matrix-matched standards for calibration purposes. All the samples were conserved in dark and cool conditions, as recommended by the manufacturer, until further analysis.

3.3. Sample Preparation

The sample preparation procedure developed by Veprikova et al. [24] was selected as a starting point and then slightly modified, as follows: 1 g of sample was weighed into a 50 mL polytetrafluorethylene (PTFE) tube and mixed with 5 mL of 1% aqueous formic acid. The mixture was placed in an SKO-D XL orbital shaker (Argo Lab, Italy) for 30 min at 294 × g. Then, 5 mL of ACN were added and the mixture was shaken for an additional 30 min at 294 × g. After that, 0.5 g of sodium chloride and 2 g of magnesium sulfate were added and the tube was shaken for 1 min by hand, followed by centrifugation at 4907 × g for 15 min in an

SL 16R centrifuge (Thermo Fisher Scientific LED GmbH, Germany). A 2 mL aliquot of the upper ACN layer was taken for dSPE cleanup in a 15 mL PTFE tube containing 100 mg of Z-Sep+ sorbent and 300 mg of magnesium sulfate. The tube was vortexed for 1 min and then centrifuged at 4907 × g for 15 min. An aliquot of the supernatant (1 mL) was collected and filtered through a 0.2 µm PTFE filter (Phenomenex, Italy) into a vial prior to UHPLC-Q-Orbitrap HRMS analysis.

3.4. UHPLC-Q-Orbitrap HRMS Analysis

The qualitative and quantitative profiles of the mycotoxins were obtained using an ultra-high-pressure liquid chromatograph (UHPLC, Thermo Fisher Scientific, Waltham, MA, USA) equipped with a degassing system, a Dionex Ultimate 3000, a Quaternary UHPLC pump working at 1250 bar, an auto sampler device and a thermostated (T = 30 °C) Luna Omega 1.6 µm (50 × 2.1 µm) column.

The eluent consisted of two different phases: A (H_2O containing 0.1% formic acid and 5 mM ammonium formate) and B (MeOH containing 0.1% formic acid and 5 mM ammonium formate). The gradient elution for LC-Orbitrap HRMS analyses was applied as follows: an initial 0% of phase B was held for 1 min, which linearly went up to 95% B over 1 min and held for 0.5 min. Next, the gradient decreased to 75% B over 2.5 min and then decreased again to 60% B over 1 min. Finally, the gradient turned to 0% B over 0.5 min and then the column was equilibrated for 1.5 min at 0% B. The total run time was 8 min, at a flow rate of 0.4 mL/min. A total of 5 µL of the sample was injected. Detection was performed using a Q-Exactive mass spectrometer. The mass spectrometer was operated in both positive and negative ion mode using fast polarity switching by setting two

scan events (full ion MS and all ion fragmentation (AIF)). Full scan data were acquired at a resolving power of 35,000 full width at half maximum (FWHM) at *m/z* 200.

The ion source parameters were: spray voltage 4 kV (-4 kV in electrospray ionization (ESI) – mode); capillary temperature 290 °C; S-lens RF level 50; sheath gas pressure (N_2 > 95%) 35, auxiliary gas (N_2 > 95%) 10, and auxiliary gas heater temperature 305 °C. The value for automatic gain control (AGC) target was set at 1 × 106, a scan range of *m/z* 100 to 1000 was selected and the injection time was set to 200 ms. The scan rate was set at 2 scans/s. For the scan event of AIF, the parameters in the positive and negative ion mode were: mass resolving power = 17,500 FWHM; maximum injection time = 200 ms; scan time = 0.10 s; ACG target = 1 × 105 ; scan range = 100–1000 *m/z*, isolation window to 5.0 *m/z*, and retention time window to 30 s. The Orbitrap-MS parameters were optimized in a previous work [45]. The exact mass for the studied compounds, including elemental composition, retention time (RT), theoretical masses and accurate mass errors for the detected ions are shown in Table 4. A mass error below 5 ppm, referring to the molecular ions, was set for identification. Retrospective screening was carried out on spectral data collected using a pesticide spectral library (Pesticide Spectral Library Version 1.1 for LibraryView™ Software, AB SCIEX, Framingham, USA). For accurate mass measurement, identification and confirmation were performed at a mass tolerance of 5 ppm for the molecular ion and for both fragments at the intensity threshold of 1000. Data analysis and processing were performed using the Xcalibur software, v. 3.1.66.10.

Table 4. Retention times, accurate mass and mass accuracy of mycotoxins evaluated.

Analyte	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (Δ ppm)
NEO	4.25	$C_{19}H_{26}O_8$	$[M+NH_4]^+$	400.1966	400.1963	-0.67
AFG2	4.50	$C_{17}H_{14}O_7$	$[M+H]^+$	331.0812	331.0808	-1.36
AFG1	4.52	$C_{17}H_{12}O_7$	$[M+H]^+$	329.0656	329.0655	-0.27
AFB2	4.58	$C_{17}H_{14}O_6$	$[M+H]^+$	315.0863	315.0862	-0.51
AFB1	4.62	$C_{17}H_{12}O_6$	$[M+H]^+$	313.0707	313.0705	-0.42
HT-2	4.74	$C_{22}H_{32}O_8$	$[M+NH_4]^+$	442.2435	442.2432	-0.7
α-ZEL	4.83	$C_{18}H_{24}O_5$	$[M-H]^-$	319.1551	319.1550	-0.31
T-2	4.85	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.2541	484.2543	0.39
β-ZEL	4.97	$C_{18}H_{24}O_5$	$[M-H]^-$	319.1551	319.1550	-0.31
ZAN	4.98	$C_{18}H_{24}O_5$	$[M-H]^-$	319.1551	319.1549	-0.6
ZEN	5.01	$C_{18}H_{22}O_5$	$[M+H]^+$	317.1395	317.1393	-0.54
ENN B	5.56	$C_{33}H_{57}N_3O_9$	$[M+NH_4]^+$	657.4433	657.4435	0.26
ENN B1	5.68	$C_{34}H_{59}N_3O_9$	$[M+NH_4]^+$	671.4599	671.4594	-0.76
BEA	5.73	$C_{45}H_{57}N_3O_9$	$[M+NH_4]^+$	801.4433	801.4432	-0.16
ENN A1	5.82	$C_{35}H_{61}N_3O_9$	$[M+NH_4]^+$	685.4746	685.4745	-0.18
ENN A	5.99	$C_{36}H_{63}N_3O_9$	$[M+NH_4]^+$	699.4903	699.4899	-0.56

3.5. Validation Parameters

An in-house validation study was conducted following the EU Commission Decision 2002/657/EC [36]. The parameters evaluated were selectivity, specificity, linearity, trueness, repeatability (intra-day precision), within-reproducibility (inter-day precision), limit of quantification (LOQ) and limit of detection (LOD). The selectivity and specificity of the method were evaluated by analyzing both standard solutions and samples, comparing the retention time of the peaks corresponding to the analytes of interest alongside the determination of its precursor and product ion, with a mass error below 5 ppm. For linearity, standard solutions built in neat solvent and matrix-matched calibration were analyzed by spiking blank samples at eight concentration levels from 0.2 to 100 ng/g. The slopes of each linear calibration function were compared in order to detect a SSE effect due to the matrix interference. This effect was quantified following the equation: SSE (%) = matrix-matched calibration slope/solvent calibration slope x 100. An SSE value of 100% was interpreted as no matrix interference in the concentration range evaluated. An SSE value above 100% revealed signal enhancement whereas a value below 100% indicated signal suppression. For trueness, recovery studies were evaluated by spiking three blank samples at three different levels. Additionally, a lower spike level was used only for aflatoxins. RSD_r was expressed as the relative standard deviation after three determinations in a single day (n = 3). Inter-day precision was calculated by repeating the measurements in triplicate on three non-consecutive days (n = 9) and expressed as RSD_R. The LOD was defined as the minimum concentration where the molecular ion can be identified by the instrument (mass error value below 5 ppm)

and the LOQ as the minimum concentration where a linear response (mass error value below 5 ppm) can be observed with an accuracy and precision of ≤ 20%.

3.6. Statistical Analysis

Validation experiments were performed in triplicate and the results expressed as the average values alongside relative standard deviation (RSD, %). The Saphiro–Wilk test was applied to evaluate normality and multivariant analysis was performed using a non-parametric Kruskal–Wallis test, considering p values < 0.05 as significant. Analysis of data was carried out using IBM SPSS version 25 statistical software package (SPSS, Chicago, IL, USA).

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3.2. *Target quantification and semi-target screening of undesirable substances in pear juices using ultra-high performance liquid chromatography-quadrupole orbitrap mass spectrometry*

1. Introduction

During the last years, diets have gravitated to higher intakes of fruits and vegetables, mainly due to its beneficial effects on health status and its protective role against chronic diseases [1]. In this line, fruit juices have become an appealing alternative, recommended as a good vitamin C source for children, and have been introduced as part of breakfast in conventional diets [2]. According to the European Fruit Juice Association (AIJN), juice consumption was 9.2 billion liters in 2017, with pear juice being one of the most consumed flavors in several countries, such as Italy [3]. The frequent intake of pear juice demands strict quality controls, especially when children become an important target group, in order to ensure safe consumption.

Several harmful compounds, originally present in pears, can also be present in marketed pear juice due to a potential carry-over during the manufacturing process. Among all the different contaminants in pears, mycotoxins and pesticide residues are some of the most impactful [4]. Mycotoxins are fungal secondary metabolites that can display several adverse effects, such as immunosuppression, carcinogenicity, or nephrotoxicity, among others [5]. In pears, the most relevant mycotoxin-producing genera are *Alternaria*, *Aspergillus*, and *Penicillium* [4], so

analytical methods have been focused on the detection of their respective mycotoxins. Patulin (PAT), produced by *Aspergillus* and *Penicillium* spp., is the most studied mycotoxin in pear juice. Diverse effects have been attributed to PAT, including hepatotoxicity or neurotoxicity, among others [6]. Furthermore, citrinin (CIT) and ochratoxin A (OTA), produced by *Aspergillus* and *Penicillium* spp. too, and even *Alternaria* mycotoxins, such as alternariol (AOH) and alternariol monomethyl ether (AME), have been studied in this matrix [7,8]. Apart from these genera, *Fusarium* has also been classified as another pathogenic fungus in pear, so its presence was recently reported for the first time, causing postharvest decay [9].

On the other hand, pesticide residues include a broad range of toxic compounds widely used to prevent crops from pests. Nevertheless, a routine application can lead to the accumulation of residues in plants meant for human consumption, causing severe adverse effects, like neurotoxicity, carcinogenicity, and reproductive and developmental disorders [10]. In pear, these products are mainly used to avoid postharvest diseases caused by fungi, so pesticides from the benzimidazole group are commonly used. Similarly, pyrethroids represent another group of pesticides routinely applied due to its insecticidal capacity [11]. Despite the accumulation of residues being due to the intended use of pesticides, there are other factors to take into consideration, such as a potential run-off from contaminated soils and waters or even cross-contamination between different crops. Consequently, the overall pesticide profile of crops can vary from what it is expected to be. In terms of regulation, MRLs have been established by the EC in pear or pear juice. Pesticide residues are brought under Regulation (EC) No.

396/2005 [12], but no specific MRLs have been set for pear juice so those corresponding to pears are applied instead. Referring to mycotoxins, Regulation (EC) No. 1881/2006 [13] covers contamination in pear juice, setting an MRL at 50 ng/mL for PAT. Several studies have reported the presence of PAT [14–16] and pesticide residues in pear juice [11,17,18], but no literature regarding *Fusarium* mycotoxins is available. Considering that *Fusarium* spp. has previously been identified as another pathogen in pears, its own mycotoxins could be expected in pear-derived products, even coexisting with other contaminants, like pesticides. Consequently, it is necessary to evaluate the contamination profile of pear juice even more when children represent one of the largest targets.

To overcome this, powerful analytical tools are needed. Concerning the extractive procedures, the most recent studies have used dispersive liquid-liquid microextraction (DLLME) [7], liquid-liquid extraction (LLE) [8,14], and QuEChERS (quick, easy, cheap, effective, rugged, and safe) combined with (DLLME) [11] for the extraction of contaminants from pear juice. Analytical methods include high-performance liquid chromatography-fluorescence detection (HPLC-FD) [7], HPLC-ultraviolet-visible detection (HPLC-UV-VIS) [8], HPLC-UV detection [14], and GC-electron capture detection (GC–ECD) [11]. Based on its high-resolution power, sensitivity, and accurate mass measurement, high-resolution mass spectrometry represents an optimal choice for evaluating trace contaminants occurring in complex matrices. Hence, the aim of the present study was to evaluate the presence of pesticide residues and mycotoxins produced by *Fusarium* spp. in 21 pear juice samples available in Italian markets, using UHPLC coupled to Q-Orbitrap HRMS. To achieve this, an extractive methodology was

validated for identifying and quantifying 14 *Fusarium* mycotoxins, followed by a screening of 283 pesticides. To the best of the authors' knowledge, this is the first multi-class analysis including *Fusarium* toxins in marketed pear juice.

2. Materials and Methods

2.1. Chemicals and Reagents

All solvents, ACN, MeOH, and water (LC-MS grade), were purchased from Merck (Darmstadt, Germany). Formic acid (MS grade) was acquired from Carlo Erba reagents (Cornaredo, Italy), whereas ammonium formate (analytical grade) was provided by Fluka (Milan, Italy). Magnesium sulfate ($MgSO_4$) (anhydrous), sodium chloride (NaCl), primary-secondary amine (PSA) (analytical grade), and C18 (analytical grade) were obtained from Sigma Aldrich (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 µm) were purchased from Phenomenex (Castel Maggiore, Italy). Conical centrifuge polypropylene tubes of 50 and 15 mL were acquired from BD Falcon (Milan, Italy).

Mycotoxin standards and metabolites (purity ≥98%), namely neosolaniol (NEO), HT-2 toxin, α-zearalanol (α-ZAL), α-zearalenol (α-ZEL), T-2 toxin, β-zearalanol (β-ZAL), β-zearalenol (β-ZEL), zearalanone (ZAN), zearalenone (ZEN), enniatins (ENNA, ENNA1, ENNB, and ENNB1), and beauvericin (BEA), were provided by Sigma Aldrich (Milan, Italy). For the preparation of individual stock solutions, 1 mg of each mycotoxin was diluted in 1 mL of methanol. A working standard solution including all the analytes was built by diluting in MeOH:H_2O (70:30 v/v, 0.1% formic acid) until the concentrations needed for the spiking

experiments were reached: 100, 20, and 10 ng/mL. The analytical standards were kept in a tightly closed container under cool dry conditions at −20 °C in a well-ventilated place as stated in the safety data sheets provided by the manufacturer.

2.2. Sampling

A total of 21 pear juices samples from different European brands were randomly purchased between January and February 2020 from different supermarkets located in Campania region, southern Italy. The sampling was limited to one product per brand. The samples were divided into organic (*n = 7*) and conventional (*n = 14*) samples as indicated on the label by the manufacturer. In all cases, the percentage of fruit in the analyzed samples was above 50%; other ingredients declared in the labels from the samples were water, sugar, glucose-fructose syrup, lemon juice, and citric and ascorbic acid. All samples were stored in a refrigerator at 4°C into their original packages and analyzed within 3 days after sample registration.

2.3. Sample Preparation

In this work, the sample preparation procedure reported by Desmarchelier et al. [19] was selected as the starting point and slightly modified. Briefly, 10 mL of sample were placed into a 50-mL Falcon tube and mixed with 5 mL of water prior to be shaken for 1 min in a vortex. Then, 10 mL of ACN were added and the mixture was horizontally shaken for 30 min at 294× *g*. After that, 4 g of magnesium sulfate and 1 g of sodium chloride were added. The mixture was shaken by hand for 1 min and centrifuged at 4907× *g* for 10 min at 15 °C in an SL

16R centrifuge (Thermo Fisher Scientific LED GmbH, Langenselbold, Germany). Then, 3 mL of the upper ACN layer were placed into a 15-mL Falcon tube containing 900 mg of magnesium sulfate, 150 mg of C18 sorbent, and 150 mg of PSA, and vortexed for 1 min. The mixture was centrifuged for 10 min at 4907× *g* at 15 °C, and 0.5 mL of the upper layer was added. Finally, the extract was evaporated to dryness under gentle nitrogen flow, reconstituted with 0.5 mL of MeOH/H_2O (70:30 *v/v;* 0.1% formic acid), and filtered through a 0.2-µm filter prior to UHPLC-Q-Orbitrap HRMS analysis.

2.4. UHPLC-Q-Orbitrap HRMS Analysis

Chromatographic and HRMS conditions were the same as described in pages 102-103 whereas parameters used for mycotoxin identification and quantification are shown in Table 1. Retrospective analysis was also based in Pesticide Spectral Library Version 1.1 for LibraryView™ Software (AB SCIEX, Framingham, MA, USA).

Table 1. UHPLC-HRMS parameters and tolerable daily intakes (TDIs) corresponding to the evaluated mycotoxins

Analyte	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (Δ ppm)	Collision Energy (eV)	Product ions (*m/z*)	TDI (ng/kg bw)
NEO	4.25	$C_{19}H_{26}O_8$	$(M+NH_4)^+$	400.19659	400.19632	−0.67	10	305.13803 141.00530	n.d.[a]
HT-2	4.74	$C_{22}H_{32}O_8$	$(M+NH_4)^+$	442.24354	442.24323	−0.7	27	263.12744 215.10641	20[b]
α-ZAL	4.81	$C_{18}H_{26}O_5$	$(M-H)^-$	321.17044	321.17065	0.65	29	259.09497 91.00272	250[c]
α-ZEL	4.83	$C_{18}H_{24}O_5$	$(M-H)^-$	319.15510	319.15500	−0.31	36	174.95604 129.01947	250[c]
T-2	4.85	$C_{24}H_{34}O_9$	$(M+NH_4)^+$	484.25411	484.25430	0.39	23	215.10603 185.09561	20[b]
β-ZAL	4.94	$C_{18}H_{26}O_5$	$(M-H)^-$	321.17044	321.17059	0.47	40	259.09497 91.00272	250[c]
β-ZEL	4.97	$C_{18}H_{24}O_5$	$(M-H)^-$	319.15510	319.15500	−0.31	36	174.95604 160.97665	250[c]
ZAN	4.98	$C_{18}H_{24}O_5$	$(M-H)^-$	319.15510	319.15491	−0.6	35	273.01187 131.05020	250[c]
ZEN	5.01	$C_{18}H_{22}O_5$	$(M+H)^+$	317.13945	317.13928	−0.54	-32	175.03989 131.05008	250[c]

Analyte	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (Δ ppm)	Collision Energy (eV)	Product ions (*m/z*)	TDI (ng/kg bw)
ENNB	5.56	$C_{33}H_{57}N_3O_9$	$(M+NH_4)^+$	657.44331	657.44348	0.26	50	214.14320 196.13280	n.d.[a]
ENNB1	5.68	$C_{34}H_{59}N_3O_9$	$(M+NH_4)^+$	671.45986	671.45935	−0.76	48	214.14343 196.13295	n.d.[a]
BEA	5.73	$C_{45}H_{57}N_3O_9$	$(M+NH_4)^+$	801.44331	801.44318	−0.16	70	262.76715 244.18239	n.d.[a]
ENNA1	5.82	$C_{35}H_{61}N_3O_9$	$(M+NH_4)^+$	685.47461	685.47449	−0.18	48	228.15900 210.14847	n.d.[a]
ENNA	5.99	$C_{36}H_{63}N_3O_9$	$(M+NH_4)^+$	699.49026	699.48987	−0.56	43	228.15900 210.14847	n.d.[a]

[a] not determined; [b] sum of T-2 and HT-2; [c] sum of ZEN and its forms α-ZEL, β-ZEL, α-ZAL, β-ZAL, and ZAN in terms of ZEN equivalents being 60, 0.2, 4, 2, and 1.5, respectively, its molar potency factors. Neosolaniol (NEO), HT-2 toxin, α-zearalanol (α-ZAL), α-zearalenol (α-ZEL), T-2 toxin, β-zearalanol (β-ZAL), β-zearalenol (β-ZEL), zearalanone (ZAN), zearalenone (ZEN), enniatins (ENNA, ENNA1, ENNB, and ENNB1), and beauvericin (BEA)

2.5. Validation Parameters

In-house validation was carried out according to the guidelines established by the EU Commission Decision 2002/657/EC [21] and the SANTE criteria (SANTE/12682/2019) [22]. The method validation was based on the following parameters: Selectivity, trueness, repeatability (intra-day precision), reproducibility (inter-day precision), linearity, LOD, and LOQ. The selectivity of the method was evaluated by analyzing blank samples ($n = 10$) to detect signals that could interfere with the analytes. The peaks for the analytes of interest in the samples were confirmed by comparing the retention times of the peak with those of standard solutions and also identifying both the precursor and product ions, with a mass tolerance below 5 ppm. To determine the linearity (R^2), standard solutions built in neat solvent and matrix-matched calibration were compared by spiking blank samples with selected mycotoxins at eight concentration levels over a range of 0.4–100 ng/mL. Calibration curves were prepared in triplicate. In order to reveal the presence of matrix effects, the slopes of each linear calibration function were evaluated. The SSE due to matrix effects was determined according to the following equation:

$$\%SSE = S_m / S_s \times 100 \quad (1)$$

where S_m is the matrix-matched calibration slope and S_s is the solvent calibration slope. An SSE of 100% indicates that no matrix effect occurred in the concentration range evaluated. An SSE value higher than 100% revealed signal enhancement, whereas there was signal suppression if the SSE value was below

100%. Trueness was determined by spiking three blank samples at three different levels (100, 20, and 10 ng/mL) during three non-consecutive days and the results were expressed as percentage of recovery. Values in the range 70–120% in relation to the theoretical concentrations were considered as satisfactory Intraday precision (repeatability) was expressed in terms of the relative standard deviation (RSD_r) after comparing the recoveries from three determinations in a single day ($n = 3$) for each fortification level. Inter-day precision (reproducibility) was expressed as the relative standard deviation (RSD_R) of a triplicated determination on three non-consecutive days ($n = 9$) for each fortification level. LODs were set considering the lowest concentration where the molecular ion could be identified (mass error value below 5 ppm) and LOQs were defined as the minimum concentration inside the linear range (mass error value below 5 ppm) with deviation below 20%.

2.6. Quality Assurance/Quality Control

For a proper confirmation of the peaks, the retention times corresponding to each analyte in the samples were compared to those in standard solutions at a tolerance of ± 2.5%. A mass error of 5 ppm was set for identification of both the precursor and product ions. Referring to the quality assurance/quality control (QA/QC) procedure, a reagent blank, a sample blank, and a replicate sample were put at the beginning and end of each sample batch in order to evaluate the efficacy and stability of the system throughout the whole batch. A potential carry-over was also evaluated through blank samples ($n = 10$) injected right after the

highest calibration value, with 100, 20, and 10 ng/mL being the concentrations chosen for the analytical quality control.

2.7. Exposure Assessment

The exposure assessment was performed following a deterministic approach. Data reported by the Italian National Food Consumption Survey INRAN-SCAI 2005-06 was considered so five different age groups were made: Infants (0.1–2.9 years), mean consumption of juice of 150 mL/day; children (3–9.9 years), mean consumption of 127 mL/day; teenagers (10–17.9 years), mean consumption of 122 mL/day; adults (18–65 years), mean consumption 58 mL/day; and elderly (>65 years), mean consumption 50 mL/day. The body weight assigned to each group was 11.3, 26.1, 52.6, 69.7, and 70.1 kg, respectively. For the calculation of the probable daily intake (PDI) corresponding to each mycotoxin, the consumption data provided by the Survey INRAN-SCAI 2005–06 was combined with the contamination data here obtained, following the next equation:

$$PDI_m = (C_m \times I) / bw, \quad (2)$$

where PDI_m is the probable daily intake (ng/kg bw/d) corresponding to each mycotoxin, *m*; C_m is the average content of a certain mycotoxin in pear juice (ng/mL); *I* represents the intake of juice (mL); and *bw* is the body weight attached to each age group (kg). After PDI_m was calculated, the risk characterization, considered as the percentage of relevant TDI_m, was evaluated by dividing the resultant PDI_m by its TDI_m value (Table 1). Since ENNB, ENNB1, ENNA, ENNA1, and

BEA do not have an assigned TDI, a theoretical 20 ng/kg bw value was used, corresponding to the lowest one for a *Fusarium* toxin:

$$\%TDI_m = PDI_m / TDI_m \times 100 \quad (3)$$

2.8. Statistical Analysis

The Mann–Whitney U test was used to evaluate differences between juice typology considering p values < 0.05 as significant. Statistical analysis of the results was carried out using IBM SPSS version 25 statistical software package (SPSS, Chicago, IL, USA).

3. Results and Discussion

3.1. Analytical Method Validation

The method was validated in order to extract and quantify 14 different *Fusarium* mycotoxins in pear juice. The results are shown in Table 2 All the analytes showed good linearity, with the regression coefficients (R^2) above 0.990 in the range evaluated (0.4–100 ng/mL), and a deviation ≤20% for each level of the calibration curves. SSE as a consequence of matrix interference was evaluated by comparing the curves built in neat solvent and blank matrix, with a minimal deviation (≤17%) being observed. Therefore, the external calibration curves were considered for quantification purposes. Sensitivity was assessed through the limits of quantifications (LOQs), that ranged from 0.4 to 3.1 ng/mL. To evaluate trueness, recovery studies were carried out in triplicate at three different spiking levels. Values corresponding to fortification at 100 ng/mL ranged between 70% and 106%, from 72–106% at 20 ng/mL, and from 70–103% at 10 ng/mL, meaning

an efficient extraction procedure even at low concentrations levels. Precision was evaluated through both RSD_r and RSD_R, showing values below 19% for all the mycotoxins analyzed. These results fulfill the criteria set by the European EU Commission Decision 2002/657/EC [21] established for a reliable quantification. This methodology, based on a simple QuEChERS extraction, stands as a powerful tool for detecting *Fusarium* mycotoxins in pear juice at low levels, reaching a higher sensitivity for several of the analytes than previous methods developed for detecting a single analyte in pear juice and carried out by Spadaro, Garibaldi, and Gullino [16] (PAT, LOQ = 1.7 ng/mL); Bonerba, Ceci, Conte, and Tantillo [15] (PAT, LOQ = 1 ng/mL); and Pan et al. [23] (AOH = 1.3 ng/mL).

Table 2. Method performance: linearity, matrix effect, recovery and limit of quantification.

Analyte	Linearity (R^2)	SSE (%)	Recovery (%)			Precision (%) (RSD_r, (RSD_R)			LOQ (ng/mL)
			100 ng/mL	20 ng/mL	10 ng/mL	100 ng/mL	20 ng/mL	10 ng/mL	
NEO	0.9971	98	99	99	91	5 (13)	8 (18)	16 (12)	1.6
HT-2	0.9967	104	88	89	72	13 (13)	15 (13)	14 (17)	1.6
α-ZAL	0.9944	83	84	77	72	10 (8)	10 (8)	7 (7)	3.1
α -ZEL	0.9967	90	97	93	102	15 (19)	8 (18)	10 (19)	3.1
T-2	0.9998	105	106	106	103	11 (14)	16 (18)	7 (17)	1.6
β-ZAL	0.9941	113	93	85	89	9 (12)	13 (16)	12 (16)	1.6
β-ZEL	0.9997	112	81	87	77	10 (8)	8 (7)	8 (6)	0.8
ZAN	0.9993	118	83	85	77	7 (5)	13 (9)	6 (5)	0.4
ZEN	0.9994	117	84	91	87	5 (5)	4 (4)	6 (8)	0.4
ENNB	0.9995	103	73	76	71	5 (8)	7 (5)	10 (7)	0.8
ENNB1	0.9980	94	76	81	75	10 (9)	8 (9)	8 (8)	0.4
BEA	0.9977	96	78	84	78	5 (5)	10 (6)	15 (10)	1.6
ENNA1	0.9994	101	70	74	71	3 (6)	7 (5)	13 (9)	0.8
ENNA	0.9994	103	70	72	70	3 (2)	7 (5)	6 (4)	0.8

%SSE: % signal suppression/enhancement effect; LOQ = limit of quantification; RSD_r: repeatability relative standard deviation; RSD_R: reproducibility relative standard deviation

3.2. Analysis of Real Samples

Up to nine different *Fusarium* mycotoxins were detected in the analyzed pear juice samples. In total, 20 out of 21 samples showed contamination with at least one mycotoxin, generally at low levels or even below the LOQ, as shown in Table 3. ZEN was the most frequently detected compound, present in 67% of the samples, with concentrations ranging from below the LOQ to 1.5 ng/mL. T2 was also a relevant mycotoxin in the analyzed samples, showing an incidence of 33% at concentrations from <LOQ up to 2.0 ng/mL. Similarly, HT-2 was detected in 33% of the samples, ranging from below <LOQ to 7.0 ng/mL. The main enniatins were also detected: ENNB and ENNA1 were present in 19% of the samples, ranging from <LOQ to 0.8 ng/mL and 1.2 ng/mL, respectively; ENNB1 was found in 14% of the samples at concentrations going from <LOQ up to 0.5 ng/mL; and ENNA was only found in one sample (5%) at 1.0 ng/mL. Lastly, ZEN metabolites were also observed. ZAN was present in 10% of the samples at concentrations below the LOQ, whereas α-ZAL was quantified in 14% of the samples, ranging from <LOQ to 10.5 ng/mL. To date, several studies have only evaluated the presence of PAT in Italian pear juice as a consequence of *Penicillium expsansum* contamination, which is the main fungus causing postharvest diseases. Spadaro, Garibaldi, and Gullino [16] reported an incidence of 64% (*n = 39*), with 17 samples showing a contamination below 10 ng/mL and 8 samples above 10 ng/mL. Similarly, Bonerba, Ceci, Conte, and Tantillo [15] found patulin in 40% of the pear juice samples (*n = 35*) at concentrations ranging from 5 to 92 ng/mL.

Table 3. Incidence and range of concentrations of the mycotoxins detected in conventional and organic pear juice samples.

Juice typology	ZEN		ZAN		α-ZAL		T-2		HT-2		ENNB		ENNB1		ENNA		ENNA1	
(n)	I (n(%))	R (ng/mL)	I (n (%))	R (ng/mL)	I (n (%))	R (ng/mL)	I (n (%))	R (ng/mL)	I (n (%))	R (ng/mL)	I (n (%))	R (ng/mL)	I (n (%))	R (ng/mL)	I (n (%))	R (ng/mL)	I (n (%))	R (ng/mL)
Conventional juice (14)	7 (50)	<L - 1.5	0 (0)	nd	1 (7)	10.5	2 (14)	<L	4 (29)	<L - 7.0	0 (0)	nd	1 (7)	<L	1 (7)	1.0	3 (21)	<L - 1.2
Organic juice (7)	7 (100)	<L - 0.6	2 (29)	<L	2 (29)	<L - 3.5	5 (71)	<L - 2.0	3 (43)	<L - 1.6	4 (57)	<L - 0.8	2 (29)	<L - 0.5	0 (0)	nd	1 (14)	0.8
Total	14 (67)	<L - 1.5	2 (10)	<L	3 (14)	<L - 10.5	7 (33)	<L - 2.0	7 (33)	<L - 7.0	4 (19)	<L - 0.8	3 (14)	<L - 0.5	1 (5)	1.0	4 (19)	<L - 1.2

nd= not determined; I = Incidence; R = Range; L = LOQ

Recently, *Alternaria* mycotoxins have been studied in pear and pear-derived foodstuffs. Pan, Sun, Pu, and Wei [23] investigated *Alternaria* toxin AOH in fresh pears (*n = 5*), observing an absence of contamination despite having good sensitivity (LOQ = 1.3 ng/mL). A specific methodology for detecting AOH and AME in pear juice has been developed by Ruan, Diao, Zhang, Zhang, and Liu [7]. The results obtained here show that the *Fusarium* toxin ZEN extensively occurred in pear juice samples, having a larger incidence than the one reported for PAT or AOH in the mentioned studies, whereas other less detected mycotoxins, such as T2 or HT-2, also had a considerable impact.

Bearing in mind the type of sample, significant differences ($p < 0.05$) were found when comparing the occurrence of mycotoxins in both organic and conventional juice, being more frequent in organic samples, as expected. Additionally, co-occurrence of mycotoxins was observed in high frequency in organic juice samples. Up to 65% of the conventional juice samples (*n = 14*) showed contamination with only one mycotoxin, whereas four or more mycotoxins co-occurred in the majority of organic samples (71%, *n = 7*). The most common associations were ZEN alongside its two metabolites, and ZEN, T-2, and ENNB, which seems to be a frequent mixture in several plant-based foodstuffs [24,25]. Furthermore, ENNB1 and ENNA1 co-occurred with ENNB in organic samples, and the combination ENNA and ENNA1 was also observed in one conventional juice sample. The presence of multiple mycotoxins could affect its toxicological potential, deriving into synergic or additive effects as observed in *in vitro* assays [26]. Based on the above-discussed points and considering the

popular trend of organic and environment-friendly products, *Fusarium* mycotoxins should also be taken into consideration in exposure assessment studies involving pear and pear juices. Moreover, further toxicological knowledge in terms of the combination of food contaminants is needed in order to ensure safe consumption.

3.3. Exposure Assessment

As reflected by the Italian National Food Consumption Survey INRAN-SCAI 2005-06, juices are mostly consumed by the young population, so a bigger intake accounts for a higher risk. Considering that *Fusarium* mycotoxins have not been studied in pear juice, the exposure to these mycotoxins might be underestimated, so an exposure assessment and risk characterization were performed.

Table 4. Risk characterization of mycotoxins found in pear juice samples according to the tolerable daily intake values

Mycotoxins	C_m (ng/mL)	Probable Daily Intake (PDI) (ng/kg bw/d)					Risk Characterization (%TDI)				
		Infants	Children	Teenager	Adult	Elderly	Infants	Children	Teenager	Adult	Elderly
ZEN + α-ZAL	2.88	38.24	14.01	6.69	2.3	2.04	15.3	5.6	2.68	0.92	0.82
T-2 + HT-2	0.88	11.68	4.28	2.04	0.7	0.62	58.40	21.40	10.20	3.50	3.10
ENNB + ENNB1 + ENNA + ENNA1	0.25	3.32	1.22	0.58	0.2	0.18	16.60	6.10	2.90	1.00	0.90

Table 4 summarizes the risk characterization of the mycotoxins found in the juice samples. The mean content of mycotoxins was 2.88, 0.88, and 0.25 ng/mL for ZEN + α-ZAL, T-2 + HT-2, and enniatins, respectively. Among the different age groups, the probable daily intake strongly varied. Infants were identified as the group with the highest PDIs due to a heavier consumption of juice and a

lower body weight. Values corresponding to the rest of the groups ranged from 2 to 20 times lower in comparison with the infants' results. These PDIs values are below the TDIs established by the Scientific Committee on Food of the EC, set as 250 ng/kg bw/day for the sum of ZEN and its derived products, 20 ng/kg bw/day for the sum of T-2 HT-2, and a theoretical value of 20 ng/kg bw/day for the sum of enniatins. Considering the results, the pear juices analyzed here account for 0.78% to 14.65% of the TDI for ZEN + α-ZAL, from 3% to 55.95% of the TDI set for T-2 and HT-2, and from 0.85% to 15.90% for enniatins. This suggests that the exposure to *Fusarium* mycotoxins as a consequence of juice consumption might not represent a health concern, but the intake of mycotoxins by infants due to regular consumption could be of importance. Therefore, the results suggest having a watchful attitude in order to ensure safe consumption.

3.4. Identification of Non-Target Compounds through Retrospective Analysis in Studied Samples

The semi-target screening was performed using a mass spectral library, allowing the detection of 283 different pesticide residues in the analyzed samples. The pesticides present in more than 25% of the samples are shown in Figure 1. Up to 77 pesticide residues were tentatively identified, but the presence of several compounds that have not yet been approved by the EU should be noted. Ethoxyquin was detected in 64% of the samples (*n = 21*), being the third most frequently found residue. This pesticide acts as a fungicide during the postharvest stage of the crops through its scald-preventive properties [27]. Currently, the use of ethoxyquin is suspended by Commission Decision 2011/143/EU [28].

Triazophos is an insecticide, which was found in 55% of the samples (*n = 21*). The use of products containing this compound is not allowed under Regulation No. 1107/2009 [29] due to its toxicity, as reported by the EFSA [30].

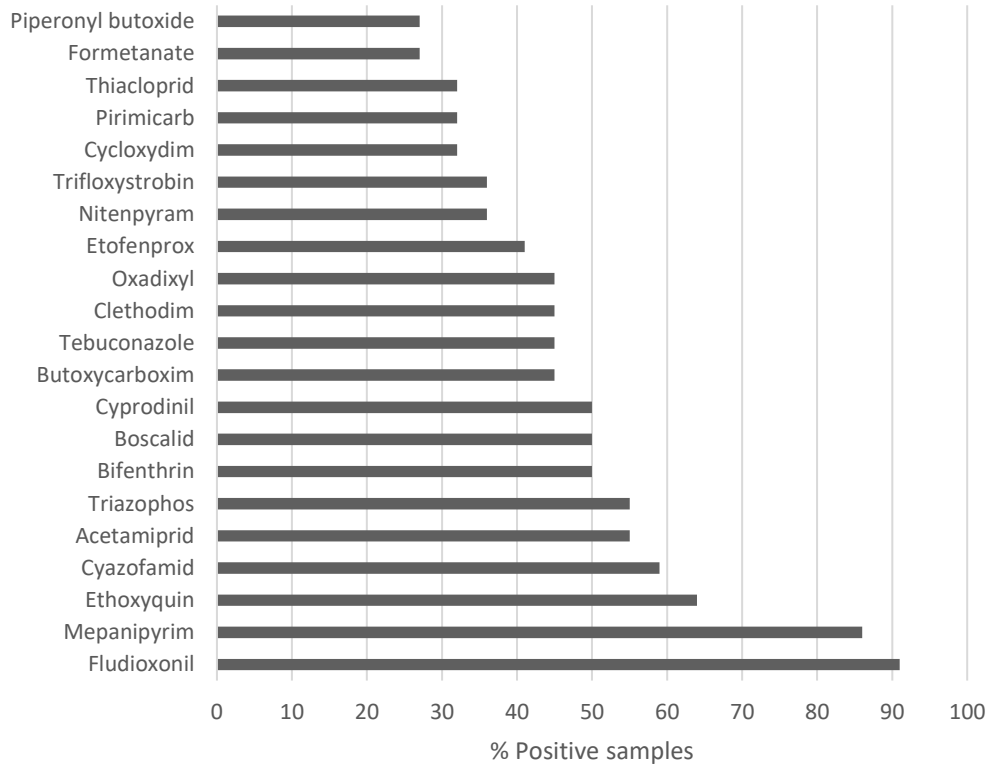

Figure 3. Occurrence of non-target pesticides in pear juice samples after retrospective screening

Similarly, the insecticide bifenthrin was also detected in 50% of the samples (*n = 21*). Although bifenthrin would fulfil the safety requirements according to the last update of report SANCO/12946/2011 released in 2018, it has not received any authorization yet. Oxadixyl was another relevant compound in the analyzed samples. This fungicide was found in 46% of the samples (*n* = 21) despite its use not allowed being in pears, as brought under Regulation (EC) No. 2076/2002 [31].

According to the mentioned legislation, butoxycarboxim was established as another forbidden insecticide, but it was present in 46% of the samples (*n = 21*). Considering that pesticide residues occurred in all samples, organic juice samples showed significantly less ($p < 0.05$) residues than conventional samples, as expected. This fact could also explain the extensive contamination of organic samples in comparison with conventional samples. Therefore, the presence of non-approved pesticides in the analyzed samples indicates the necessity of monitoring potential contaminants of pears and pear-derived products.

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3.3. *Occurrence and exposure assessment of mycotoxins in ready-to-eat tree nut products through ultra-high performance liquid chromatography coupled with high resolution q-orbitrap mass spectrometry*

1. Introduction

Tree nuts have become a popular alternative to unhealthy snacks due to its attributed benefits. The intake of tree nuts has been related to a lower risk of suffering from cardiovascular diseases through several mechanisms and can also act as antioxidant suppliers [1-4]. According to the International Nut and Dried Fruit Council (INC), the annual production of tree nuts products has increased over the last ten years, especially almonds, walnuts and pistachios, reaching a maximum of 4.6 million metric tons in 2019 and remarking a global trend in tree nuts consumption [5].

Nevertheless, tree nuts are susceptible to fungal growth that can occur for several reasons related to environmental factors, such as moisture and temperature. In addition, improper post-harvest practices and storage conditions can also promote fungal contamination [6]. As a consequence of these mentioned factors, mycotoxins could also be expected in crops. These are secondary metabolites produced by several filamentous fungi, mainly *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium* and *Penicillium* spp., that can exert severe adverse effects including neurotoxicity, nephrotoxicity, immunosuppression or carcinogenesis [7]. According to the carcinogenic potential, some mycotoxins

have been included in the list of human carcinogens released by the International IARC.

In order to control the content of potentially dangerous mycotoxins in ready-to-eat tree nuts products, the EC released the Regulation (EC) 1881/2006 [8] amended by Regulation (EU) 165/2010 [9] setting MLs for certain mycotoxins. Almonds and pistachios cannot exceed 8 µg/kg for aflatoxin B1 and 10 µg/kg for the sum of aflatoxin B1, B2, G1 and G2, whereas MLs in walnuts were set at 2 µg/kg and 4 µg/kg, respectively. However, different mycotoxins could be expected due to the susceptibility of tree nuts to fungal contamination. In this line, *Aspergillus* and *Fusarium* genera have been characterized as other major pathogens in tree nuts, so their secondary toxic metabolites could also be expected [6, 10, 11]. Alongside aflatoxins, relevant toxins from *Fusarium* genus included in the IARC classification, such as T-2 or zearalenone, have been evaluated in tree nuts products [12-17]. However, zearalenone-derived forms have been scarcely studied and, recently, the EFSA remarked the necessity to include these metabolites in risk assessment studies due to their oestrogenic activity [18]. Furthermore, the presence of other mycotoxins-producing fungi such as *Alternaria*, able to produce the genotoxic compounds alternariol (AOH) and alternariol monomethyl-ether (AME), has been detected in tree nuts [11]. According to the EFSA CONTAM Panel, these mycotoxins have been scarcely studied, so the toxicological potential is still unknown, and seem to be major contributors to mycotoxin exposure in several commodities [19]. Therefore, there is a necessity to develop analytical methods able to detect and quantify these less

studied mycotoxins, since they could also contribute towards overall exposure when consuming ready-to-eat nuts products.

To overcome this, sensitive methods are required in order to detect low levels of mycotoxins occurring in tree nuts products. In addition, environmental conditions, geographical area or harvest practices can lead to a strong variety among products regarding mycotoxins occurrence, so constant monitoring based on sensitive and multi-analytes methods are required for having proper mycotoxin profiles. The most recent methods for detecting mycotoxins in tree nuts are based on LC coupled to MS/MS [12-17]. However, other alternatives are able to provide a more precise detection and quantification. The use of high-resolution mass spectrometry stands as the best alternative when performing simultaneous determinations of analytes. Identification based on full scan-AIF analysis represents an improvement in mass accuracy when compared to traditional multiple reaction monitoring analysis based on QqQ [20]. Furthermore, Q-Orbitrap offers a better performance than other high-resolution mass spectrometers for low *m/z* compounds, as most mycotoxins. Hence, the aim of this work was to evaluate the presence of eighteen mycotoxins from different genera in ready-to-eat tree nuts products (n = 54), including almonds, walnuts and pistachios from Italian markets through UHPLC coupled to Q-Orbitrap HRMS. To achieve this, a single QuEChERS-based extraction was validated in the three commodities. In addition, the risk characterization resulting from mycotoxin contamination in ready-to-eat tree nuts products was performed in Italian population for the first time.

2. Results and discussion

2.1 Analytical Method Validation

The proposed methodology was validated for the simultaneous detection and quantification of 18 mycotoxins in almonds, walnuts and pistachios. Results are shown in Tables 1a, 1b and 1c. Linearity, expressed through correlation coefficient (R2), ranged from 0.9908 to 0.9998 for all compounds analyzed. Matrix effect was quantified through the percentage of SSE ranging from 77 to 120%, from 74 to 148% and from 82 to 149% in almond, walnut and pistachio, respectively. There were only two analytes out of the range 80-120%: AME showed a %SEE of 148% and 149% in pistachio and almond, respectively, whereas %SEE for ENNB were 77% and 74% in almond and pistachio. In order to avoid miscalculation, matrix-matched calibration curves were used for quantification purposes. Despite the complexity of those matrices consisting mainly of proteins and fatty acids, a simple ACN-based extraction including a clean-up step with C18 was enough to almost completely remove the matrix interference. The method also displayed a high sensitivity, with limits of quantification (LOQs) ranging from 0.20 to 0.78 µg/kg for all the analytes in each matrix. The recovery studies showed satisfactory results. For almonds, values ranged from 81 to 106% at the highest fortification level (20 µg/kg), from 73 to 95% at a medium level (5 µg/kg) and from 71 to 95% at the lowest level (1 µg/kg). Similarly, walnuts showed values from 79 to 105%, 70 to 99% and 71 to 100% for the highest, medium and lowest levels respectively. Finally, recovery values for mycotoxins in pistachios ranged from 80 to 113%, from 70 to 105% and 72 to 107% for the highest, medium and lowest

fortification levels. The precision evaluated through RSD_r and RSD_R was below 20% for all the analytes at three spiking level

Table 1a. Method performance in almonds.

		Recovery (%) (RSD_R (%))			
Analyte	SSE (%)	20 ng/g	5 ng/g	1 ng/g	LOQ (ng/g)
NEO	106	83 (12)	77 (16)	88 (7)	0.780
AFG2	101	88 (16)	82 (19)	78 (14)	0.195
AFG1	111	83 (20)	78 (20)	82 (13)	0.390
AFB2	106	93 (15)	94 (16)	83 (12)	0.195
AFB1	117	100 (10)	95 (15)	95 (5)	0.390
HT-2	115	105 (8)	89 (17)	79 (8)	0.780
α-ZAL	102	88 (11)	92 (12)	83 (10)	0.390
α-ZEL	109	92 (7)	88 (9)	87 (6)	0.780
AOH	105	81 (14)	73 (14)	71 (14)	0.195
T-2	114	104 (16)	87 (11)	89 (13)	0.780
β-ZAL	118	96 (10)	95 (13)	89 (10)	0.780
β-ZEL	98	95 (9)	90 (15)	76 (9)	0.195
ZEN	120	94 (18)	81 (17)	86 (18)	0.195
AME	149	94 (15)	85 (16)	81 (15)	0.780
ENNB	77	94 (6)	90 (9)	98 (10)	0.780
ENNB1	106	106 (14)	84 (13)	74 (10)	0.780
ENNA1	111	87 (3)	86 (5)	95 (18)	0.390
ENNA	102	86 (12)	83 (13)	89 (12)	0.780

Table 1b. Method performance in walnuts.

Analyte	SSE (%)	Recovery (%) (RSD^R (%))			LOQ (ng/g)
		20 ng/g	5 ng/g	1 ng/g	
NEO	91	85 (9)	81 (14)	92 (10)	0.780
AFG2	93	85 (5)	78 (9)	82 (10)	0.195
AFG1	91	79 (5)	70 (8)	76 (11)	0.780
AFB2	103	91 (4)	84 (8)	79 (12)	0.390
AFB1	97	102 (6)	83 (12)	78 (10)	0.390
HT-2	107	97 (9)	88 (11)	76 (9)	0.780
α-ZAL	103	99 (10)	81 (12)	100 (9)	0.780
α-ZEL	110	88 (7)	78 (15)	75 (9)	0.780
AOH	105	83 (4)	71 (6)	74 (8)	0.195
T-2	96	86 (10)	84 (13)	79 (8)	0.780
β-ZAL	105	95 (5)	91 (11)	99 (12)	0.780
β-ZEL	97	94 (7)	85 (7)	95 (12)	0.195
ZEN	100	81 (7)	78 (7)	73 (5)	0.195
AME	114	80 (7)	74 (9)	81 (15)	0.780
ENNB	74	101 (5)	90 (9)	84 (16)	0.780
ENNB1	99	88 (8)	89 (10)	76 (8)	0.780
ENNA1	96	105 (8)	99 (9)	76 (13)	0.780
ENNA	107	99 (8)	87 (15)	86 (8)	0.780

Table 1c. Method performance in pistachios.

Analyte	SSE (%)	Recovery (%) (RSD_R (%))			LOQ (ng/g)
		20 ng/g	5 ng/g	1 ng/g	
NEO	103	86 (10)	75 (14)	72 (8)	0.780
AFG2	82	106 (10)	82 (14)	82 (9)	0.390
AFG1	91	80 (11)	70 (11)	73 (6)	0.780
AFB2	87	113 (10)	103 (11)	97 (7)	0.390
AFB1	94	87 (18)	82 (18)	79 (13)	0.390
HT-2	105	91 (14)	85 (17)	88 (14)	0.780
α-ZAL	86	99 (13)	99 (16)	88 (17)	0.780
α-ZEL	97	83 (15)	85 (15)	89 (7)	0.780
AOH	83	84 (16)	77 (17)	83 (13)	0.390
T-2	85	92 (11)	98 (14)	85 (18)	0.780
β-ZAL	96	87 (10)	89 (15)	81 (9)	0.780
β-ZEL	112	92 (9)	92 (13)	89 (12)	0.780
ZEN	118	104 (9)	105 (9)	107 (13)	0.195
AME	148	87 (10)	77 (16)	75 (14)	0.780
ENNB	115	99 (14)	92 (16)	83 (15)	0.780
ENNB1	116	102 (6)	94 (8)	96 (16)	0.780
ENNA1	118	105 (13)	99 (16)	96 (13)	0.780
ENNA	114	100 (14)	92 (17)	96 (15)	0.780

Several multi-mycotoxins methods for tree nuts products based on QuEChERS methodology have been recently published, as shown in Table 2. Although methodologies are focused on detecting aflatoxins since they are the only regulated mycotoxins in tree nuts, less attention have been put into *Fusarium* and *Alternaria* mycotoxins, which are common genera causing fungal contamination in almonds, pistachios and walnuts, as stated by Marín and Ramos [10] and Escrivá, Oueslati, Font and Manyes [11]. In addition, ZEN-derived forms have also been here validated for its simultaneous detection, as recommended by the EFSA [18]. The main feature of the present methodology lies in its high sensitivity when compared to previous ones, with LOQs ≤ 0.78 µg/kg. Sensitivity also plays a key role when performing exposure assessment studies, allowing a more realistic analysis and avoiding underestimation of mycotoxins as highlighted by the EFSA.

Table 2. Quantitative methods for multi-mycotoxin detection in several tree nuts products.

Analytes (n)	Method	Sample treatment	Sensitivity (µg/kg)	References
Aflatoxins (B1, B2, G1, G2), CIT, DON, FB1, FB2, FUS-X, HT-2, OTA, T-2, STE, ZEN (14)	UHPLC-MS/MS	QuEChERS-DLLME	0.61-150	Arroyo-Manzanares *et al.*, 2013 [12]
Aflatoxins (B1, B2, G1, G2), BEA, DAS, enniatins (A, A1, B, B1), FB1, FB2, FB3, HT-2, OTA, T-2 (16)	LC-MS/MS	QuEChERS-SPE cartridge	0.2-45	Azaiez *et al.*, 2014 [13]
Aflatoxins (B1, B2, G1, G2), DAS, 3AC-DON, 15AC-DON, DON, FB1, FB2, FUS-X, HT-2, NEO, OTA, T-2, ZEN (16)	LC-MS/MS	QuEChERS-Z-Sep$^+$ + C18	1.25 - 5	Cunha *et al.*, 2018 [14]
Aflatoxins (B1, B2, G1, G2), AME, AOH, BEA, enniatins (A, A1, B, B1), OTA, OTB, T-2, TEN, ZEN (16)	UPLC-MS/MS	QuEChERS-C18	0.1-5	Wang *et al.*, 2018 [15]
3-ADON, aflatoxins (B1, B2, G1, G2, M1), DAS, ERGC1, ERGC2, FB1, FB2, GLI, HT-2, OTA, T-2, α-ZEL, ZEN (17)	Nano flow LC-HRMS	QuEChERS EMR-Lipid	0.05-5	Alcantara *et al.*, 2019 [16]
Aflatoxins (B1, B2, G1, G2), α-ZEL, ZEN (6)	UHPLC-MS/MS	QuEChERS-C18	0.5-1	Hidalgo *et al.*, 2019 [17]
Aflatoxins (B1, B2, G1, G2), AME, AOH, enniatins (A, A1, B, B2), HT-2, NEO, T-2, α-ZAL, α-ZEL, β-ZAL, β-ZEL, ZEN (18)	UHPLC-HRMS	QuEChERS-C18	0.2-0.78	Present work

AME: alternariol monomethyl-ether; AOH: alternariol; BEA: beauvericin; CIT: citrinin; DAS: diacetoxyscirpenol; DLLME: dispersive liquid-liquid microextraction; 3AC-DON: 3-acetyl-deoxynivalenol; 15AC-DON: 15-acetyl-deoxynivalenol; DON: deoxynivalenol; EMR: enhanced matrix removal; ERGC1: ergocornine 1; ERGC2: ergocornine 2; FB1: fumonisin B1; FB2: fumonisin B2; FB3: fumonisin B3; FUS-X: fusarenon-X; GLI: gliotoxin; HRMS: high-resolution mass spectrometry; LC: liquid chromatography; MS/MS: tandem mass spectrometry; NEO: neosolaniol; OTA: ochratoxin A; OTB: ochratoxin B; STE: sterigmatocystin; TEN: tentoxin; UHPLC: ultra-high performance liquid chromatography; UPLC: ultra performance liquid chromatography; α-ZAL: alpha-zearalanol; α-ZEL: alpha-zearalenol; β-ZAL: beta-zearalanol; β-ZEL: beta-zearalenol; ZEN: zearalenone

2.2 Analysis of Real Samples

Up to nine different mycotoxins were detected and quantified in the here-analyzed samples. Results are shown in Table 3. At least one mycotoxin occurred in 33 out of 54 nuts products. The most commonly found mycotoxins belong to *Fusarium* species, whereas *Alternaria* metabolites were also found in all the studied commodities.

Table 3. *Occurrence of mycotoxins in the tree nuts analyzed*

			Range (µg/kg)	
Analyte	Incidence (n, (%))	Mean (µg/kg)	Min	Max
Almonds (n = 17)				
AFB1	1 (6)	0.45	-	-
α-ZAL	3 (18)	3.99	3.70	4.54
α-ZEL	1 (6)	1.40	-	-
β-ZEL	2 (12)	0.54	0.46	0.62
AOH	2 (12)	0.35	0.34	0.37
Walnuts (n = 22)				
α-ZAL	2 (12)	2.18	2.13	2.24
β-ZAL	3 (18)	3.13	1.67	5.24
β-ZEL	4 (24)	0.39	0.3	0.55
ZEN	3 (18)	0.44	<LOQ	0.93
AOH	9 (53)	0.67	0.29	1.65
AME	3 (18)	1.63	1.13	1.95
ENN B1	1 (6)	1.30	-	-
Pistachios (n = 15)				
α-ZAL	2 (12)	25.75	2.16	49.35
β-ZAL	1 (6)	11.86	-	-
α-ZEL	2 (12)	1.50	1.26	1.74
β-ZEL	10 (59)	3.42	0.96	8.60
AOH	1 (6)	7.75	-	-

Referring to almonds (*n* = 17), five mycotoxins were identified in 41% of samples. The most relevant compound was α-ZAL, a *Fusarium* toxin that results from the metabolism of its parental mycotoxin, ZEN. This toxin was present in 18% of the samples ranging from 3.70 to 4.54 µg/kg. β-ZEL, another product from the metabolization of ZEN, was found in 12% of samples at low level going from 0.46 up to 0.62 µg/kg. ZEN has also been studied in almonds, and contamination at 1.2 and 3.48 µg/kg was reported by Škrbić, et al. [21] in the two analyzed samples. According to the present results, ZEN metabolites resulted to be the major contaminants in almonds and scarce literature regarding them is available. Since ZEN appears to be a common toxin in tree nuts, its metabolites could also be expected, as observed in this study. Despite not being a major fungus in almonds, *Alternaria* toxins have been usually reported. AOH was also quantified in 12% of samples ranging from 0.34 to 0.37 µg/kg. In a previous work conducted by Varga, et al. [22], AOH was found in one sample (*n* = 8) at 1.5 µg/kg, more than three times the contamination here-reported. Wang, Nie, Yan, Li, Cheng and Chang [15] reported AOH contamination in four samples (*n* = 25) with a maximum level of 54.24 µg/kg. On the contrary, aflatoxins are one the most studied toxins in every nut typology. AFB1 was found in one sample at 0.45 µg/kg, complying with the regulation regarding MLs. Liao, et al. [23] reported contamination with AFB1 at 0.3 µg/kg in one almond sample (*n* = 9), similar to the concentration found in this study. Due to the toxicological potential of aflatoxins, special treatments are used in order to eliminate fungal contamination or to inactivate the toxins, such as roasting, sorting and physical segregation [10], so it is not usual finding

aflatoxins at concentrations above the MLs (8 µg/kg for AFB1 and 10 µg/kg for the sum of aflatoxins).

Walnuts (*n* = 22) showed contamination with seven mycotoxins, finding at least one in 59% of samples. ZEN was quantified in 18% of samples ranging from <LOQ to 0.93 µg/kg. Several metabolites were also detected: β-ZEL was the most common one, present in 24% of samples varying from 0.3 to 0.55 µg/kg; β-ZAL was found in 18% of samples and, quantitatively, meant the most relevant one at levels going from 1.67 to 5.24 µg/kg; lastly, α-ZAL was present in 12% of samples at 2.13 and 2.24 µg/kg. ENNB1, an emerging *Fusarium* mycotoxin, was quantified at 1.3 µg/kg in one sample (6%). As previously observed in almonds, walnut samples also contained ZEN and ZEN metabolites. In the same study conducted by Wang, Nie, Yan, Li, Cheng and Chang [15] a high contamination with ZEN was observed in one sample (*n* = 35) at 49.35 µg/kg, whereas Arroyo-Manzanares, Huertas-Pérez, Gámiz-Gracia and García-Campaña [12] reported the presence of ZEN in one sample (*n* = 6) at 221.8 µg/kg. These levels of contamination strongly vary from the here-obtained, with concentrations below 1 µg/kg. Furthermore, ZEN metabolites were quantitatively more relevant than ZEN, remarking the necessity of taken into consideration these mycotoxins when performing contaminants analysis in walnuts.

Alternaria toxins are not common mycotoxins included in tree nuts studies. Nevertheless, AOH was extensively found at low concentrations ranging from 0.29 to 1.65 µg/kg in 53% of samples. AME was also present in 18% of samples at slightly higher levels, going from 1.13 up to 1.95 µg/kg. Therefore, sensitive

analytical methods are required in order to understand the incidence of these toxins. In this line, Wang, Nie, Yan, Li, Cheng and Chang [15] quantified AOH and AME in 23% and 31% of the walnut samples analyzed (*n* = 35), respectively, with a LOQ of 2 µg/kg. AOH was quantified in a range of 5.78-142.9 µg/kg and AME ranged from 1.53 to 110.5 µg/kg. Similar to the here-presented data, *Alternaria* toxins resulted to be the most common toxins occurring in walnuts.

Lastly, five mycotoxins were quantified in pistachios samples (*n* = 15), with at least one occurring in 80% of them. Among the matrices analyzed, pistachios resulted to be the most contaminated. β-ZEL was the most prevalent mycotoxin, being detected in 59% of samples ranging from 0.96 to 8.6 µg/kg. α-ZEL and α-ZAL were both quantified in 12% of samples, ranging from 1.26 to 1.74 µg/kg and from 2.16 to 49.35 µg/kg, respectively. β-ZAL was only found in one sample at 11.86 µg/kg and, similarly, AOH was found at 7.75 µg/kg.

Quantitatively, pistachios showed a significant heavier contamination ($p < 0.05$) when compared to almond and walnuts. In addition, the highest levels of AOH and ZEN metabolites were detected in pistachios. Although aflatoxins or ZEN have been analyzed in this matrix [22, 24, 25] there is scarce literature regarding the here-found toxins. Furthermore, the available validated procedures for ZEN metabolites cannot reach a high sensitivity as the here-obtained (LOQ = 0.78 µg/kg), with Spanjer, et al. [26] establishing the LOQs at 40 µg/kg and Hidalgo-Ruiz, Romero-González, Martínez Vidal and Garrido Frenich [17] at 1 µg/kg for α-ZEL. Sensitivity becomes a crucial feature in analytical procedures, even more when the contamination reported only reach a few µg/kg. In this line,

Alcántara-Durán, Moreno-González, García-Reyes and Molina-Díaz [16] did not found α-ZEL occurring in pistachios despite having a low LOQ (0.05 µg/kg). *Alternaria* toxins have not been deeply studied in pistachio neither. Varga, Glauner, Berthiller, Krska, Schuhmacher and Sulyok [22] developed a procedure for AOH with the LOQ at 9.6 µg/kg, which would not have been sensitive enough for detecting the contamination here-reported (7.75 µg/kg).

There is a strong variability referring to the content of mycotoxins depending on several parameters including temperature, moisture or pre and post-harvest practices, among others. This variability observed remarks the necessity of constantly monitoring this kind of products using highly sensitive analytical procedures in order to ensure a safe consumption. Furthermore, an investigation carried out by the EFSA [18] considered the application of potency factors for ZEN metabolites ranging from 0.2 to 60 times the toxicity associated to ZEN. Considering the high uncertainty related to these metabolites, the analytical procedures should include these mycotoxins that could account for future exposure assessment studies.

2.3 Exposure Assessment

An exposure assessment and risk characterization were performed taking into consideration the left-censored data for the more prevalent mycotoxins detected in the samples, including ZEN-derived forms, AOH and AME. Results are showed in Table 4.

The tree nuts consumption did not vary much throughout the age groups so children, with the lowest body weight, showed the heaviest exposure. The percentages of relevant TDI or TTC calculated for children were two fold higher than those for teenagers and adults, but below the maximum tolerable values established by the EFSA CONTAM Panel at 0.25 μg/kg bw/day for the sum of ZEN and its metabolites and 0.0025 μg/kg bw/day for both AOH and AME.

Under the worst-case scenario, the percentages of relevant TDI or TTC calculated for children meant between a tenth and a fifth of the established safety levels. Nevertheless, it might be a concern since ZEN, its derived forms, AOH and AME can be found in different commodities such as cereals and other vegetal products, which are more commonly consumed by children.

The results evidence a negligible exposure of these mycotoxins due to ready-to-eat nuts products, but these scarce studied mycotoxins might be of importance when performing risk assessment through total diet studies.

Table 4. Risk characterization of detected mycotoxins in different population groups based on the percentage of tolerable daily intake considering four different scenarios per group: mean and 95th percentile consumption value combined with lower and upper bound of contamination

				Risk Characterization (%TDI or %TTC)															
				Children				Teenager				Adult				Elderly			
		C (µg/kg)		Mean		P95th		Mean		P95th		Mean		P95th		Mean		P95th	
Mycotoxins	TDI or TTC (µg/kg bw/day)	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB	LB	UB
Σ ZEN_d	0.25	7.25	59.70	0.7	5.9	1.9	15.6	0.4	3.2	1.2	10.1	0.4	3.3	1.0	8.5	0.5	3.8	1.3	10.5
AOH	0.0025	0.10	0.48	0.8	4.8	2.8	12.4	0.4	2.4	1.6	8.0	0.4	2.8	1.6	6.8	0.8	3.2	1.6	8.4
AME	0.0025	0.25	0.82	2.4	8.0	6.4	21.2	1.2	4.4	4.4	14.0	1.2	4.4	3.6	11.6	1.6	5.2	4.4	14.4

Σ ZEN_d: sum of ZEN and its derived forms α-ZAL, α-ZEL, β-ZAL, β-ZEL; TDI: tolerable daily intake; TTC: threshold of toxicological concern; *C*: contamination; LB: lower bound; UP: upper bound; P95th: 95th percentile.

3. Materials and Methods

3.1. Chemicals and Reagents

ACN, MeOH, water and formi acid (FA) for LC mobile phase (HPLC grade) were acquired from Merck (Darmstadt, Germany). Ammonium formate (NH4HCO2), sodium chloride (NaCl), magnesium sulfate (MgSO4) and C18 were acquired from Sigma Aldrich (Milan, Italy). Eighteen mycotoxin standards (purity >98%) including aflatoxins (AFB1, AFB2, AFG1, and AFG2), HT-2 toxin, T-2 toxin, neosolaniol (NEO), zearalenone (ZEN), α-zearalanol (α-ZAL), α-zearalenol (α-ZEL), β-zearalanol (β-ZAL), enniatins (A, A1, B, and B1), alternariol monomethyl ether (AME) and alternariol (AOH) were obtained from Sigma-Aldrich (Milan, Italy).

Individual stock solutions were built by dissolving 1 mg of solid reference standard in 1 mL of methanol. An intermediate mixed solution containing all the mycotoxins at a concentration of 30 µg/mL was obtained after mixing individual stock solutions and diluting in MeOH:H2O (70:30 v/v) 0.1% formic acid. Working standard solutions at 1.6, 0.4, 0.08 µg/mL were used for spiking experiments (fortification levels at 20, 5 and 1 µg/kg). All solutions were kept in safe conditions at −20 °C in screw-capped glass vials.

3.2. Sampling

Fifty-four commercially available nuts products were randomly purchased from different supermarkets in the Campania region of Southern Italy. Products were classified as walnuts (n = 22), pistachios (n = 15) and almonds (n = 17) and

shipped to the laboratory in their original packages. All the samples were conserved in dark and cool conditions as recommended by the manufacturer, and analysis were carried out within five days after receiving them.

3.3. Sample Preparation

The sample preparation was based on a procedure previously developed by Cunha, Sá and Fernandes [14], with some modifications. Briefly, 10 g of homogenized sample was introduced into a 50 mL Falcon tube (Conical Polypropylene Centrifuge Tube; Thermo Fisher Scientific, Milan, Italy) and 5 mL of distilled water and 5 mL of ACN containing 1% formic acid (v/v) were added. The sample was vortexed (ZX3; VEPL Scientific, Usmate, Italy) for 2 min. Then, 0.5 g of sodium chloride and 2.0 g of anhydrous sulfate sodium were added. The tube was manually shaken for 1 min and then centrifuged (X3R Heraeus Multifuge; Thermo Fisher Scientific, Kalkberg, Germany) for 10 min at 4907 × g at room temperature. The supernatant (1.5 mL) was transferred to a 15 mL Falcon tube containing 50 mg of C18 sorbent, then vortexed for 1 min and centrifuged for 3 min at 4907 x g at room temperature. Lastly, 0.4 mL were collected, filtered through a 0.22 µm filter and injected into the UHPLC-Q-Orbitrap HRMS instrument.

3.4. UHPLC-Q-Orbitrap HRMS Analysis

Chromatographic and HRMS conditions were the same as described in pages 102-103 whereas parameters used for mycotoxin identification and quantification are shown in Table 5.

Table 5. UHPLC-HRMS parameters corresponding to the here-analyzed mycotoxins

Analyte	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (Δ ppm)	Collision Energy (eV)	Product ions (*m/z*)
NEO	4.25	$C_{19}H_{26}O_8$	$(M+NH_4)^+$	400.19659	400.19632	-0.67	10	305.13803
								141.00530
AFG2	4.52	$C_{17}H_{14}O_7$	$(M+H)^+$	331.08123	331.08078	-1.36	37	313.07010
								245.08032
AFG1	4.55	$C_{17}H_{12}O_7$	$(M+H)^+$	329.06558	329.06549	-0.27	40	243.06467
								200.04640
AFB2	4.60	$C_{17}H_{14}O_6$	$(M+H)^+$	315.08631	315.08615	-0.51	36	287.09064
								259.05945
AFB1	4.64	$C_{17}H_{12}O_6$	$(M+H)^+$	313.07066	313.07053	-0.42	36	285.07489
								269.04373
HT-2	4.74	$C_{22}H_{32}O_8$	$(M+NH_4)^+$	442.24354	442.24323	-0.70	27	263.12744
								215.10641
α-ZAL	4.81	$C_{18}H_{26}O_5$	$(M-H)^-$	321.17044	321.17065	0.65	29	259.09497
								91.00272
α-ZEL	4.83	$C_{18}H_{24}O_5$	$(M-H)^-$	319.15510	319.15500	-0.31	36	174.95604
								129.01947
T-2	4.84	$C_{24}H_{34}O_9$	$(M+NH_4^+$	484.25411	484.25430	0.39	23	215.10603
								185.09561
AOH	4.85	$C_{14}H_{10}O_5$	$(M-H)^-$	257.04555	257.04581	1.01	−32	215.03490
								213.05569
β-ZAL	4.94	$C_{18}H_{26}O_5$	$(M-H)^-$	321.17044	321.17059	0.47	40	259.09497
								91.00272
β-ZEL	4.97	$C_{18}H_{24}O_5$	$(M-H)^-$	319.15510	319.15500	-0.31	36	174.95604
								160.97665
ZEN	5.01	$C_{18}H_{22}O_5$	$(M+H)^+$	317.13945	317.13928	-0.54	-32	175.03989
								131.05008
AME	5.13	$C_{15}H_{12}O_5$	$(M-H)^-$	271.06120	271.06140	0.74	−36	256.03751
								228.04276
ENN B	5.56	$C_{33}H_{57}N_3O_9$	$(M+NH_4)^+$	657.44331	657.44348	0.26	50	214.14320
								196.13280
ENN B1	5.68	$C_{34}H_{59}N_3O_9$	$(M+NH_4)^+$	671.45986	671.45935	-0.76	48	214.14343
								196.13295
ENN A1	5.82	$C_{35}H_{61}N_3O_9$	$(M+NH_4)^+$	685.47461	685.47449	-0.18	48	228.15900
								210.14847
ENN A	5.99	$C_{36}H_{63}N_3O_9$	$(M+NH_4)^+$	699.49026	699.48987	-0.56	43	228.15900
								210.14847

3.5. Validation Parameters

An in-house validation study was carried out for the three different matrices here-analyzed following the EU Commission Decision 2002/657/EC guidelines in terms of linearity, selectivity, trueness, intra-day precision (repeatability), inter-day precision (reproducibility) and sensitivity expressed as LOQs [28]. Linearity was determined by injecting a series of neat solvent and matrix-matched calibration curves at eight concentrations levels in the range from 0.2 to 200 ng/mL with a deviation of ≤ 20% for each calibration level. The coefficient of determination was calculated using the means of the least square approach. For evaluating a potential interference of the matrix, the slopes corresponding to each linear function were compared. The %SSE occurred when a deviation ≥ 20% was observed after comparing both slopes. The selectivity of the method was assessed by injecting ten blank samples, observing no peaks that could interfere in the same retention time area that the analytes, considering a mass error of 5 ppm. Trueness was evaluated through recovery studies, spiking three blank samples at three different fortification levels: 1, 5 and 20 µg/kg. The measurements were made during three non-consecutive days. Values ranging from 70 to 120% of recovery were considered as optimal. Precision was assessed in terms of repeatability (expressed as the relative standard deviation after three determinations in a single day, RSD_r) and reproducibility (calculated by repeating the measurements in triplicate on three non-consecutive days, RSD_R). Sensitivity was determined through the LOQ for each analyte, which was established as the minimum concentration with a linear response that can be observed with a deviation ≤ 20% considering a mass error of 5 ppm.

3.6. Exposure Assessment

A deterministic approach was followed for performing the exposure assessment. The latest data consumption published by the Italian National Food Consumption Survey INRAN-SCAI 2005-06 were considered [29]. The commodities here-analyzed were all included in "nuts" category according to the survey, so exposure assessment was performed considering both the mean and the 95th percentile values. Population was divided in four age groups: children (3-9.9 years) mean consumption 6.4 g/day, P95 17.0 g/day; teenagers (10-17.9 years) mean consumption 7 g/day, P95 22.3 g/day; adults (18-65 years) mean consumption 9.5 g/day, P95 24.7 g/day and elderly (65 < years) mean consumption 11.1 g/day, P95 30.7 g/day. The mean body weights attached to each group were 26.1, 52.6, 69.7 and 70.1 kg, respectively, as detailed in the INRAN-SCAI 2005-06 survey. The PDI values were obtained following the next equation:

$$PDI = C \times I / bw, \quad (1)$$

where C represents the contamination (for the lower or upper bound) of each mycotoxin (μg/kg); I accounts for the mean or 95th percentile consumption established for each age group (g/day) and bw means the body weight assigned to each age group (kg). Once the PDIs were calculated, the tolerable daily intakes (TDIs) corresponding to the detected mycotoxins were considered for performing the risk characterization. In case of finding a mycotoxin without any TDI assigned by the EFSA yet, a TTC was used. The risk characterization was calculated following the next equation.

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

Because of the high proportion of left-censored data, two scenarios of exposure were defined considering negative samples as zero or LOQ for the lower-bound and upper-bound, respectively.

3.7. Statistical Analysis

Validation experiments were conducted in triplicate and expressed as mean values alongside the corresponding relative standard deviation (RSD, %). The normality was evaluated through Saphiro-Wilk test and multivariant analysis was carried out using the Kruskal-Wallis test in order to compare the contamination levels among different matrices. A p-value < 0.05 was considered as significant. The statistical software package IBM SPSS version 25 was used for data analysis.

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3.4. Citrinin dietary exposure assessment approach through human biomonitoring high-resolution mass spectrometry-based data

1. Introduction

Mycotoxins are secondary metabolites produced by several fungi genera, primarily *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria* and *Claviceps*. These compounds can be found in cereal grains, food commodities and animal feed under propitious environmental conditions or because of bad practices at any point from the preharvest interval to the storage [1-2]. Once ingested, mycotoxins can display a wide variety of adverse effects including immunosuppression, neurotoxicity or carcinogenicity [3-4]. In consequence, regulatory authorities set MLs in certain foodstuffs for several hazardous mycotoxins, in light of tolerable daily intake (TDIs) derived by Scientific Committees, e.g. the EFSA CONTAM Panel or the Joint FAO/WHO Expert Committee on Food Additives [5]. Over the last years, citrinin (CIT) has become a relevant compound, due to its occurrence in grains and grain-products and its toxicity [6-7], but the EFSA noted that occurrence data are insufficient to conduct dietary exposure assessments for humans [8].

CIT is produced by several *Aspergillus*, *Penicillium* and *Monascus* species and it can be found in stored grain and other plant products like fruits, herbs and spices, showing a wide distribution throughout different geographical areas around the world and occurring at concentration ranges from a few ng/g up to 1500 ng/g depending on the commodity [7, 9-13]. This toxin has also been identified

co-occurring with other toxins produced by these fungi, especially ochratoxin A (OTA) [7, 14]. Nonetheless, only maximum level for citrinin in food supplements based on rice fermented with red yeast *Monascus purpureus* has been set to date[15]. CIT is a quinone with a planar and conjugated structure that targets primarily the kidney, resulting in necrosis of renal tubules [8, 16]. Although the mechanism responsible for its toxicity is not fully understood, it could be related to the production of ROS linked to apoptotic processes [17-18]. Moreover, CIT has genotoxic properties and can induce micronuclei (mainly aneugenic) and chromosomal aberration in several animal and human cell lines [8, 19-20]. The EFSA CONTAM Panel concluded that the combined effect of OTA and CIT is mainly additive [8]. In combination with OTA, a synergistic effect has been reported after *in vitro* assays, displaying a higher nephrotoxic [21] and genotoxicity [22] potential. Nevertheless, the limited toxicological data available is insufficient to evaluate its carcinogenicity potential, so CIT has been placed into group 3 within the classification released by International IARC [23].

Referring to the metabolism of CIT, data are scarce on the sites of its bioconversion and the enzymes involved. The main product of CIT metabolism is dihydrocitrinone (DH-CIT), firstly detected in rat urine by Dunn, et al. [24]. This compound showed lower cytotoxic and genotoxic potential, so the conversion of CIT to DH-CIT could be considered as a detoxification process [22]. As regards the bioavailability, little is known in humans. The only toxicokinetic study carried out in humans determined a half-life of 6.7 h and 8.9 h for CIT and DH-CIT, respectively, and a rapid absorption of CIT with at least a 40% of the initial dose being excreted in urine [25]. After metabolization of the parent toxin, the urinary

levels of DH-CIT are strongly variable among individuals, with concentrations ranging between three and seventeen times greater in relation to the parent compound [26].

According to the toxicological potential of CIT, the EFSA CONTAM Panel derived a provisional tolerable daily intake (PTDI) of 0.2 μg/ kg bw per day that corresponds to the level of no concern for nephrotoxicity in order to characterize the risk of citrinin [8]. However, considering the lack of data regarding the occurrence of CIT in feed and foodstuffs, a reliable exposure assessment cannot be performed. A complementary approach to assess mycotoxin exposure is biomonitoring, which involves the analysis of parent compounds and/or their metabolites in human biological samples [27]. In this line, the sum of CIT and DH-CIT in urine has been proposed as an effective biomarker to assess the exposure to CIT [25-26, 28-29]. Several biomonitoring surveys have reported the occurrence of CIT and DH-CIT in urines from different human cohorts from Belgium [30-32], Czech Republic [33], Portugal [34-35], Germany [36-39], Haiti [39], Bangladesh [39-43], Nigeria [44], Turkey [36] and Tunisia [45]. Biomarkers should be measured by sensitive and specific analytical methods able to detect even a low level of exposure. Currently, HRMS stands as a suitable method for providing accurate measurements at low levels, and its high resolving power ensures a very specific detection in complex mixtures. Hence, the aim of this study was to evaluate the presence of citrinin and dihydrocitrinone in 300 urine samples from Italian population in order to assess the exposure. For quantification purposes, an UHPLC-Q-Orbitrap HRMS-based methodology was developed.

2. Material and methods

2.1. Chemicals, Reagents and Materials

MeOH, ACN and water for LC mobile phase (LC-MS grade) were purchased from Merck (Darmstadt, Germany). Ammonium formate (analytical grade) was acquired from Fluka (Milan, Italy) and formic acid (MS grade) was provided by Carlo Erba reagents (Cornaredo, Italy). Sodium chloride (NaCl) and C18 (analytical grade) were purchased from Sigma Aldrich (Milan, Italy). Conical centrifuge polypropylene tubes of 15 mL were obtained from BD Falcon (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 µm) were acquired from Phenomenex (Castel Maggiore, Italy).

Analytical standard of CIT and DH-CIT (HPLC purity > 98%) were acquired from Sigma-Aldrich (Milan, Italy) and Analyticon Discovery GmbH (Potsdam, Germany), respectively. Stock solutions were prepared diluting 1 mg of each mycotoxin in 1 mL of MeOH. Working solutions were built from the stock, diluting in MeOH/H_2O (70:30 *v/v*) 0.1% formic acid until reaching the desired concentrations for spiking experiments. The solutions were stored in tightly closed containers at -20°C in a well-ventilated place as specified by the manufacturer.

2.2. Urine Sample Collection

First-spot morning urine (50 mL) samples from 300 volunteers living in Campania region (South Italy) and aged between 2 and 91 years old were collected into sterile plastic vessels during January and February 2018. After collection, each sample was aliquoted and kept at -20°C until analysis due to

stability issues. Volunteers were randomly recruited among students, academic and non-academic staff from the Faculty of Pharmacy of University of Naples Federico II who complied the following exclusion criteria: (i) only one member per family was allowed; (ii) people exposed to a large number of mycotoxins in a way other than food, such as farmers and veterinarians, were excluded; (iii) people with severe problems in liver, bile or kidney could not participate due to related risk of interferences with the metabolism of mycotoxins. The use of medication was not an exclusion criterion since scarce information regarding interferences with mycotoxins is available. The participants were not subjected to any diet restriction before and during the sampling. All volunteers provided a written consent in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects and the project was approved by the University of Naples Federico II Institutional Human Research Committee. The sample size *(n* = 300) selected is consistent with previous pilot biomonitoring studies and recommendations from the International Federation of Clinical Chemists (IFCC) [46].

All samples were anonymous but participants were asked to write down their gender and age in the vessel for sample classification purpose. The sampling tried to maintain the gender parity (male: 45.7%, female: 54.3%). Three age groups were considered: <18 years old (*n* = 20), from 18 to 65 years old *(n* = 170), and >65 years old *(n* = 110). Samples with undetectable levels of mycotoxins were used for recovery studies.

2.3. Sample Preparation

The sample preparation was performed following a previously developed method by Rodríguez-Carrasco, et al. [47]. In short, 1.5 mL of sample was placed into a 2 mL Eppendorf Safe-Lock Microcentrifuge tube and centrifuged at 3926 *x g* for 3 min. Next, 1 mL of the supernatant was collected and transferred into a 15 mL screw cap test tube with conical bottom and 1 mL of ACN was added. The mixture was vortexed for 30 s and a mixture of 0.3 g sodium chloride and 30 mg of C18 sorbent was added. The solution was vortexed for 30 s and centrifuged at 3926 *x g* for 3 min at 4°C. Finally, the upper layer was collected and evaporated to dryness under nitrogen flow at 45°C, reconstituted with 0.5 mL of MeOH/H_2O (70:30 *v/v*) 0.1% formic acid and filtered through a 0.2 µm filter prior to UHPLC-Q-Orbitrap HRMS analysis.

2.4. UHPLC-Q-Orbitrap HRMS Analysis

Chromatographic and HRMS conditions were the same as described in pages 102-103. Identification parameters for CIT and DH-CIT were optimized by injection of analytical standards using a solution at 1 µg/mL in both positive and negative ESI modes. Data analysis was carried out using Quan/Qual Browser Xcalibur v.3.1.66 (Thermo Fisher Scientific, Waltham, USA).

2.5. Method Validation

In-house validation was carried out in accordance with the EU Commission Decision 2002/657/EC [48]. The assessed parameters were linearity, selectivity, trueness, repeatability, within-laboratory reproducibility, LOD and LOQ. Linearity

(r^2) was determined through both neat solvent and matrix-matched calibration curves ranging from 25 to 0.01 ng/mL and considering a deviation < 20% for each concentration level. In order to evaluate the interference of the matrix, the slopes of both calibration curves were used to calculate the percentage of SSE through the following equation:

$$\%\mathrm{SSE} = \frac{\mathrm{Sm}}{\mathrm{Ss}} x100$$

where S_m represents the matrix-matched calibration slope and S_s is the solvent calibration slope. An %SSE below 100% indicated signal suppression whereas values above 100% meant signal enhancement in the range of concentrations previously assayed. Trueness was assessed through recovery experiments, spiking blank urine samples at three different concentrations (5, 1 and 0.5 ng/mL). Experiments were performed in triplicate on three non-consecutive days and expressed as intra-day (repeatability, RSD_r) or inter-day (within-laboratory reproducibility, RSD_R) relative standard deviation. LODs were established as the lowest concentration where the molecular ion could be distinguished from the background noise (S/N=3) whereas LOQs were set as the lowest concentration where the molecular ion could be identified inside the linear range, considering a mass error below 5 ppm.

Selectivity was also studied in order to determine the presence of potential coelutants in the matrix, so blanks (n = 10) were injected right after the highest calibration sample. For confirmation criteria, the retention times of the analytes in standards and samples were compared.

2.6. Quality Control/Quality Assurance

Chromatographic and spectra data were used for proper confirmation of the analytes. Retention times corresponding to the analytes were compared in both positive samples and standards in neat solvent at a tolerance of ± 2.5% of the total run time (8 min). Data quality was monitored using a comprehensive range of quality assurance and quality control procedures. Therefore, a reagent blank, a procedural blank, a replicate sample and a matrix-matched calibration were included in each batch of samples in order to assess the stability and robustness of the instruments throughout the whole analysis.

2.7. Creatinine Analysis

Urinary levels of creatinine (Crea) were determined through a spectrophotometric methodology previously reported by Rodríguez-Carrasco, et al. [49]. Briefly, 3.5 mM picric acid was mixed with 1000 mM NaOH to obtain alkaline picrate. The resultant solution was kept in dark conditions in an amber glass container. Urine samples were diluted using ultrapure water (1:10, *v/v*) and 1 mL was reacted with 1 mL of alkaline picrate solution. The optical density was determined after 30 min at 500 nm Shimadzu mini 1240 spectrophotometer (Shimadzu Corp; Kyoto, Japan). Mycotoxin concentrations were then correlated to the Crea content of the corresponding sample and expressed as ng/mg Crea.

2.8. Statistical Analysis

For comparison of categorical data, the Pearson chi-square and Fisher exact test were performed in order to assess whether the occurrence of CIT and

DH-CIT throughout the different subgroups was significantly different, whereas Kruskal-Wallis test was used for detecting quantitative differences. A confidence level of 95% was chosen for examining data and a *p*-value < 0.05 was considered as significant.

3. Results and Discussion

3.1. Evaluation Of UHPLC-Q-Orbitrap HRMS Conditions

The optimization of the compound-dependent parameters was carried out by injecting analytical standards of CIT and DH-CIT at a concentration of 1 µg/mL. The Q-Orbitrap spectrometer was operated in both positive and negative ESI modes, in order to identify the ions with the higher intensity. Table 1 shows the analytical parameters of CIT and DH-CIT referring to elemental composition, retention time, adduct ion, theoretical mass, measured mass and accuracy. Retention times were 4.78 min and 4.97 min for DH-CIT and CIT, respectively. As expected, DH-CIT eluted first, meaning it has a more polar character. Comparing the ionization modes, CIT offered higher base peak intensity when using positive ESI mode whereas DH-CIT showed a better performance in negative ESI mode. The chosen ions displayed high accuracy when compared to the theoretical masses, with mass errors within the acceptable range (< 5 ppm).

Table 1. UHPLC-Q-Orbitrap HRMS parameters corresponding to the analytes.

Analyte	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (Δ ppm)
DH-CIT	4.78	$C_{13}H_{14}O_6$	$[M-H]^-$	265.07243	265.07241	-0.08
CIT	4.97	$C_{13}H_{14}O_5$	$[M+H]^+$	251.09140	251.09129	-0.44

3.2. Method Performance

The proposed method was validated in terms of sensitivity, selectivity, trueness, repeatability (intra-day precision), reproducibility (inter-day precision), linearity, LODs and LOQs as specified in Commission Decision 2002/657/EC [48]. Results are shown in Table 2. Both compounds showed correlation coefficients > 0.990 for both neat solvent and matrix-matched calibration curves. A slight signal suppression was calculated and therefore quantitation based on neat solvent calibration curves was carried out. Recovery results revealed a suitable performance, with values within the acceptable accuracy range of 70-120% at three assayed concentrations, and relative standard deviation <16% for intra-day (RSD_r) and inter-day (RSD_R) precision studies were obtained. LODs were established at 0.003 ng/mL and 0.017 ng/mL for CIT and DH-CIT, respectively, whereas LOQs were set at 0.01 ng/mL and 0.05 ng/mL for CIT and DH-CIT, respectively. Lastly, the absence of coelutants was confirmed since no peaks were observed in the same retention time zones. Hence, the proposed method was selective, sensitive and accurate enough for a reliable quantification of CIT and DH-CIT at low ppt levels in urine samples.

Table 2. Method performance parameters for CIT and DH-CIT

Analyte	Linearity (r^2)	SSE (%)	Recovery (%)			Precision (%) [RSD_r, (RSD_R)]			LOD (ng/mL)	LOQ (ng/mL)
			5 ng/mL	1 ng/mL	0.5 ng/mL	5 ng/mL	1 ng/mL	0.5 ng/mL		
CIT	0.9987	89	82	86	70	6 (16)	16 (16)	10 (12)	0.003	0.01
DH-CIT	0.9947	94	83	72	72	10 (15)	9 (12)	11 (16)	0.017	0.05

In literature, there have recently been published analytical methods for the determination of CIT and DH-CIT in human urine, as reviewed in Table 3. The most common extraction procedure is based on IACs, that offer high selectivity for a specific analyte. Nevertheless, considering their cost and the high amount of samples used for a HBM study, a simpler and more affordable sample preparation that still fits performance parameters is preferred, as the salting-out liquid-liquid extraction proposed in the present study. In addition, considering the low concentrations reported in those previous studies, very sensitive analytical methods are required in order to have an accurate overview of CIT and DH-CIT in urine samples. In this line, LC coupled to QqQ mass spectrometry has been applied elsewhere for CIT and DH-CIT quantification. HRMS methodologies are becoming more usual when analyzing contaminants in complex biological matrices due to its high resolving power and accurate mass measurement [50-51]. The present study based on HRMS methodology was in-house validated for

quantification of CIT and DH-CIT in human urine samples for the first time and applied to determine the occurrence of the studied analytes in 300 human urine samples.

3.3. Urinary levels of CIT and DH-CIT in human urines

The number of biomarker data for CIT is rather limited compared to its structurally related nephrotoxic mycotoxin ochratoxin A, thus the detection of CIT in biological samples is of great interest considering the reported synergistic effects when both toxins co-occur [8, 21-22]. Table 3 reviews the occurrence data and the concentration of CIT biomarkers in human urines published during the last decade. In this study CIT was detected in 142 out of the 300 analyzed samples (47%) at concentrations ranging from >LOD to 4.00 ng/mg Crea and showing a mean value of 0.29 ng/mg Crea; whereas DH-CIT was detected in 64 out of 300 samples (21%) at levels from >LOD up to 2.48 ng/mg Crea, presenting an average value of 0.39 ng/mg Crea. By age, the excretion ratio CIT:DH-CIT varied from 0.3 (below 18 years old) to 0.9 (between 18 and 65 years), whereas the incidence values of CIT and DH-CIT were comparable throughout the studied age groups. Nonetheless, the DH-CIT average excretion levels (1.04 ng/mg Crea for children, 0.37 ng/mg Crea for adults, and 0.26 ng/mg Crea for elderly) were greater than those CIT levels (0.32, 0.35 and 0.19 ng/mg Crea for children, adults and elderly, respectively).

Table 3. Human biomonitoring studies of CIT biomarkers in urine samples during the last decade.

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Belgium	na	40	5.76	na	2.5	na	nd-4.5[a]	na	na	na	LLE with SAX SPE clean-up	UHPLC-MS/MS (QQQ)	2012	Njumbe Ediage, et al. [30]
Turkey	Infants (< 2 years)	6	0.05	0.1	100	100	<LOQ-0.20	<LOQ-1.12	na	na	IAC extraction	HPLC-MS/MS (QQQ)	2013	Blaszkewicz, et al. [36]
Germany	Adults (20-58 years)	4			100	100	<LOQ-0.07	<LOQ-0.34	na	na				

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Belgium	Adults	32	0.003	0.03	59	66	<LOQ-0.12	<LOQ-0.21	0.026	0.035	IAC extraction	UHPLC-MS/MS (QQQ)	2015	Huybrechts, et al. [31]
Belgium	Children (3-12 years)	155	0.003	0.03	72	6	<LOQ-0.42	0.27-2.03	0.04	0.81	Filter and shoot	UHPLC-MS/MS (QQQ)	2015	Heyndrickx, et al. [32]
	Adults (19-65 years)	239			59	12	<LOQ-1.49	0.09-2.12	0.07	0.74				

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Germany	Adults	50	0.05	0.1	82	84	nd-0.19	nd-0.55	0.03	0.1	IAC extraction	HPLC-MS/MS (QQQ)	2015	Ali, et al. [37]
Germany	Adults	50	na	0.02	na	28	na	<LOQ-0.33	na	0.09	Dilute and shoot	HPLC-MS/MS (Q-TRAP)	2015	Gerding, et al. [39]
Haiti	Adults	142			na	14	na	<LOQ-4.34	na	0.28				
Bangladesh	Adults	95			na	75	na	<LOQ-58.82	na	3.12				

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Bangladesh	Adults (rural area)	32	0.05	0.1	97	91	nd-1.22	nd-7.47	0.14	0.97	IAC extraction	HPLC-MS/MS (QQQ)	2015	Ali, et al. [40]
	Adults (urban area)	37			92	54	nd-0.45	nd-0.36	0.06	0.08				
Bangladesh	Pregnant women (rural area)	32	0.05	0.1	84	84	na	na	0.60	0.70	IAC extraction	HPLC-MS/MS (QQQ)	2016	Ali, et al. [42]
	Pregnant women (suburban area)	22			91	86	na	na	0.39	0.57				

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Germany	Controls (males)	13	0.05	0.1	100	100	<LOQ-0.20	<LOQ-0.57	0.06	0.14	IAC extraction	HPLC-MS/MS (QQQ)	2016	Föllmann, et al. [38]
	Workers in grain mills (males)	12			100	100	<LOQ-0.06	<LOQ-0.72	0.03	0.19				
	Workers in grain mills (females)	5			100	100	<LOQ-0.06	<LOQ-0.38	0.03	0.14				

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Bangladesh	Adults (rural area, summer)	30	0.05	0.1	97	93	nd-1.22	nd-5.39	0.53	2.81	IAC extraction	HPLC-MS/MS (QQQ)	2016	Ali, et al. [41]
	Adults (rural area, winter)	30			93	97	nd-3.51	nd-46.44	1.10	7.23				
	Adults (urban area, summer)	32			90	50	nd-0.45	nd-0.31	0.20	0.31				
	Adults (urban area, winter)	32			91	97	nd-5.03	nd-4.64	0.85	2.86				

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Portugal	Controls	19	na	na	12	2	na	na	na	na	Dilute and shoot	HPLC-MS/MS (Q-TRAP)	2018	Viegas, et al. [34]
	Workers of bread dough company	21			6	3	na	na	na	na				
Nigeria	Children (≤ 8 years), teenagers (9-19 years), adults (20 ≤ years)	120	na	na	66	58	0.015-241.46	0.05-16.89	5.96	2.39	Dilute and shoot	UHPLC-MS/MS (Q-TRAP)	2018	Šarkanj, et al. [44]

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Czech Republic	Kidney tumor patients (40-81 years)	50	0.05	0.1	91	100	<LOQ-0.087	<LOQ-0.160	0.02	0.08	IAC extraction	HPLC-MS/MS (QQQ)	2019	Malir, et al. [33]
Portugal	Adults	94	1	na	2	na	nd-1.20	na	na	na	QuEChERS-based extraction	UHPLC-MS/MS (QQQ)	2019	Martins, et al. [35]

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Tunisia	Controls	50	0.2	na	72	na	<LOQ-5.72	na	0.53	na	QuEChERS -based extraction	UHPLC-MS/MS (QQQ)	2020	Ouhibi, et al. [45]
	Colorectal cancer patients	50			76	na	<LOQ-2.94	na	0.95	na				
Bangladesh	Infants (< 1 years)	49	0.05	0.1	33	82	0.03-0.33	0.06-6.78	1.24	4.59	IAC extraction	HPLC-MS/MS (QQQ)	2020	Ali and Degen [43]
	Children (1-6 years)	105			65	98	0.03-3.54	0.06-22.91	1.60	6.15				

Provenance	Cohort	n	LOQ (ng/mL)		Incidence (%)		Range (ng/mg Crea)		Mean (ng/mg Crea)		Sample treatment	Analytical method	Year	Reference
			CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT	CIT	DH-CIT				
Italy	Children, teenagers (< 18)	20	0.01	0.05	50	25	0.02-1.48	0.41-1.94	0.32	1.04	SALLE	UHPLC-HRMS (Q-Orbitrap)	2021	Present study
	Adults (18-65)	170			52	25	0.01-4.00	0.05-2.48	0.35	0.37				
	Elderly (65 <)	110			42	16	0.01-1.39	0.05-1.16	0.19	0.26				
	Total	300			47	21	0.01-4.00	0.05-2.48	0.29	0.39				

[a]Values without creatinine normalization (ng/mL); na = not available; nd = not detected; na = not available; LOQ = limit of quantification; LLE = liquid-liquid extraction; SAX = strong anionic exchange; SPE = solid phase extraction; IAC = immunoaffinity column; SALLE = salting-out assisted liquid-liquid extraction; nd = not detected; HPLC= high performance liquid chromatography; UHPLC = ultrahigh performance liquid chromatography; MS/MS = tandem mass spectrometry; HRMS = high resolution mass spectrometry; Q = quadrupole; QQQ = triple quadrupole

In available literature, the metabolite DH-CIT is often present at higher average levels in urine than the parent compound, although the analyte ratios are quite variable, and this fact justifies the need to measure DH-CIT as an important additional biomarker of CIT exposure [25, 43]. It has to be highlighted that the prevalence of CIT and DH-CIT in here analyzed urine samples was lower than those values reported in literature for which an incidence of CIT biomarkers >80% were obtained, despite the comparable LOQ levels reported in surveys. However, the here reported CIT and DH-CIT incidences were similar than those reported in biomonitoring studies with sampling size over one-hundred [39, 43-44]. Likewise, average values and range of CIT and DH-CIT obtained in these biomonitoring surveys (>100 samples) were comparable with the data obtained in the present work (*n* = 300 samples) (Table 3). Figure 1 shows the chromatograms and MS/MS spectra extracted from a human urine sample containing citrinin (1.24 ng/mg Crea) and dihydrocitrinone (2.48 ng/mg Crea).

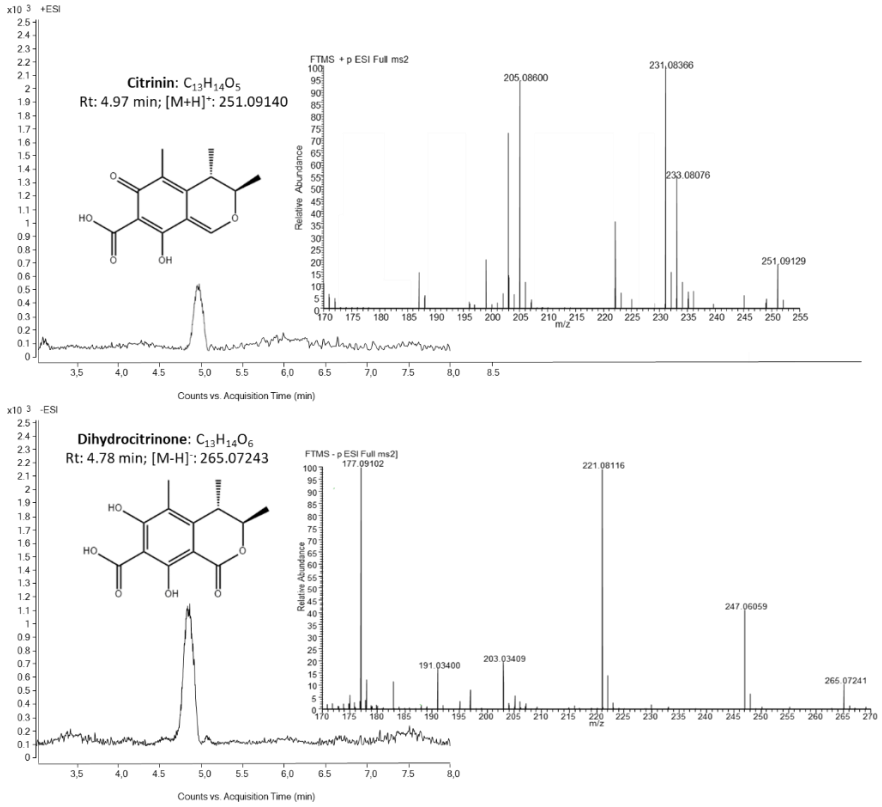

Figure 1. Extracted ion chromatogram and the secondary mass (MS/MS) spectra of a human sample containing citrinin (1.24 ng/mg Crea) and dihydrocitrinone (2.48 ng/mg Crea)

3.4. Estimated Exposure Approach through CIT Biomarkers in Urine

An exposure assessment approach to CIT through urinary data was conducted taken into account the CIT kinetics in humans reported by Degen, et al. [25] who determined that the median value for the excretion of the sum of CIT and DH-CIT was 40.2%. Hence, the following equation was used to calculate the PDI of CIT:

$$PDI\ \left(\frac{\mu g}{kg} bw\right) = \frac{C_T\ x\ V_T}{bw\ x\ EF}\ x\ 100$$

were C_T is the individual urinary total CIT biomarker concentration (ng/mg Crea) obtained in this study; V_T is the average volume of urine excreted in 24h of 1.6 L [26]; bw is the body weight provided in the inform consent from each participant, and EF is the daily urinary CIT excretion rate of 40.2% (the median fraction of an oral CIT dose excreted within 24h) [25].

Based on the above, CIT PDI for each participant was calculated and PDI values were compared with the PTDI of 0.2 µg/kg bw, the level of no concern for nephrotoxicity set by EFSA [8]. Results are shown in Table 4. Matching the calculated data with the CIT PTDI value, it comes out that the resulting Italian average exposures represents a range between 8% and 40%, being children the most exposed population group. However, 6.4% of the positive tested subjects (*n* = 142) had biomarkers levels which were indicative of a CIT exposure comparable to half the value considered as PTDI, and four individuals surpassed the limits suggested by EFSA.

Table 4. Exposure assessment approach to CIT based on CIT urinary biomarkers

Population group	CIT biomarkers (CIT+DH-CIT, ng/mg Crea)			Probable daily intakes (PDI, µg/kg bw)			Percentage of provisional tolerable daily intakes (PTDI)		
	CIT_{min}	[a]CIT_{mean}	CIT_{max}	PDI_{min}	PDI_{mean}	PDI_{max}	$PTDI_{min}$	$PTDI_{mean}$	$PTDI_{max}$
Children (<18 years)	0.003	0.842	3.412	0.069	0.095	0.242	0.2	40	113
Adults (≥18 and ≤65 years)	0.008	0.526	4.723	0.007	0.030	0.268	0.2	15	134
Elderly (> 65 years)	0.003	0.293	1.391	0.011	0.017	0.071	0.1	8	40

[a]Average value based on positive samples only. CITmin and CITmax indicate the lowest and highest concentration of CIT total found in urines according to each population group; CITmean indicates the mean CIT total values for each population group; PDImin and PDImax indicate the range of CIT PDIs; PDImean indicates the CIT PDI based on mean CIT total levels found in urine; PTDImin and PTDImax indicate the range of exposure to CIT for each population group; PTDImean indicate exposure to CIT based on mean CIT total levels found in urine.

As individual data from other surveys were not available to us, it was not possible to calculate their CIT PDIs. Nonetheless, average CIT and DH-CIT urinary levels reported by some European studies were used to estimate exposure to CIT through biomarkers data (Table 3). Heyndrickx, et al. [32] conducted a Belgian biomonitoring study and they reported average levels of CIT (0.040 ng/mg Crea and 0.074 ng/mg Crea) and DH-CIT (0.810 ng/mg Crea and 0.739 ng/mg Crea)

for children (*n* = 155) and adults (*n* = 239), respectively. Those data were used to calculate CIT total as an approach to obtain CIT PDI for both population groups. In this approach, body weight values of 70 kg and 21.7 kg were assumed for adults (> 18 years) and children (3-10 years) as suggested by EFSA [8]. Based on these assumptions, calculated exposures were 23% CIT PTDI for adults and 53% CIT PTDI for children. Similarly, exposure estimates for German adults (*n* = 50) were calculated according to CIT (0.034 ng/mg Crea) and DH-CIT (0.102 ng/mg Crea) urinary data reported by Ali, et al. [37], corresponding to an exposure to CIT equivalent to 23% PTDI. Hence, the obtained results showed a widespread human exposure to CIT. Non-negligible exposure levels to CIT were highlighted in this study and data was comparable to other European biomonitoring studies. In addition, the safe levels (PTDI) surpassed by some individuals could raise concern and should trigger more efforts to analyze and monitor the CIT levels in foodstuffs in future studies. Results here reported are based on a CIT excretion rate assumption and thus, inter-individual variations, derived from different metabolism activities, should be taken into account. Moreover, the excretion rate may also vary in the same subject.

To conclude, a biomonitoring study on CIT and its metabolite DH-CIT in 300 urine samples from Italian population was carried out through an UHPLC coupled to Q-Orbitrap HRMS methodology for the first time. Samples were extracted using a salting-out assisted liquid-liquid extraction alongside a simple clean-up step based on C18. This procedure was validated according to EU Commission Decision 2002/657/EC in terms linearity, selectivity, trueness, repeatability, within-laboratory reproducibility, LOD and LOQ. CIT was detected

in 47% of the samples (*n* = 300) at concentrations ranging from >LOD to 4.0 ng/mg Crea (mean value = 0.29 ng/mg Crea), whereas DH-CIT was detected in 21% of samples *(n* = 300) at levels from >LOD up to 2.5 ng/mg Crea (mean value = 0.39 ng/mg Crea). These results are comparable with previous biomonitoring studies including a large sampling (*n > 100*). The exposure of Italian population to CIT was estimated using the sum of CIT and DH-CIT as a biomarker. Considering the different age groups, CIT average exposure ranged from 8% to 40% of the PTDI, being children the most exposed group, whereas four individuals surpassed the limits suggested by the EFSA. A similar approach was used for estimating the exposure using data from previous European biomonitoring studies, showing similar PTDI values. Hence, these results revealed non-negligible exposure levels to CIT within the Italian population and comparable to previous European studies. The surpassing of the safety levels could raise concern, encouraging further CIT investigation in foodstuffs monitoring studies.

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3.5. *Human biomonitoring of T-2 toxin, T-2 toxin-3-glucoside and their metabolites in urine through high-resolution mass spectrometry*

1. Introduction

Mycotoxins are toxic metabolites resultant from the secondary metabolism of several species belonging to *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps* and *Alternaria* genera. Under certain conditions, these fungi can colonize a broad variety of crops that eventually lead to accumulation of mycotoxins. Among the mentioned fungal genera, *Fusarium* species represents the major mycotoxin-producing pathogens of warm areas from America, Europe and Asia, especially affecting cereal grains and their derived products [1]. Throughout the consumption of contaminated materials, mycotoxins can cause severe adverse health effects in humans such as immunotoxic, neurotoxic or even carcinogenic effects [2]. Therefore, regulatory authorities have established MLs) in susceptible foods and foodstuffs alongside TDI values for certain mycotoxins, setting a maximum level of dietary exposure for avoiding the appearance of toxic effects.

Traditional ways for estimating dietary exposure are mainly based on the combination of consumption surveys and occurrence data throughout total diet studies or meta-analysis approaches [3,4]. Nevertheless, the outcomes can be considerably biased due to inter-individual differences in consumption patterns or assuming an homogeneous contamination within the same food category. In order to obtain a more reliable estimation of the exposure, HBM studies

represents an ideal alternative [5]. These studies consist on the measurement of parent mycotoxins and/or their respective metabolites in biological samples, preferably urine considering its easy and non-invasive collection. In this line, a considerable amount of literature about HBM studies of *Fusarium* toxins and their metabolites occurring in human urine is available: deoxynivalenol (DON), nivalenol (NIV), fumonisins B1 and B2 (FB1 and FB2), zearalenone (ZEN), enniatin B (ENNB) and B1 (ENNB1) [6–10]. Nevertheless, the extensive metabolism of T-2 toxin (T-2), a major *Fusarium* toxin included in group 3 of the International Agency for Cancer Research (IARC), has been scarcely studied in biological samples.

T-2 is a type-A trichothecene containing an epoxy group between C12 and C13, a double bond between C9 and C10 and variable acetoxy groups. This toxin mainly occurs in wheat, maize and oat, although it can be found in other cereal grains and derived commodities [11]. Once ingested, T-2 can represent a health concern for being a major cause of alimentary toxic aleukia, affecting the mucosa and immune system [12]. At cellular level, T-2 binds the 60S ribosomal subunit inhibiting protein synthesis, causes oxidative stress, impairs the mitochondrial function by altering the electron transport chain and stimulates apoptosis after activating several MAPK and caspases [13–15]. Nevertheless, the toxicity of T-2 highly depends on its metabolites, considering the intensive and rapid metabolization reported after *in vitro* assays with human liver microsomes. In this line, numerous metabolic pathways have been described by Yang et al. [16] for the biotransformation of T-2 into its major metabolic products: hydrolysis for the production of HT-2 toxin (HT-2) and neosolaniol (NEO); hydroxylation,

generating 3'-hydroxy-T-2 (3'-OH-T-2) and 3-hydroxy-HT-2 (3'-OH-HT-2, also known as T-2 triol) and glucuronidation for the production of T-2-3-glucuronic acid (T-2-3-GlcA), HT-2-3-glucuronic acid (HT-2-3-GlcA) and HT-2-4-glucuronic acid (HT-2-4-GlcA).

Additionally, T-2 can also be modified while occurring in plants by the addition of polar molecules, such as glucose, producing T-2-3-glucoside (T-2-3-Glc) as a result [17]. This modified form has also been repeatedly reported in cereal grains [18], so, as T-2, humans could also be exposed to this T-2 modified form through dietary intake. Exposure to T-2-3-Glc represents a health concern considering that it can be deconjugated into its free parent toxin within the intestinal tract, but other metabolites have been recently characterized after *in vitro* assays with human liver microsomes conducted by Yang et al. [19]. Among them, major metabolites resulted to be the hydrolyzed forms HT-2-3-glucoside (HT-2-3-Glc) and NEO-3-glucoside (NEO-3-Glc) and the hydroxylated 3'-hydroxy-T-2-3-Glc (3'-OH-T-2-3-Glc) and 4'-hydroxy-T-2-3-Glc (4'-OH-T-2-3-Glc).

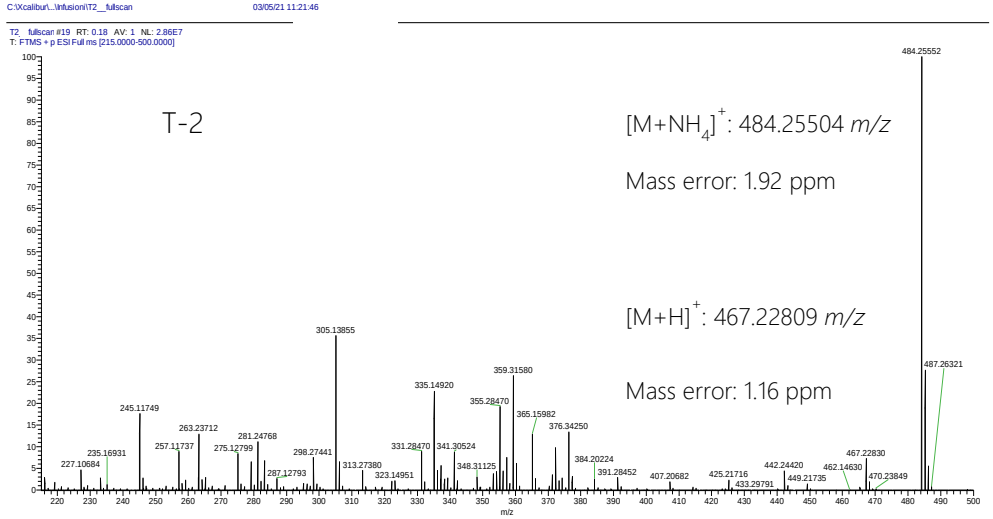
Although Gerding et al. [20] and, recently, De Ruyck et al. [21] have investigated the presence of HT-2-4-GlcA and T-2 triol, respectively, in human urine samples, the complete metabolic profile of major biotransformation products remains unexplored. To overcome the lack of analytical standards, HRMS stands as an optimal tool for characterizing the metabolic profiles of these parent toxins, that become of important consideration when facing exposure assessment studies. Therefore, the present study aimed to investigate the

presence of T-2, T-2-3-Glc and their respective major metabolites in human urine samples (*n* = 300) collected in South Italy through an UHPLC coupled to Q-Orbitrap-HRMS methodology. A combined strategy consisting in targeted quantification of T-2 and HT-2 and suspect screening based on exact mass measurement was applied.

2. Results and discussion

2.1. Optimization of Q-Orbitrap HRMS Parameters

Analytical standards of T-2 and HT-2 diluted at 1 µg/mL in methanol were directly infused into the Q-Orbitrap HRMS system at a constant flow rate of 8 µL/min in order to evaluate the MS parameters of both analytes. The system operated in both positive and negative ESI mode to determine the best ionization pattern and exact mass measurements were compared to theoretical masses for assessing the accuracy, as shown in Figure 1. Both mycotoxins formed stable ammonium adducts in positive ESI mode with high accuracy (mass error < 2 ppm) that were further used for targeted quantification in urine samples. Protonated adducts were also observed for both mycotoxins but in at lower relative intensity, whereas negative ionization showed not only lower relative intensity but also lower exact mass accuracy.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T2 fullscan #19 RT: 0.18 AV: 1 NL: 2.86E7
T: FTMS + p ESI Full ms [215.0000-500.0000]
T-2
[M+NH4]+: 484.25504 m/z
Mass error: 1.92 ppm
[M+H]+: 467.22809 m/z
Mass error: 1.16 ppm
484.25552
487.26321
305.13855
359.31580
335.14920
355.28470
365.15982
376.34250
245.11749
263.23712
281.24768
257.11737
275.12799
235.16931
227.10684
298.27441
331.28470
341.30524
313.27380
348.31125
323.14951
384.20224
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433.29791
462.14630
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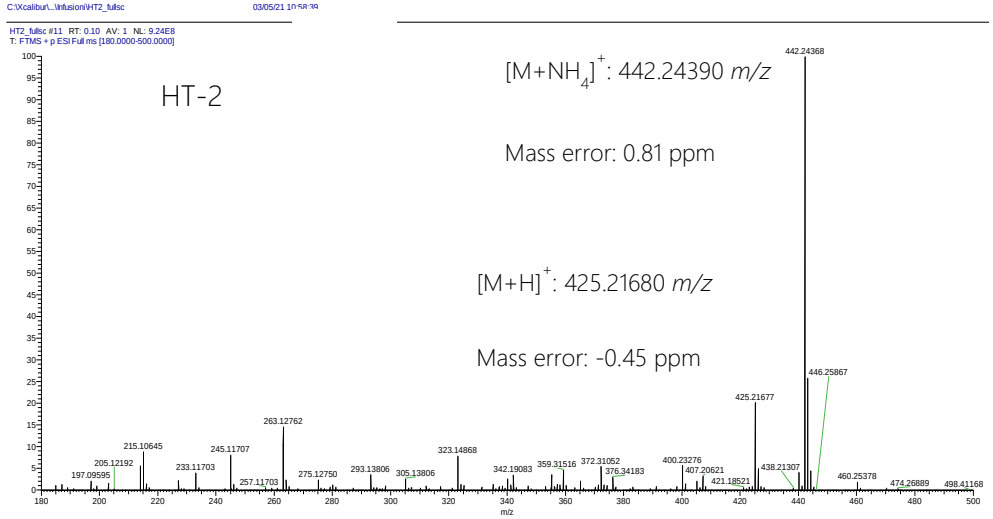
470.23849
m/z


C:\Xcalibur\...\Infusion\HT2_fullsc
HT2_fullsc #11 RT: 0.10 AV: 1 NL: 9.24E8
T: FTMS + p ESI Full ms [180.0000-500.0000]
HT-2
[M+NH4]+: 442.24390 m/z
Mass error: 0.81 ppm
[M+H]+: 425.21680 m/z
Mass error: -0.45 ppm
442.24368
446.25867
425.21677
263.12762
215.10645
245.11707
323.14868
205.12192
233.11703
197.09595
257.11703
275.12750
293.13806
305.13806
342.19083
359.31516
372.31052
376.34183
400.23276
407.20621
421.18521
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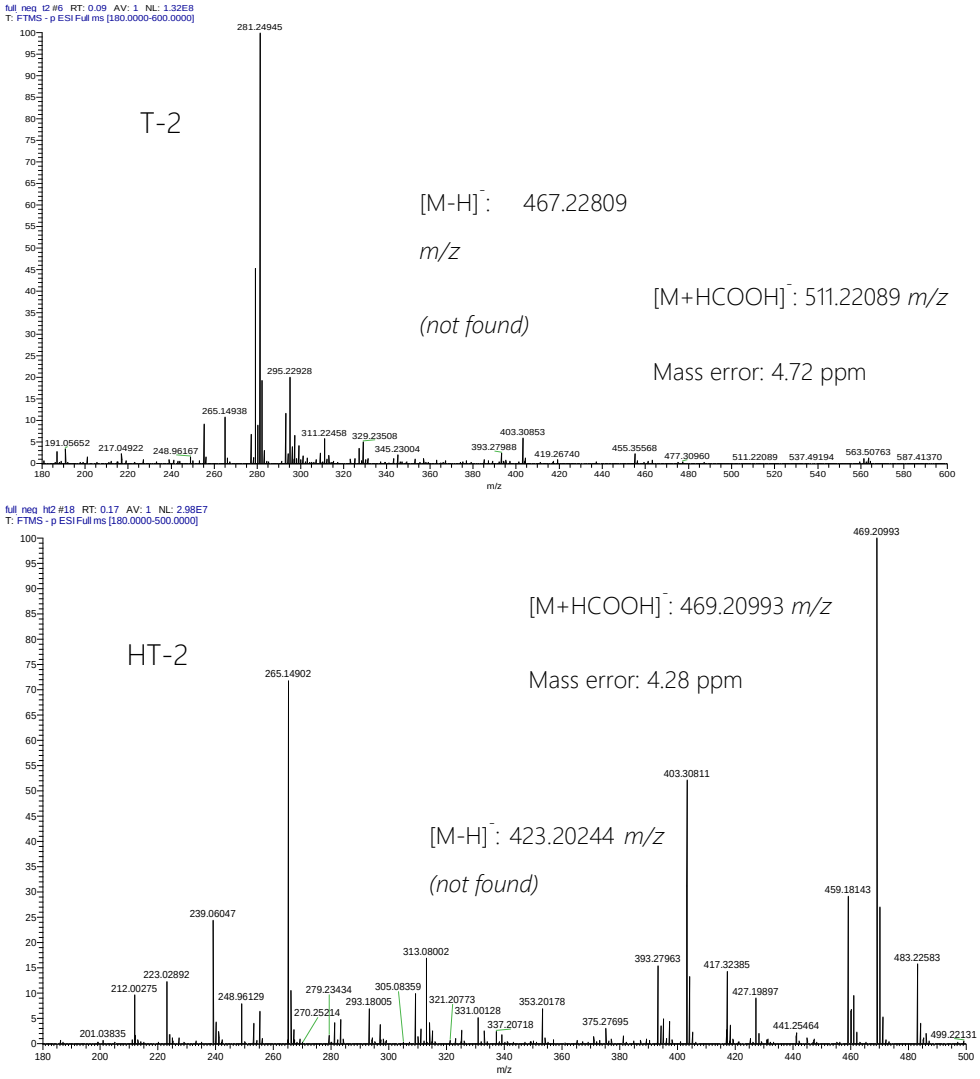
460.25378
474.26889
498.41168
m/z

(a)



(b)

Figure 1. Mass spectra of T-2 and HT-2 in (a) positive ionization mode and (b) negative ionization mode after full scan.

Previous *in vitro* studies with human liver microsomes have tentatively identified the major biotransformation products of T-2 and T-2-3-Glc and elucidated their structure through mass spectrometry analysis [16,19]. Therefore, the following T-2 metabolites were targeted for retrospective analysis: hydrolysis products (NEO and 4-deAc-NEO); hydroxylation products (3′-OH-T-2 and T-2 triol) and phase II metabolites (T-2-3-GlcA, HT-2-3-GlcA and HT-2-4-GlcA). In addition, the modified form T-2-3-Glc was also included alongside its hydrolyzed (NEO-3-Glc and HT-2-3-Glc) and hydroxylated (3′-OH-T-2-3-Glc and 4′-OH-T-2-3-Glc) metabolic products. Despite the biotransformation of T-2-3-Glc could also originate T-2 and its corresponding products, only glucoside forms have been included as T-2-3-Glc metabolites for classification purposes. Tentative identification was carried out following a suspect screening approach based on exact mass measurements. Both protonated and ammonium adducts of each analyte were initially targeted in order to select the fragment with the higher intensity for further analysis. In addition, the order of elution was compared to previous methodologies that used reverse-phase chromatography, considering that a more polar character implies a sooner elution, also allowing a proper differentiation of isomeric forms. A stringent mass error of < 2 ppm was also set aiming for a more accurate identification in urine samples. Data for retention times, theoretical mass, observed mass and mass error for all the assayed compounds is shown in Table 1.

Table 1. UHPLC-Q-Orbitrap-HRMS parameters for targeted and non-targeted analytes.

Analyte	Retention Time (min)	Molecular Formula	Adduct Ion	Exact Mass (*m/z*)	Observed Mass (*m/z*)	Mass Error (ppm)
Parent mycotoxin						
T-2	4.82	C24H34O9	$[M + NH4]^+$	484.25411	484.25504	1.92
Phase I metabolites—Hydrolyzed group						
4-deAc-NEO[a]	-	C17H24O7	$[M + NH4]^+$	358.18602	-	-
			$[M + H]^+$	341.15950	-	-
HT-2	4.79	C22H32O8	$[M + NH4]^+$	442.24354	442.24390	0.81
NEO	4.25	C19H26O8	$[M + NH4]^+$	400.19669	400.19659	−0.25
Phase I metabolites—Hydroxylated group						
T-2 triol	4.7	C20H30O7	$[M + H]^+$	383.20642	383.20662	0.52
3′-OH-T-2	4.78	C24H34O10	$[M + NH4]^+$	500.24902	500.24926	0.48
Phase II metabolites—Conjugated group						
T-2-3-GlcA	4.69	C30H42O15	$[M + H]^+$	643.25964	643.25974	0.16
HT-2-3-GlcA	4.67	C28H40O14	$[M + H]^+$	601.24908	601.24998	1.5
HT-2-4-GlcA	4.39	C28H40O14	$[M + H]^+$	601.24908	601.24998	1.5
Parent mycotoxin						
T-2-3-Glc	4.38	C30H44O14	$[M + NH4]^+$	646.30693	646.30793	1.55
Phase I metabolites—Hydrolyzed group						
HT-2-3-Glc	4.59	C28H42O13	$[M + NH4]^+$	604.29636	604.29616	−0.33
NEO-3-Glc	3.85	C25H36O13	$[M + NH4]^+$	562.24941	562.24897	−0.78
Phase I metabolites—Hydroxylated group						
3′-OH-T-2-3-Glc	3.91	C30H44O15	$[M + NH4]^+$	662.30184	662.30121	−0.95
4′-OH-T-2-3-Glc	4.04	C30H44O15	$[M + NH4]^+$	662.30184	662.30121	−0.95

[a]Neither the protonated nor the ammonium adducts were observed.

2.2. Method Validation

The proposed methodology for analysis of urine samples was in-house validated according to the in-force legislation [22]. Results from validation experiments are shown in Table 2. Calibration curves built in neat solvent and blank matrix showed good linearity (r2 > 0.990) throughout the assayed range of concentrations (20–0.1 ng/mL). After comparing both slopes of the calibration curves, a negligible matrix interference was detected (98–102%), so external calibration based on neat solvent curve was used for quantification purposes. Spiking experiments determined a proper recovery of analytes that ranged from 79% to 116% for the selected fortification levels (5, 1 and 0.5 ng/mL). Maximum relative standard deviations of 19% and 17% after intra-day (RSD_r) and inter-day (RSD_R) precision studies were respectively obtained. The absence of coelutant peaks in the matrix after analysis of blank samples (n = 10) confirmed the selectivity of the proposed methodology. LOQs corresponding to T-2 and HT-2 were set at 0.2 ng/mL and 0.4 ng/mL, respectively. Therefore, the proposed methodology fulfilled validation criteria and was further applied for quantification of T-2 and HT-2 in human urine samples.

Table 2. Method performance for T-2 and HT-2.

			Recovery (%)			Precision (%) [RSD_r, (RSD_R)]			
Analyte	Linearity (r^2)	SSE (%)	5 ng/mL	1 ng/mL	0.5 ng/mL	5 ng/mL	1 ng/mL	0.5 ng/mL	LOQ (ng/mL)
HT-2	0.9901	102	116	86	85	11 (9)	5 (6)	12 (17)	0.4
T-2	0.9944	98	116	87	79	8 (7)	11 (17)	19 (14)	0.2

Several methodologies have been validated for the quantification of T-2 and HT-2 in urine samples, as shown in Table 3. LC coupled to tandem LRMS appears to be the gold standard, although GC has also been used. Similarly, to the present study, it has to be highlighted the use a Q-Orbitrap mass spectrometer in the recent study conducted by Ndaw et al. [23], which remarks the rising use of HRMS techniques in HBM studies. Apart from quantification, HRMS allows retrospective data analysis for untargeted compounds and suspect screenings. Therefore, HRMS could stand as the main tool for future HBM studies considering the complex metabolome occurring in biological samples [24]. In terms of sensitivity, most of the available analytical techniques have reported LOQs ≤ 1 ng/mL for T-2, whereas sensitivity to HT-2 seems to be critically impacted not by the analytical technique but the sample treatment. In this line, direct approaches such as dilute and shoot or filter and shoot have shown a strong variability in sensitivity between both analytes.

Although these procedures represent a good alternative in HBM studies, based on an easy and quick workflow, these are unable to selectively remove any interference present in the matrix, thus reducing the performance of the methodology. It can be observed how other sample treatments including a clean-up step, such as QuEChERS or SALLE procedures, obtained better sensitivities for HT-2 when compared to direct approaches. The here-presented methodology, based on a simple SALLE + clean up procedure and later UHPLC-HR-Q-Orbitrap-MS/MS showed suitable sensitivity for detecting both analytes at low ng/mL levels.

Table 3. Previous methodologies for the quantification of T-2 and HT-2 in human urine samples.

		LOQ a (ng/mL)		
Sample Treatment	Analytical Method	T-2	HT-2	Reference
Dilute and shoot	UHPLC-Q-TRAP-MS/MS	0.5	4	Gerding et al. [20]
SALLE	UPLC-QqQ-MS/MS	0.013	0.036	De Ruyck et al. [21]
Clean-up	UHPLC-HR-Q-Orbitrap-MS/MS	1	0.5	Ndaw et al. [23]
LLE	HPLC-Q-TRAP-MS/MS	6.7	67	Abia et al. [25]
QuEChERS	GC-QqQ-MS/MS	1	2	Rodríguez-Carrasco et al. [26]
Dilute and shoot	UHPLC-Q-TRAP-MS/MS	1	40	Ezekiel et al. [27]
LLE	HPLC-Q-TRAP-MS/MS	1	40	Warth et al. [28]
Filter and shoot	UHPLC-QqQ-MS/MS	0.03	0.5	Heyndrickx et al. [29]
LLE	UHPLC-QqQ-MS/MS	0.1	0.84	
LLE	HPLC-Q-TRAP-MS/MS	0.2	9	Gerding et al. [30]
LLE	UHPLC-Q-TRAP-MS/MS	0.1	0.5	Fan et al. [31]
IA-SPE	UHPLC-QqQ-MS/MS	0.013	0.031	Gratz et al. [32]
DLLME	GC-QqQ-MS/MS	1	2	Niknejad et al. [33]
Dilute and shoot	HPLC-Q-TRAP-MS/MS	10	5	Duringer et al. [34]
SALLE + clean-up	UHPLC-HR-Q-Orbitrap-MS/MS	0.2	0.4	Present study

Values considering dilution/concentration factor of each methodology; LLE = liquid-liquid extraction; QuEChERS = quick, easy, cheap, effective, rugged and safe; IA = immunoaffinity; SPE = solid-phase extraction; DLLME = dispersive liquid-liquid microextraction; SALLE = salt-assisted liquid-liquid extraction; HPLC = high performance liquid chromatography; Q = quadrupole; MS/MS = tandem mass spectrometry; GC = gas chromatography; QqQ = triple quadrupole; UHPLC = ultra-high performance liquid chromatography; HR = high resolution.

2.3. Urinary Levels of T-2 and HT-2

The validated methodology was applied to 300 human urine samples for assessing urinary levels of T-2 and HT-2. In the present study, T-2 was quantified in 21% of samples at a mean concentration of 1.34 ng/mg Crea (range: 0.22–6.54 ng/mg Crea), whereas HT-2 showed a slightly higher prevalence, occurring in 30% of samples at a similar mean concentration of 1.23 ng/mg Crea. No significant differences were observed when comparing concentration levels of both analytes throughout the whole dataset. Nevertheless, a statistically significant positive association between the occurrence of T-2 and HT-2 within the same sample was revealed (p-value < 0.01, phi coefficient = 0.479). This co-exposure might be explained by the also correlated occurrence of both toxins in foodstuffs, which has been extensively reported in literature [11,35,36]. Across age and gender groups, both mycotoxins reflected alike distribution in terms of prevalence and concentration, supported by statistical analysis which determined non-significant differences.

Table 4. Previous human biomonitoring studies with positive urine samples for T-2 and/or HT-2.

Provenance	Cohort (age)	Samples (n)	LOQ (ng/mL)[a]		Prevalence (%)		Range of Concentration (ng/mg Crea)		Mean (ng/mg Crea)		Reference
			T-2	HT-2	T-2	HT-2	T-2	HT-2	T-2	HT-2	
Germany	Adults (20–30)	101	0.5	4	1	nd	<LOQ	nd	na	nd	Gerding et al. [20]
Belgium, Czech Republic, Netherlands, and Norway	Adults (45–65)	188	0.013	0.036	21.8	6.4	<LOQ—0.77	<LOQ—4.60	0.05[c,d]	0.48[c,d]	De Ruyck et al. [21]
France	Adults, grain elevator workers (19–56)	18	1	0.5	4	4	<LOQ—2.73	<LOQ—3.29	na	na	Ndaw et al. [23]
Spain	Children (8–14)	16			nd	6.2	nd	12.6 [b]	nd	12.6	Rodríguez-Carrasco et al. [26]
	Young adults (18–28)	16			nd	nd	nd	nd	nd	nd	
	Adults (>28)	22			nd	13.6	nd	15.8 [b]	nd	14.3	
	Total	54	1	2	nd	7.4	nd	15.8 [b]	nd	na	
China	Adults (18–66)	260	0.1	0.5	2.3	nd	0.392–4.23	nd	1.75	nd	Fan et al. [31]
United Kingdom	Children (2–6)	21	0.013	0.031	5	5	0.03	6.13	0.03	6.13	Gratz et al. [32]

Provenance	Cohort (age)	Samples (n)	LOQ (ng/mL)[a]		Prevalence (%)		Range of Concentration (ng/mg Crea)		Mean (ng/mg Crea)		Reference
			T-2	HT-2	T-2	HT-2	T-2	HT-2	T-2	HT-2	
Iran	Adults, esophageal cancer patients (50–92)	17			6	18	na	na	44.7	29.09	Niknejad et al. [33]
	Adults, control group (20–46)	10	1	2	nd	10	nd	na	nd	16.81	
Uganda	Children, nodding syndrome patients (5–18)	50			74	nd	0–288 [c]	nd	29 [c]	nd	Duringer et al. [34]
	Children, control group (5–18)	50			70	nd	0–425 [c]	nd	49 [c]	nd	
	Total	100	10	5	72	nd	0–425 [c]	nd	39 [c]	nd	
Italy	Children, teenagers and adults (≤30)	94			20	32	0.42–2.37	0.44–2.32	1.26	1.19	Present study
	Adults (31–59)	72			19	28	0.33–6.54	0.46–2.75	1.48	1.48	
	Elderly (≥60)	134			22	30	0.22–2.51	0.44–2.39	1.4	1.13	
	Total	300	0.2	0.4	21	30	0.22–6.54	0.44–2.75	1.34	1.23	

[a] Values considering dilution/concentration factor of each methodology; [b] Only maximum value available; [c] Values expressed as ng/mL without applying; creatinine correction; [d] Referring to median values instead of mean; nd = not detected; na = not available

Table 4 reviews previous studies with positive urine samples for any of both mycotoxins alongside the here obtained results. Scarce literature about T-2 or HT-2 occurring in urine samples is available. Additionally, most of the published studies focused on specific cohorts compiling several criteria in terms of age range, occupational exposure or diseases potentially related to T-2 exposure, as shown in Table 4. In general, a low rate of positive samples has been reported when considering healthy cohorts, with T-2 occurring in a higher number of samples than HT-2 (T-2 < 21.8%; HT-2 < 13.6%). Quantitatively, HT-2 seemed to occur at higher concentrations when compared to T-2 (T-2 < 1.75 ng/mg Crea; HT-2 < 16.81 ng/mg Crea). In contrast with these previous studies, the here presented results remarked a higher prevalence rate for both mycotoxins but at lower concentration values. These inconsistencies in urinary levels could be due to several factors. Firstly, the analytical performance of the above-mentioned studies displayed differences in terms of sensitivity for each mycotoxin, with LOQs being between 3–8 times higher for HT-2. This could directly translate into lower rates of positive samples and higher mean concentration values. Secondly, both mycotoxins can be found in foods and foodstuffs, so variable levels could be expected according to dietary habits. In this line, the latest data on food supply quantity by the FAO remarked a higher consumption of cereals and cereal-based products in Italy (160.97 kg/capita/year) when compared to Germany (115.03 kg/capita/year), United Kingdom (126.75 kg/capita/year) Belgium, Czech Republic, Netherlands and Norway (<102.12 kg/capita/year) [37], so higher prevalence could also be expected in the present study. Additionally, results obtained in HBM studies that focused on a specific age segment (see Table 4,

Gratz et al. [32], 2–6 years old; Gerding et al. [20], 20–30 years old) may not be comparable to studies with larger sample sizes due to the potential bias generated by age-related consumption patterns. Lastly, although little is known about toxicokinetics of T-2 and HT-2 in human, *in vivo* studies with rats and pigs have reported low half-life values for both toxins in urine, as reviewed by Schelstraete et al. [38], so this might hamper the understanding of urinary levels in human samples too. Therefore, these results provide even more evidence about the occurrence of T-2 in human urine and support the development of more sensitive analytical techniques for its application in HBM studies in order to clarify the impact of T-2 in humans.

2.4. Retrospective Analysis of Urine Samples

Data collected after UHPLC-Q-Orbitrap-HRMS analysis were manually examined in Xcalibur Qual Browser v.3.1.66 to evaluate the tentative presence of NEO, 4-deAc-NEO, 3′-OH-T-2, T-2, T-2-3-GlcA, HT-2-3-GlcA, HT-2-4-GlcA, T-2-3-Glc, NEO-3-Glc, HT-2-3-Glc, 3′-OH-T-2-3-Glc and 4′-OH-T-2-3-Glc in 300 human urine sample. Identification was based on exact mass identification in, at least, two of the three replicates with a stringent mass error of 2 ppm using the molecular formulas previously reported in literature [16,19], thus corresponding to a level 5 of certainty as established by Schymanski et al. [39]. Results are shown in Figure 2.

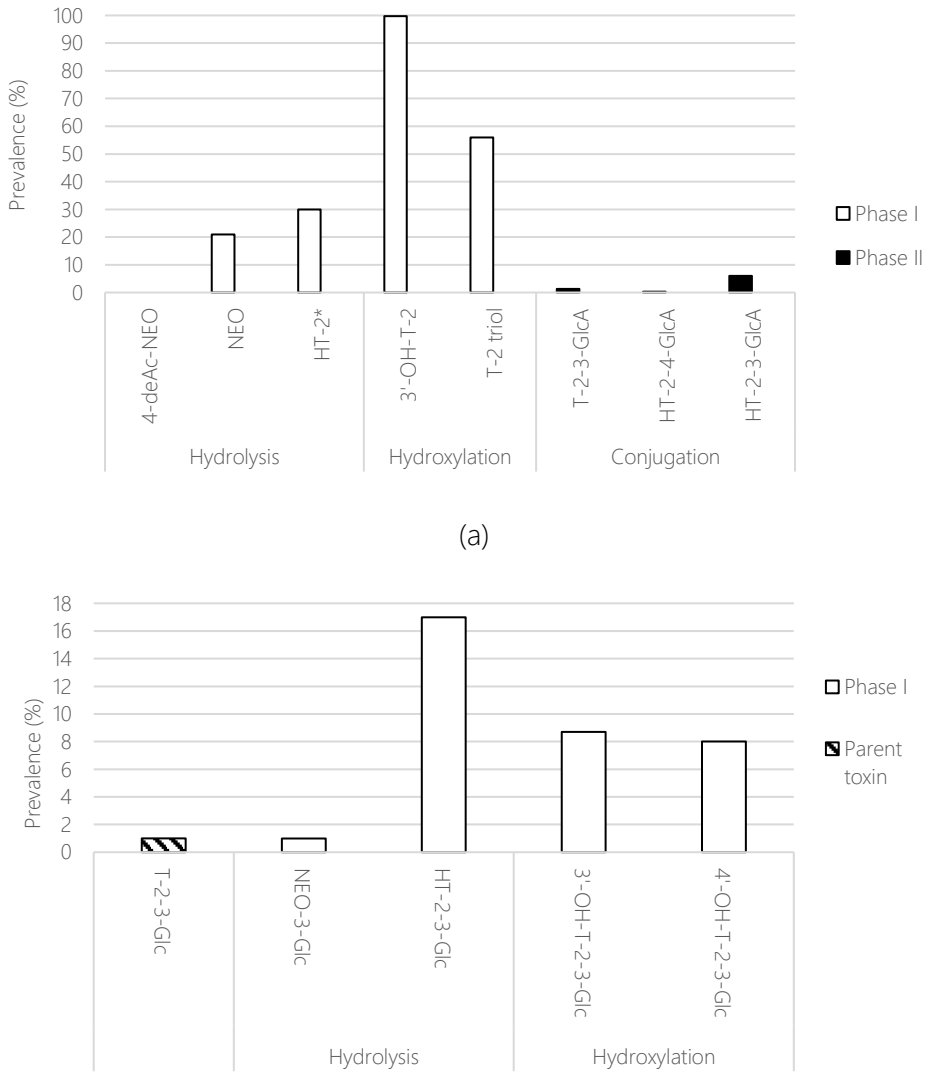


Figure 2. *Prevalence of (a) T-2 metabolites and (b) T-2-3-Glc and its phase I metabolites in 300 human urine samples. Although HT-2 was assessed through a targeted methodology, it was introduced in this section for being a major T-2 metabolite.*

Almost all the major T-2 metabolites previously characterized *in vitro* were tentatively found in human urine within the following order of prevalence: 3′-OH-T-2 (99.7%) > T-2 triol (56%) > HT-2 (30%) > NEO (21%) > HT-2-3-GlcA (6%) > T-2-3-GlcA (1.3%) > HT-2-4-GlcA (0.3%) > 4-deAc-NEO (0%). These results could point to phase I metabolism as the preferential biotransformation pathway, as evidenced by the high proportion of 3′-OH-T-2 and T-2 triol and the low relevance of conjugated metabolites, although dietary exposure could also take part on these outcomes. Furthermore, among phase I metabolites, statistical analysis revealed a significantly higher frequency of hydroxylated metabolites when compared to the hydrolyzed products (p-value < 0.01). Therefore, hydroxylation seemed to be the main biotransformation pathway of T-2. Although the presence of HT-2 and NEO could point to hydrolysis as an alternative reaction, their prevalence could be partly due to dietary exposure considering that they extensively occur in foodstuffs too [40]. The low relevance of conjugated metabolites in human urine has been previously reported by Gerding et al. [20], that did not find any positive sample for HT-2-4-GlcA. These results are in contrast with the human *in vitro* assays conducted by Yang et al. [16], which remarked HT-2 as the most predominant metabolites whereas T-2 triol was the preferred hydroxylation product. Nevertheless, different metabolic profiles have been observed after comparing *in vivo* and *in vitro* data from other species. Similarly, to human *in vitro* data, other previous assays with liver microsomes of chickens and rats revealed a predominance of hydrolyzed metabolites, whereas *in vivo* experiments in both species remarked hydroxylation as the main biotransformation pathway and conjugation was not observed [16,41],

in line with the here observed findings. The high relevance of hydroxylated compounds, especially 3'-OH-T-2, could represent a concern considering that it does not exert a significantly lower toxicity, or might even display faintly higher toxicity when compared to its parent mycotoxin [42,43].

Regarding the T-2-3-Glc metabolic profile, low prevalence of the parent mycotoxin (1%) and its metabolites were observed: HT-2-3-Glc (17%) > 3'-OH-T-2-3-Glc (8.7%) > 4'-OH-T-2-3-Glc (8%) > NEO-3-Glc (1%). The observed low urinary prevalence is in agreement with findings reported by Yang et al. [19], who observed that T-2-3-Glc and its metabolites were mainly excreted in faeces whereas only traces were observed in urine after oral administration of T-2-3-Glc to rats. Nevertheless, only T-2, HT-2 and 3'-OH-T-2-3-Glc were reported in urine, whereas the here obtained results highlighted HT-2-3-Glc as the major T-2-3-Glc metabolite, being tentatively detected at a significantly higher frequency when compared to the rest of products (p-value < 0.01) This discrepancy could be addressed by considering not only *in vitro/in vivo* and inter-species differences but also a potential dietary exposure. Although the presence of HT-2-3-Glc has been scarcely studied in foodstuffs, the available studies reported a high prevalence in oats, wheat and barley samples [44].

The vast occurrence of metabolites in human urine samples indicated an extensive biotransformation of T-2 and T-2-3-Glc. Although scarce information is available about *in vivo* metabolism, a similar pattern across species has been observed with a preferential metabolism through phase I reactions for T-2 and low-to-no relevance of conjugation reactions.

Attending to the large number of samples tested positive for T-2 or its metabolites, this study also remarked a frequent exposure to T-2 although there is a considerable variability in available HMB studies using urine samples. Thus, more sensitive analytical techniques should be validated for their application in biological matrices in order to clarify the impact of T-2 in humans. Additionally, considering the frequent exposure to several metabolites that can also occur in foodstuffs, such as T-2 triol or HT-2-3-Glc, analytical methodologies in food analysis should incorporate them. This could help to elucidate whether the presence of those metabolites is due to dietary exposure or to T-2/T-2-3-Glc metabolism.

3. Material and Methods

3.1. Chemicals, Reagents and Materials

Water for LC mobile phase (LC-MS grade), ACN and MeOH were acquired from Merck (Darmstadt, Germany). Formic acid (MS grade) was supplied by Carlo Erba reagents (Cornaredo, Italy). Ammonium formate (analytical grade) was purchased from Fluka (Milan, Italy). C18 (analytical grade) and sodium chloride (NaCl) were obtained from Sigma Aldrich (Milan, Italy). Conical centrifuge polypropylene tubes of 15 mL were provided by BD Falcon (Milan, Italy). Syringe filters with polytetrafluoroethylene membrane (PTFE, 15 mm, diameter 0.2 µm) were acquired from Phenomenex (Castel Maggiore, Italy).

Analytical standard of T-2 and HT-2 (HPLC purity > 98%) were supplied by from Sigma-Aldrich (Milan, Italy). Stock solutions were built by diluting 1 mg of

each standard mycotoxin in 1 mL of MeOH. Then, working solutions were prepared by properly diluting with MeOH/H2O (70:30 v/v) 0.1% formic acid for reaching the concentrations needed for spiking experiments (5, 1 and 0.5 ng/g). The solutions were kept in securely closed vials at −20 °C.

3.2. Sampling

First-spot morning urine (50 mL) samples from 300 volunteers were collected during January and February 2018 in Campania region (Southern Italy) and stored in sterile plastic vessels. Samples were aliquoted and storage at −20 °C until further analysis to avoid stability issues. Volunteers were selected among students, academic and non-academic staff of the Faculty of Pharmacy of University of Naples "Federico II" considering the following exclusion criteria: (i) susceptible people to occupational exposure, such as veterinarians and farmers, were excluded; (ii) only one member per family was allowed; (iii) people with serious problems in bile, kidney or liver were not eligible due to potential interferences with the metabolism of mycotoxins. No diet limitations were established during the sampling. All participants provided a written consent in accordance with the Helsinki Declaration on ethical principles for medical research involving human subjects. This project was approved by the University of Valencia Ethics Committee. The sample size (n = 300) selected is consistent with previous HBM studies of food contaminants [5].

Participants were asked to specify their age and gender in the vessel for further data treatment. The sampling tried to maintain the gender parity (male: 45.7%, female: 54.3%). Three age groups were considered: ≥60 years old (n =

134), from 31 to 59 years old (*n* = 72) and ≤31 years old (*n* = 94). Samples with undetectable levels of mycotoxins were used for recovery studies.

3.3. Extraction Procedure

Sample preparation procedure was conducted following a methodology previously developed by Rodríguez-Carrasco et al. [8]. Briefly, 1.5 mL of urine sample was transferred into a 2 mL Eppendorf Safe-Lock Microcentrifuge tube and centrifuged at 3926× g for 3 min. Afterwards, 1 mL of the supernatant was collected and placed into a 15 mL screw cap test tube with conical bottom alongside 1 mL of ACN. The mixture was vortexed for 30 s. Then, a blend of 30 mg of C18 sorbent and 0.3 g of NaCl were added and vortexed for 30 s and centrifuged at 3926× g at 4 °C for 3 min. Finally, the upper layer was transferred into another 15 mL screw cap test tube with conical bottom and evaporated to dryness under nitrogen flow at 45°C, reconstituted with 0.5 mL of MeOH/H_2O (70:30 *v/v*) 0.1% formic acid and filtered through a 0.2 µm filter prior to UHPLC-Q-Orbitrap HRMS analysis.

3.4. UHPLC-Q-Orbitrap HRMS Analysis

Chromatographic and HRMS conditions were the same as described in pages 102-103. Data analysis was carried out using Quan/Qual Browser Xcalibur v.3.1.66 (Thermo Fisher Scientific, Waltham, MA, USA).

3.5. Method Validation

In-house validation was conducted following the in-force legislation [22] in terms of linearity, matrix-induced deviations, selectivity, trueness, within-

laboratory reproducibility, repeatability and limits of quantification (LOQs). Linearity (r^2) was obtained after building neat solvent and matrix-matched calibration curves using T-2 and HT-2 analytical standards. Concentrations ranged from 20 to 0.1 ng/mL, with each level of the calibration curves showing a relative standard deviation (RSD) < 20% compared to the theoretical concentration. The comparison of both calibration curves throughout their corresponding slopes allowed to assess the SSE, following the next equation:

$$\%SSE = Sm/Ss \times 100$$

being Sm the matrix-matched calibration slope and Ss the solvent calibration slope. An %SSE below 100% was translated into signal suppression whereas values above 100% meant signal enhancement in the range of concentrations assayed. Trueness was evaluated through recovery experiments, spiking known blank samples at three different levels (5, 1 and 0.5 ng/mL). Experiments were carried out in triplicate on three non-consecutive days and reflected as inter-day (within-laboratory reproducibility, RSD_R) or intra-day (repeatability, RSD_r) relative standard deviation. Selectivity was assessed for determining the potential presence of coelutants in the matrix, so blank samples (n = 10) were injected immediately after the highest calibration sample. For confirmation criteria, the retention times of the analytes in standards and samples were compared. LOQs were considered as the lowest concentration where the molecular ion could be identified inside the linear range, considering a mass error below 5 ppm.

3.6. Quality Control/Quality Assurance

Spectral and chromatographic data were combined for correct identification of the analytes. Retention times attached to the assayed analytes were compared in both positive samples and standards in neat solvent at a tolerance of ±2.5% of the total run time (8 min). Data quality was verified through the inclusion of a comprehensive range of quality assurance and quality control procedures. Each batch of samples contained a reagent blank, a procedural blank and a matrix-matched calibration in order to evaluate the robustness and stability of the system throughout the whole analysis.

3.7. Creatinine Analysis

Urinary levels of Crea were calculated throughout a spectrophotometric assay previously performed by Rodríguez-Carrasco et al. [23]. In brief, 1000 mM NaOH was mixed with 3.5 mM picric acid to obtain alkaline picrate. The solution was stored in dark conditions in an amber glass container. Urine samples were then diluted using ultrapure water (1:10 v/v) and 1 mL was reacted with 1 mL of alkaline picrate solution. The optical density was measured after 30 min at 500 nm using a Shimadzu mini 1240 spectrophotometer. Finally, concentrations of mycotoxins were then related to the Crea content of the corresponding sample and expressed as ng/mg Crea.

3.8. Statistical Analysis

Statistical data treatment was carried out in software package IBM SPSS v.25. Mann–Whitney U test was used for detecting quantitative differences

between T-2 and HT-2 in the assayed samples according to gender and age groups. Categorical data, as the prevalence of T-2, T-2-3-Glc and its corresponding metabolites across gender and age groups, were compared throughout the Pearson chi-square tests. A confidence level of 95% was settled for data treatment and a p-value < 0.01 was considered as significant.

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3.6. *Novel quadrupole-time of flight-based methodology for determination of multiple mycotoxins in human hair*

1. Introduction

Mycotoxins are toxic metabolites resulting from secondary metabolism of several fungi genera, mainly *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps*, that are estimated to occur in 50-90% of foods worldwide, depending on the food group, although at very low levels. Ingestion of contaminated foodstuffs represents a health concern due to the reported toxic effects derived from chronic exposure to mycotoxins, including immunosuppression, carcinogenicity, nephro- or hepatotoxicity [1]. Therefore, regulatory authorities have set TDI values for some mycotoxins in order to prevent from those adverse effects, so there is a crucial necessity for controlling exposure to mycotoxins within the provided framework. Currently, the most accurate approach for assessing dietary exposure to mycotoxins is HBM through the direct measurement of either parent toxins or their metabolites in biological matrices such as urine, blood, plasma or serum [2]. However, these traditional matrices fail to provide information about exposure over long periods of time due to their rapid excretion rates and short half-lives [3].

In this context, human hair emerges as a novel matrix able to provide long-term information about exposure to mycotoxins and whose use is widely extended in exposure assessments of other dietary contaminants and drugs.

Although the mechanisms remain unclear, these molecules seem to incorporate into the hair matrix throughout diffusion from either bloodstream to hair follicle or biological secretions, such as sweat, to developing or developed hair [4]. Hair provides a highly stable chemical environment based on the interactions that melanin and the keratin matrix establish with the incorporated contaminants, that could presumably include covalent bonds, among other [5]. Therefore, a wider surveillance window can be provided since contaminants remain stable when bounded to the hair matrix. In fact, considering a standard growth ratio of 1 cm/month, exposure can be estimated within a desired time frame depending on the length of the sample [6].

Accumulation of mycotoxins in human hair has been previously reported [7-9], but only Bordin et al. [8] provided a validated methodology following Commission Decision 2002/657/E [10] for the quantification of fumonisin B1 (FB1) in human hair samples. Although these previous methodologies have proven the feasibility of hair analysis in the mycotoxins field, the current HBM context is evolving towards more flexible methodologies able to extract and quantify multiple mycotoxins occurring in biological samples.

Extraction of multiple mycotoxins from biological samples requires from versatile procedures mainly based on LLE or SPE that usually incorporate a clean-up step [11, 12]. Miniaturization is another important feature, considering the large number of samples required for HBM, implying little expense of reagents and time. Regarding hair analysis, a pretreatment stage based on washing and later digestion (acidic, basic or enzymatic) for the release of analytes from the hair

matrix is also implemented [4]. In the mycotoxin context, an enzymatic digestion is commonly applied to plasma or serum samples utilizing Pronase E, a proteolytic enzyme that operates in aqueous solutions at pH 7 [13]. Later extractive procedures must be compatible with enzymatic solutions considering that most solvents for extraction of mycotoxins, such as ACN or ethyl acetate, are miscible with water. Therefore, phase separation has to be induced through the addition of a salt.

Referring to the analytical methodologies for the quantification of mycotoxins in biological samples, LC-MS/MS represents the gold standard for multi-mycotoxin studies [11, 12], enabling a sensitive and accurate quantification of mycotoxins occurring at ultra-trace levels. Nevertheless, MS methodologies can be strongly affected by matrix interferences that can alter the ionization of analytes providing misleading measurements, so the effectiveness of methodologies also lies on the ability to remove matrix co-elutants prior to MS analysis. A recent trend in HBM is the use of HRMS, that not only provides quantitative measurements of known compounds but also can identify untargeted compounds through the acquisition of full scan spectra and exact mass measurements, also allowing retrospective data analysis [14]. These HRMS-based methodologies can operate through data-dependent acquisition (DDA) or data-independent acquisition (DIA), both equally used for the quantification of multiple mycotoxins in biological samples [15]. Considering that hair still remains as an scarcely unexplored matrix in the mycotoxin field, the aim of the present study was to develop and validate a multi-mycotoxin methodology, based on a pretreatment stage and later SALLE followed by high performance liquid

chromatography coupled to high resolution quadrupole-time of flight mass spectrometry (HPLC-Q-TOF-HRMS), for the determination of aflatoxins (AFs), beauvericin (BEA), enniatins (ENs) and T-2 toxin (T-2) in human hair. Then, the proposed methodology was applied to 10 human hair samples for evaluate its usefulness. To the best of author's knowledge, this is the first study proposing an analytical methodology for the determination of multiple mycotoxins in human hair.

2. Materials and Methods

2.1. Chemical and Reagents

MeOH (HPLC grade), ethyl acetate (HPLC grade), ACN (HPLC grade) and absolute ethanol were acquired from Merck (Darmstadt, Germany). Deionized water used as the mobile phase (resistivity > 18 MΩ) was obtained from a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA). Formic acid (> 99%) was purchased from Acros Organics (New Jersey, NJ, USA). Sodium chloride (NaCl) (ACS grade) was acquired from VWR Chemicals (Leuven, Belgium). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Zirconium-based sorbent Z-Sep+ for extract clean-up was purchased from Supelco (St Quentin Fallavier, France). 1,4-dithiothreitol (DTT) (≥ 99%), tris(hydroxymethyl) aminomethane hydrochloride (TRIS HCl) (≥ 99%) and non-ionic detergent Tween® 20 were obtained from Sigma Aldrich (St. Louis, MO, USA). Pronase E (4000 U/mg) was supplied by Millipore (Darmstadt, Germany).

Two working solutions were prepared for enzymatic digestion: DTT at 12 mg/mL and Pronase E at 2 mg/mL in TRIS HCl buffer solution 0.05M at pH 7.2.

Analytical standards of AFB1, AFB2, AFG1, AFG2, ENNA, ENNA1, ENNB, ENNB1, BEA and T-2 toxin were provided by Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each toxin were prepared by properly diluting in MeOH to reach 1000 ng/mL. Then, working standard solutions for spiking experiments were built by mixing individual stock solutions and diluting in MeOH:H_2O (70:30, *v/v*) containing 0.1% formic acid and ammonium formate 5 mM until reaching 100 ng/mL, 50 ng/mL and 10 ng/mL. Ethanol was used for submerging spiked hair samples in order to achieve an homogeneous fortification prior to washing.

2.2. Sampling

A total of 10 hair samples were collected from laboratory staff. Stainless steel scissors were used for hair collection from the vertex posterior region of the scalp. Each hair sample was asked to be 5 cm long and, approximately, 7 mm diameter, accounting for a total weight between 0.4-0.8 g. No exclusion criteria were set and volunteers provided a signed informed consent following the Helsinki Declaration on ethical principles for medical research. This investigation was approved by the University of Valencia Institutional Human Research Committee.

2.3. Evaluation of Hair Pretreatment

Hair was subjected to a pretreatment in two steps in order to: (i) remove contaminants from the hair surface and (ii) achieve a complete release of mycotoxins embedded into the hair matrix.

2.3.1. Washing Procedure

Hair samples were washed with 5 mL of deionized water and 5 mL of Tween® 20 diluted in deionized water (25:75, *v/v*) and manually shaken. Hair was successively rinsed with distilled water until complete removal of Tween® 20. Afterwards, hair was dried at 60°C in heater (Binder BD 115; Binder, Tuttlingen, Germany) and then cut into fragments (< 5 mm) using stainless steel scissors for further processing. Washing procedure was evaluated according to two different events: (i) the effect over the total burden of mycotoxins and (ii) the effectiveness removing interferents present in the hair matrix.

Firstly, blank hair samples (n = 3) previously analyzed and hair samples spiked at 100 ng/g (n = 3) were covered in 20 mL of ethanol and left at room temperature until complete evaporation, in order to achieve a proper homogenization of mycotoxins. Samples were subjected to washing and aqueous fractions obtained from the successive rinses were collected to investigate the presence of mycotoxins through a dispersive liquid-liquid microextraction (DLLME) protocol [16]. Briefly, 1 mL of aqueous rinse was placed into a 15 mL PTDI tube alongside 0.3 g NaCl. Then, 1 mL of ACN and 100 µL of ethyl acetate were quickly added. The mixture was vortexed for 1 min and centrifuged at 2880*g* for

3 min for a proper phase separation. The supernatant was collected and placed into another 15 mL PTFE tube for evaporation using a TurboVap LV Evaporator (Zymark, Hoptikinton, MA, USA). The extract was later reconstituted in 140 µL of MeOH:H_2O (70:30, *v/v*) containing 0.1% formic acid and 5 mM ammonium formate and filtered through a 0.2 µm filter prior to HPLC-Q-TOF analysis.

Secondly, the effect of washing for obtaining cleaner extracts was compared throughout the SSE. Therefore, washed samples (*n* = 3) and unwashed samples (*n* = 3) were subjected to enzymatic digestion, spiked at 100 ng/g and brought under SALLE procedure for later HPLC-Q-TOF analysis.

2.3.2. Enzymatic Digestion Procedure

Enzymatic digestion was applied over washed, dried and cut hair samples. Initially, 50 mg of each sample were weighted in 15 mL Pyrex glass tubes with screw cap and subjected to enzymatic digestion using a slightly modified protocol developed by Míguez-Framil et al. [13]. In brief, 0.5 mL of solution DTT at 12 mg/mL were firstly added and left at 40°C for 1 h; then, 0.5 mL of solution Pronase E at 2 mg/mL were added and left at 40°C overnight.

The effect of enzymatic digestion was evaluated through the analysis of known positive samples. Digested replicates (*n* = *3*), non-digested replicates (*n* = *3*) and non-digested replicates spiked at 100 ng/g (*n* = 3) were subjected to SALLE extraction and later HPLC-Q-TOF analysis.

2.4. Mycotoxin Extraction from Hair Samples

For extraction purposes, a SALLE methodology was developed as follows: 3 mL of ACN and 0.3 g of NaCl were added to the digested samples. Then, the samples were vortexed (Vortex-vib; Selecta, Barcelona, Spain) for 1 min and later centrifuged at 2880*g* (Centrifuge 5810 R; Eppendorff, Hamburg, Germany) for 3 min. The supernatant was transferred to a 15 mL PTFE tube containing 30 mg of Z-Sep$^+$ and the mixture was vortexed for 30 s. Afterwards, the sample was centrifuged again at 2880*g* for 3 min and the supernatant was placed in a 15 mL PTFE tube and evaporated to dryness using a TurboVap LV Evaporator (Zymark, Hoptikinton, MA, USA). The extract was finally resuspended in 140 µL of MeOH:H_2O (70:30, *v/v*) containing 0.1% formic acid and 5 mM ammonium formate and filtered through a 0.2 µm filter prior to HPLC-Q-TOF analysis.

2.5. HPLC-Q-TOF-HRMS Analysis

Chromatographic analysis was performed through an Agilent 1200 Infinity Series LC system (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent Technologies 6540 UHD Accurate-Mass Q-TOF equipped with an Agilent Technologies Dual Jet Stream electrospray ionization (Dual AJS ESI).

Chromatographic separation was performed through a Gemini-NX column C18 (110 Å, 3 µm, 150 mm × 4.6 mm) (Phenomenex; Torrance, CA, USA) with temperature set at 20 °C. The binary gradient consisted in the elution of water (phase A) and MeOH (phase B) both containing 0.1% formic acid and 5 mM ammonium formate. The separation gradient consisted in: 50% phase B was held

for 6 min; then, it linearly went up to 100% phase B in 1 min and held for 5 min. Next, the gradient decreased to 50% phase B in 1 min and held for 7 min for column re-equilibration. Total run time was 20 min with a flow rate set at 0.2 mL/min an injection volume of 5 µL.

The Q-TOF mass spectrometer operated AutoMS/MS setting both positive and negative ionization mode with the following settings: drying gas flow (N2), 10 L/min; drying gas temperature, 330 °C; nebulizer pressure, 30 psi; sheath gas flow, 9 L/min; sheath gas temperature, 350 °C. The ion source parameters were: capillary voltage, 3500 V; nozzle voltage 500 V, fragmentor voltage 160 V, skimmer voltage 30 V and octopole RF peak, 750 V. Fragmentation events were conducted at fixed collision energies (10 and 40 V). Acquisition was performed in the range 100-1000 *m/z* for MS and at 40-700 *m/z* for MS/MS acquisition, at a scan rate of 5 spectra/s. Internal mass correction was carried out through two reference masses: 121.0509 and 922.0098 m/z. Data processing was conducted using Agilent MassHunter Workstation software B.08.00 (Agilent Technologies).

2.6. Method Validation

The proposed methodology was in-house validated following the guidelines established in the Commission Decision 2002/657/EC [10] in terms of linearity, matrix effect, selectivity, trueness, repeatability, within-lab reproducibility and LOQ. Firstly, calibration curves were built in neat solvent and blank matrix within the range 100-0.4 ng/mL. %SSE was evaluated in order to detect a potential interference of the matrix, so the slopes of both curves were compared following Equation 1:

$$\%SSE = S_m / S_s \times 100 \quad (1)$$

being S_m the slope of the matrix-matched calibration curve and S_s the slope of the calibration curve built in neat solvent. A %SSE below 80% or above 120% indicated non-negligible signal suppression or enhancement, respectively. Selectivity was determined through the analysis of blank samples (n = 10) for detecting signals in the same retention time areas of the analytes. LOQ was defined as the lowest concentration of the analyte at which the concentration could be determined with accuracy and precision ≤20%. Trueness was assessed through recovery studies at three fortification levels (100, 50 and 10 ng/g). Spiked samples were extracted in triplicate over three non-consecutive days in order to determine the precision in terms of repeatability (intra-day precision, RSD_r) and within-lab reproducibility (inter-day precision, RSD_R). Accuracy was defined as a combination between trueness and precision following Equation 2:

$$\%Accuracy = \sqrt{(100 - recovery)^2 + {RSD_R}^2} \quad (2)$$

2.7. Quality Control / Quality Assurance

Retention times corresponding to analytes present in samples were compared to those in standard solutions at a maximum deviation of ±2.5% of total run time (20 min). Alongside biological samples, a set of quality assurance/quality control samples including a reagent blank, a procedural blank, a replicate sample and a matrix-matched calibration were introduced at the beginning and end of each batch to evaluate the efficacy and stability of the system throughout the whole batch.

2.8. Statistical Analysis

Statistical analysis of data was performed using IBM SPSS version 25 statistical software package (SPSS, Chicago, IL, USA). Mann-Whitney U test was applied to evaluate differences between solvents in terms of %SSE and accuracy. A p-value < 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Evaluation of HPLC-Q-TOF HRMS Conditions

The HPLC-Q-TOF HRMS parameters (retention time, adduct ion, measured mass, mass error and product ion) were evaluated through the injection of mycotoxins analytical standards at a concentration of 1 µg/mL. Results are shown in Table 1. A satisfactory separation was observed in the chromatographic profile of the studied analytes with the gradient used. The 10 mycotoxins investigated eluted from 4.23 to 15.39 min.

AFs showed stable and abundant protonated ion $[M+H]^+$ whereas ENNs, BEA and T-2 showed ammonium adducts $[M+NH4]^+$ with greater intensity than sodium and protonated ones. Measurements showed a high precision with a mass error below 5 ppm, indicating optimal selectivity for all the assayed mycotoxins. For confirmatory purposes, products ions were chosen according to the two most abundant fragments at both collision energies previously set, that also showed a similar ion ratio between the measurements in analytical standard and matrix-matched calibration.

Table 1. HPLC-Q-TOF-HRMS parameters

Analyte	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (m/z)	Measured mass (*m/z*)	Mass error (Δ ppm)	Product ion
AFB1	7.33	$C_{17}H_{12}O_6$	$[M+H]^+$	313.0706	313.0707	-0.2	285.0749; 269.0437
AFB2	5.88	$C_{17}H_{14}O_6$	$[M+H]^+$	315.0856	315.0863	-2.3	287.0906; 259.0595
AFG1	5.15	$C_{17}H_{12}O_7$	$[M+H]^+$	329.0652	329.0656	-1.2	243.0647; 200.0464
AFG2	4.23	$C_{17}H_{14}O_7$	$[M+H]^+$	331.0808	331.0812	-1.3	313.0701; 245.0803
BEA	14.9	$C_{45}H_{57}N_3O_9$	$[M+NH_4]^+$	801.4433	801.4436	-0.4	262.7672; 244.1824
ENNA	15.39	$C_{36}H_{63}N_3O_9$	$[M+NH_4]^+$	699.4923	699.4903	2.9	228.1590; 210.1485
ENNA1	15.14	$C_{35}H_{61}N_3O_9$	$[M+NH_4]^+$	685.4773	685.4746	3.9	228.1590; 210.1485
ENNB	14.64	$C_{33}H_{57}N_3O_9$	$[M+NH_4]^+$	657.4483	657.4463	3.0	214.1432; 196.1328
ENNB1	14.92	$C_{34}H_{59}N_3O_9$	$[M+NH_4]^+$	671.4639	671.4609	4.5	214.1434; 196.1329
T-2	12.78	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.2530	484.2541	-2.3	215.1060; 185.0956

3.2. Optimization of hair preparation procedure

The lack of knowledge about hair analysis in the mycotoxins field imply the absence of standardized protocols for their extraction from the hair matrix. Therefore, current and common techniques in hair analysis, such as washing

procedure and enzymatic digestion, were evaluated for their further applicability to mycotoxins. Similarly, in order to achieve a multi-mycotoxin feature, different extraction solvents were assessed and compared.

3.2.1. Evaluation of the influence of washing in the content of mycotoxins

The lack of reference material for mycotoxin analysis in hair sample hampered the understanding of how the washing process influenced the total content of mycotoxins. Therefore, an assessment was carried out in order to evaluate two events derived from the washing procedure: (i) the potential loss of bounded mycotoxins throughout the analysis of rinses and (ii) the effectiveness removing interferents after analyzing washed and unwashed hair samples.

Firstly, blank samples and samples spiked at 100 ng/g were subjected to washing and the subsequent rinses were later extracted with a DLLME procedure and analyzed. Results showed a removal between 20-85% of the initial fortification within the first rinse, whereas no mycotoxins were observed in the rinse corresponding to the blank samples. Last rinse, collected once the detergent was completely removed, showed no mycotoxins pointing to a complete elimination during the successive rinses. Therefore, washing resulted to be a safe procedure prior to extraction, since only mycotoxin present in hair surface were removed whereas those mycotoxins bounded to hair matrix remained unaltered as observed after enzymatic digestion of blank hair samples and subsequently extraction of mycotoxins.

Secondly, the effect of washing for obtaining cleaner extracts was compared throughout the %SSE after enzymatic digestion and SALLE extraction. Results are shown in Figure 1. Washing proved to reduce matrix effect by maintaining most values within the range 80-120% except for T-2 (36%) whereas strong suppression effect was observed in unwashed samples with no mycotoxins fitting the recommended range (%SSE < 58%). Significant differences were observed after statistical analysis (p-value = 0.03). The presence of contaminants occurring in unwashed samples, such as cosmetic products, appeared to be co-extracted ultimately leading to a worse efficiency in the ionization prior to mass spectrometry detection, as showed by the strong matrix effect.

Hence, washing procedure did not affect the total content of mycotoxins bounded to the hair matrix and improved the analytical performance by having extracts free from interferents.

Previous studies reporting the presence of mycotoxins in hair samples also incorporated a washing step prior to extraction of mycotoxins embedded in hair. An ethanol-based procedure was used by Sewram et al. [7] and Bordin et al. [8] for later detection of fumonisins (FBs), whereas Kintz et al. [9] applied a dicholoromethane-based washing prior to extraction of zearalenone (ZEN) and α-zearalanone (α-ZAL).

3.2.2. Evaluation of the influence of enzymatic digestion

The influence of enzymatic digestion was assessed through a known positive hair sample and later comparison among digested, non-digested, and

non-digested replicates spiked at 100 ng/g and subjected to SALLE procedure. Results showed the simultaneous presence of AFG1, BEA and ENNA1 co-occurring at 11, 12.4 and 23.3 ng/g, respectively, within the digested replicates, whereas no contamination was observed after analyzing the non-digested replicates. In addition, the spiked replicates showed concentration values between 102-105 ng/g for the mentioned mycotoxins, implying a good performance of the SALLE procedure.

This is the first methodology including a digestion stage for the quantification of mycotoxins in human hair. Despite the scarce methodologies available, an incubation process prior to extraction is commonly performed based on the use of MeOH [7, 8]. However, the extensive amount of MeOH per sample (20-150 mL) represent a great drawback for biomonitoring studies, that require from a high number of samples. Additionally, this MeOH-based incubation was carried out for the quantification of FBs only, so its applicability in multi-mycotoxin methodologies could be compromise. Considering that analysis of a non-digested sample led to a false-negative finding, mycotoxins bounded to the hair matrix may require from an exhaustive procedure for achieving a complete release. In order to avoid a great expense of reagents and aiming for a multi-mycotoxin feature, an enzymatic digestion is encouraged since no severe conditions are applied and the final extract is compatible with simple LLE procedures.

3.2.3. Evaluation of solvents

The multi-analyte approach of the present methodology requires the use of universal solvents in mycotoxin context. In this line, ACN and ethyl acetate represent the traditional choice for multi-mycotoxins studies, considering that most of them show high solubility in organic solvents [17]. Therefore, both solvents were compared in terms of matrix effect (Equation 1) and accuracy (Equation 2). Hair samples were subsequently subjected to pretreatment, fortification at 100 ng/g and extraction. Results showed comparable matrix effect in both solvents, with %SSE within the range 80-120% for most mycotoxins revealing no significant differences after statistical analysis (p-value = 0.106). Low accuracy was detected after analyzing ethyl acetate extraction, with values ranging from 13% to 141%, whereas ACN showed a better performance with accuracy ranging from 11% to 19% and significant differences were observed between the two solvents (p-value = 0.043) as regards accurate extraction of mycotoxins.

The primary chemical components of hair are proteins, mainly keratins (65-90%), water (15-35%) and lipids (1-9%). In this methodology, hair samples underwent enzymatic digestion prior to extraction, so the keratin fraction is expected to be converted into free amino acids based on the combined activity of dithiotreitol (DTT), reducing sulfide bonds within the keratin matrix, and Pronase E, breaking peptide bonds. Although similar matrix effect was observed in extracts obtained from both solvents, ethyl acetate is more likely to co-extract

other compounds considering the composition of the hair matrix. Hence, ACN was chosen for further experiments.

3.3. Method validation

The proposed SALLE methodology was in-house validated and results are shown in Table 2. Calibration curves built in neat solvent and blank matrix showed coefficients of linearity (R^2) above 0.990 within the assayed range (100-0.4 ng/mL) and a deviation below 20% for each level of the calibration curve. Slopes of both calibration curves were compared for assessing %SSE, revealing negligible interference for all analytes (80%-120%) except for T-2, so in this case matrix-matched calibration curve was used for quantification purposes. Additionally, the absence of matrix co-elutants was confirmed since no additional peaks were observed in the same retention time zones of the analytes after analysis of blank samples (n = 10), thus displaying proper selectivity. The proposed methodology showed to be sensitive enough to quantify mycotoxins occurring at low ng/g values, with LOQs established in the range 0.6-8.7 ng/g, being able to provide reliable measurements for signals within the linear range and a mass error below 5 ppm. Suitable recoveries were also observed at the three fortification levels assayed (100, 50 and 10 ng/g) with intra-day and inter-day relative precision values below 20%, providing repeatable and reproducible measurements. Hence, this in-house validated methodology for monitoring up to 10 mycotoxins was further applied ensuring the reliability of the obtained results.

Table 2. Method performance

Analyte	Linearity (r^2)	SSE (%)	LOQ (ng/g)	Recovery (%)			Precision (%) [RSD_r, (RSD_R)]		
				100 ng/g	50 ng/g	10 ng/g	100 ng/g	50 ng/g	10 ng/g
AFB1	0.9998	89	2.2	108	71	60	10 (13)	12 (12)	11 (12)
AFB2	0.9976	91	2.2	101	62	61	11 (14)	7 (13)	3 (6)
AFG1	0.9985	83	2.2	102	61	61	10 (11)	13 (18)	9 (19)
AFG2	0.9988	83	2.2	109	71	63	10 (18)	11 (19)	9 (19)
BEA	0.9993	81	2.2	102	64	68	8 (14)	11 (14)	10 (7)
ENNB	0.9924	82	0.6	100	70	68	8 (9)	16 (18)	12 (18)
ENNB1	0.9955	87	0.6	102	85	97	5 (13)	14 (19)	7 (9)
ENNA	0.9986	89	2.2	107	84	79	6 (12)	11 (18)	13 (19)
ENNA1	0.9972	82	2.2	105	81	74	8 (15)	8 (18)	8 (19)
T-2	0.9973	36	8.7	96	85	81	17 (18)	10 (17)	16 (16)

SSE: signal suppression/enhancement effect; LOQ: limit of quantification; RSD_r: intra-day precision; RSD_R: inter-day precision

A limited number of studies have investigated the presence of mycotoxins in human hair, as shown in Table 3. Considering the novelty of the matrix in the mycotoxin field, no standard procedure is currently available and methodologies strongly differ from each other according to the studied analytes. The initial amount of hair seemed to be very variable, between 20 and 1000 mg. Greater amounts of hair can maximize the influence of interferents present in the matrix and also imply higher expense of reagents, so miniaturized methodologies are preferred especially in biomonitoring studies that require great sample sizes. These previous extraction methodologies showed a good performance for only

Table 3. Available methodologies for the determination of mycotoxins in human hair

Sample weigth	Extraction	Quantification	Analytes	LOQ (ng/g)	Reference
700-1000 mg	Reflux with MeOH (150 mL) for 5 h; evaporation and resuspension in 20 mL MeOH:H_2O (70:30, *v/v*); defatting with hexane (5 mL) and SAX cartridge (pH 6)	HPLC-Q-TRAP-MS/MS	FB1 FB2 FB3	4	Sewram et al. (2003)
500 mg	Incubation with MeOH (20 mL) overnight at 45 °C; evaporation and resuspension in 20 mL MeOH:H_2O (70:30, *v/v*) + 8 mL H_2O; defatting with hexane (5 mL) and SAX cartridge (pH 6)	HPLC-Q-MS	FB1	5.5	Bordin et al. (2015)
20 mg	Incubation with MeOH (1 mL) for 1 h at room temperature	UPLC-QQQ-MS/MS	α-ZAL ZEN	5	Kintz et al. (2018)
50 mg	Enzymatic digestion with DTT (0.5 mL) + Pronase E (0.5 mL) overnight at 40°C; 3 mL ACN + 0.3 g NaCl; clean-up with 30 mg Z-Sep$^+$	HPLC-Q-TOF-HRMS	AFs ENs BEA T-2	2.2 0.6-2.2 2.2 8.7	Present study

EtOH: ethanol; MeOH: methanol; SAX: strong anion exchange; DCM: dichloromethane; DTT: di-thiothreitol; ACN: acetonitrile; HPLC: high performance liquid chromatography; Q: quadrupole; MS/MS: tandem mass spectrometry; UPLC: ultra performance liquid chromatography; QQQ: triple quadrupole; TOF: time of flight; HRMS: high resolution mass spectrometry; LOQ: limit of quantification.

recovering up to three chemically-related mycotoxins from human hair: FB1, FB2 and FB3 [7], FB1 [8] and ZEN alongside α-ZAL [9]. However, biomonitoring approaches are evolving into methodologies able to determine the presence of many mycotoxins simultaneously [11], so extraction methodologies must be

flexible enough to recover analytes with different chemical behaviors, mainly referring to their polarity, while being able to avoid co-extraction of undesired compounds that could interact with the analytes of interest.

In this context, the here-presented extraction method based on SALLE procedure was applied for the extraction of ten chemically different mycotoxins after implementing an enzymatic digestion step, that has been previously proposed as an efficient way to recover drugs from the keratin matrix [13, 18, 19]. A simple clean-up step based on Z-Sep$^+$ was also applied for successfully removing interferences from the matrix, as shown by the values of %SSE. This sorbent has been previously used in extracts with high amount of lipids and proteins [20], which are the two main chemical constituents of hair, avoiding an intermediate step of defatting with hexane as reported in some of the previously developed methods. Neither the inclusion of several mycotoxins into the same methodology nor the use of an untargeted methodology compromised the sensitivity, obtaining LOQs ranging from 0.6-2.2 ng/g (emerging *Fusarium* toxins and AFs) to 8.7 ng/g (T-2), comparable to the available studies. Nonetheless, whereas Bordin et al. [8] conducted a full in-house validation stage fitting the requirements set in current legislation, only recovery experiments were carried out in the rest of the studies, thus compromising their applicability in future biomonitoring studies. Although other toxicologically relevant mycotoxins have not been included in the present study, this work aim to be a starting point for the development of other multi-mycotoxin methodologies applicable to hair samples.

3.4. Monitoring of mycotoxins in hair samples

The validated methodology was then applied to 10 human hair samples belonging to both genders (males, *n = 6*; females, *n = 4*) aging from 25 to 44 years old. Results are shown in Table 4. At least one mycotoxin was detected in 60% of the samples at concentration levels ranging from 3.7 to 33.7 ng/g. The highest prevalence was observed for ENNA1 (50%), whereas AFB1 and AFG1 were also identified in 10% of samples. Quantitatively, ENNB showed the highest mean concentration (24.6 ng/g), whereas AFB1 (13.5 ng/g) and AFG1 (11 ng/g) were the mycotoxins occurring at the lowest mean levels. In addition, co-occurrence was observed in 30% of the samples due to the simultaneous presence of up to three toxins, accounting for a maximum sum of 46.7 ng/g within a single sample. Simultaneous presence of mycotoxins could represent a health concern considering the toxicological outcomes resulting from their interactions. Figure 2 shows LC-HRMS chromatograms corresponding to a spiked human hair sample at 50 ng/g and a naturally co-contaminated sample with AFG2 and BEA. These results showed a cumulative dietary exposure to mycotoxins over a surveillance window of 5 months, according to the initial length of the sample (5 cm) [21].

Table 4. Concentration and prevalence of mycotoxins in the assayed samples

Analyte	Positive samples (n, (%))	Range (ng/g)	Mean ± SD (ng/g)
AFB1	7 (1)	12.4-14.6	13.5 ± 1.6
AFB2	*nd*	*nd*	*nd*
AFG1	7 (1)	10.9-11.1	11 ± 0.2
AFG2	*nd*	*nd*	*nd*
BEA	13 (2)	9.3-18.3	14.3 ± 4.6
ENNA	7 (1)	26.7-30.2	28.5 ± 1.8
ENNA1	47 (7)	3.7-33.7	21.3 ± 8.4
ENNB	7 (1)	21.5-27.7	24.6 ± 4.4
ENNB1	7 (1)	29.7-30	29.8 ± 0.2
T-2	7 (1)	21.6-23	22.3 ± 1
Total	*67 (10)*	*3.2-37.1*	*19.9 ± 8.9*

nd = not detected

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3.7. *Human biomonitoring of multiple mycotoxins in hair: first large-scale pilot study*

1. Introduction

Mycotoxins are toxic metabolites produced by several fungi genera, mainly *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps*. These fungi can colonize a broad variety of crops including cereals, nuts, spices and fruits, at any point from the preharvest stage to the storage. Over the last decades, the study of mycotoxins intake has been approached by analyzing marketed foods consumed by the population in a given geographical area, which implies carrying out a large number of analysis and lowering representativeness when analyzing foods without previous culinary treatments, considering the different stability of mycotoxins against heat.

Currently, the most precise approach for estimating the exposure is HBM through the direct measurement of either parent toxins and/or their metabolites in biological matrices such as urine, blood, plasma or serum (Habschied *et al.*, 2021). In fact, an ever-expanding number of studies have reported the presence of mycotoxins such as aflatoxins (AFs), fumonisins (FBs), ochratoxin A (OTA), citrinin (CIT), T-2 toxin (T-2), deoxynivalenol (DON), zearalenone (ZEN), enniatins (ENNs) and their respective metabolites in biological samples (Al-Jaal *et al.*, 2019; Arce-López *et al.*, 2020b; Narváez *et al.*, 2021; Vidal *et al.*, 2018), whereas several biomarkers have undergone validation process for a proper interpretation of the

exposure to mycotoxins (Marín *et al.*, 2018). However, these traditional matrices fail to provide information about exposure to mycotoxins over long periods of time due to their rapid excretion rates and short half-lives (Turner and Snyder, 2021).

In this line, hair emerges as an innovative matrix in the mycotoxin field that can supply long-term information regarding chronic exposure. Despite the mechanisms have not been clarified yet, contaminants present in the bloodstream or other biological secretions seem to reach the follicle and incorporate into the hair matrix through a diffusion process (Khajuria *et al.*, 2018). After that, contaminants establish interactions, presumably including covalent bonds, with hair components such as melanin or the keratin matrix (Yu *et al.*, 2017). Therefore, the high chemical stability provided by hair enables a wider surveillance window. Considering a standard growth ratio of 1 cm/month, the exposure over an specific period of time could also be estimated (Mupunga *et al.*, 2017).

Although accumulation of mycotoxins in human hair has been previously reported, scarce information is currently available. Sewram *et al.* (2003) determined the presence of FB1, FB2 and FB3 in human hair samples for the first time, whereas Bordin *et al.* (2015) validated a procedure in order to quantify FB1 and applied to human hair samples. Kintz *et al.* (2018) detected the presence of α-zearalanol (α-ZAL) in three segments from the same hair sample in an adverse analytical finding. Although these previous methodologies have proven the feasibility of hair analysis in the mycotoxins field, the current HBM context is evolving towards more flexible methodologies able to quantify multiple

mycotoxins occurring in biological samples. These methodologies are of great toxicological relevance considering the synergistic, additive or antagonistic effects of mixtures of mycotoxins (Alassane-Kpembi *et al.*, 2017). Additionally, the great sample sizes typically used in HBM promote the development of miniaturized sample preparation procedures, implying little expense of reagents and time, whereas LC coupled to MS represent the gold standard for mycotoxin quantification (Arce-López *et al.*, 2020b). HRMS represents a recent trend in HBM that is not only able to deliver quantitative measurements of targeted compounds, but can also identify untargeted analytes through retrospective analysis of samples and exact mass measurements. This feature is especially useful in HMB studies for evaluating the presence of metabolic products, indicative of potential exposure to parent toxin. Hence, the aim of the present study was to monitor the simultaneous presence of ten major mycotoxins, including AFB1, AFB2, AFG1, AFG2, ENNA, ENNA1, ENNB, ENNB1, beauvericin (BEA) and T-2 in human hair samples (n = 100) through a high performance liquid chromatography coupled to Q-TOF high resolution mass spectrometry (HPLC-Q-TOF-HRMS). After that, a retrospective analysis was conducted for assessing the presence of untargeted mycotoxins and related metabolites. As far as authors' knowledge, this is the first multi-mycotoxin HBM study using hair as biological matrix.

2. Materials and methods

2.1. Chemical and Reagents

Deionized water used as the mobile phase (resistivity < 10 MΩ) was purchased from a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, MA, USA). MeOH (HPLC grade) and ACN (HPLC grade) were obtained from Merck (Darmstadt, Germany). Formic acid (> 99%) was acquired from Acros Organics (New Jersey, NJ, USA). Ammonium formate (99%) was supplied by Panreac Quimica S.A.U. (Barcelona, Spain). Sodium chloride (NaCl) (ACS grade) was purchased from VWR Chemicals (Leuven, Belgium). Non-ionic detergent Tween® 20 and DTT (≥ 99%), tris(hydroxymethyl) aminomethane hydrochloride (TRIS HCl) (≥ 99%) and were obtained from Sigma Aldrich (St. Louis, MO, USA). Zirconium-based sorbent Z-Sep^{+} for extract clean-up was obtained from Supelco (St Quentin Fallavier, France). Pronase E (4000 U/mg) was supplied by Millipore (Darmstadt, Germany).

Two working solutions consisting in DTT 12 mg/mL and pronase E 2 mg/mL in TRIS HCl buffer solution 0.05M at pH 7.2 were prepared for enzymatic digestion.

Analytical standards of AFB1, AFB2, AFG1, AFG2, ENNA, ENNA1, ENNB, ENNB1, BEA and T-2 toxin were supplied by Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of each toxin were built by properly diluting in MeOH until reaching 1000 ng/mL. After that, working standard solutions for spiking experiments were prepared by mixing individual stock solutions and diluting in

MeOH:H_2O (70:30, *v/v*) containing 0.1% formic acid and ammonium formate 5 mM to reach 100 ng/mL, 50 ng/mL and 10 ng/mL.

2.2. Sampling

A total of 100 hair samples were collected between August 2020 and April 2021 from local hairdressing saloons located in Valencia and Úbeda, East and South East Spain, respectively. Hair from the vertex posterior region of the scalp was collected using stainless steel scissors. Each hair sample was asked to be 5 cm long and, approximately, 7 mm diameter, accounting for a total weight between 0.4-0.8 g. Volunteers supplied their gender and age for further classification and statistical purposes. Gender parity was maintained throughout the sampling (female: 50%; male: 50%) and participants were aged from 5 to 80 years, although the majority were adults comprehended between 18 and 60 years old (78%).

No exclusion criteria were established and volunteers provided a signed informed consent following the Helsinki Declaration on ethical principles for medical research. In case of children, the informed consent was signed by their progenitors. This investigation was approved by the University of Valencia Institutional Human Research Committee.

2.3. Sample Preparation

Hair samples was treated according to an in-house developed methodology, including a pretreatment stage prior to extraction (Narváez *et al.*, personal communications). Firstly, samples were washed with 5 mL of deionized

water and 5 mL of Tween® 20 diluted in deionized water (25:75, v/v), and further rinsed with deionized water until complete removal of Tween® 20. Afterwards, hair was dried at 60°C in heater (Binder BD 115; Binder, Tuttlingen, Germany) and cut into fragments (< 5 mm) using stainless steel scissors. Secondly, 50 mg of each sample were weighted in 15 mL Pyrex glass tubes with screw cap and subjected to enzymatic digestion using a slightly modified protocol developed by Míguez-Framil *et al.* (2013). In brief, 0.5 mL of solution DTT 12 mg/mL were firstly added and left at 40°C for 1 h; then, 0.5 mL of solution pronase E 2 mg/mL were added for incubation at 40°C overnight.

Digested hair samples were subjected to SALLE. In brief, 3 mL of ACN and 0.3 g of NaCl were added to the digested samples. Afterwards, the samples were vortexed (Vortex-vib; Selecta, Barcelona, Spain) for 1 min and subsequently centrifuged at 2880*g* (Centrifuge 5810 R; Eppendorff, Hamburg, Germany) for 3 min. The upper layer was transferred to a 15 mL PTDI tube containing 30 mg of Z-Sep$^+$ and the mixture was vortexed for 30 s. Then, the sample was centrifuged again at 2880*g* for 3 min and the supernatant was placed in a 15 mL PTFE tube and evaporated to dryness using a TurboVap LV Evaporator (Zymark, Hoptikinton, MA, USA). Finally, the extract was resuspended in 140 µL of MeOH:H_2O (70:30, *v/v*) containing 0.1% formic acid and 5 mM ammonium formate and filtered through a 0.2 µm filter prior to HPLC-Q-TOF analysis.

2.4. HPLC-Q-TOF-HRMS Analysis

Chromatographic and HRMS conditions were the same as described in pages 246-247. The HPLC-HRMS parameters of the targeted mycotoxins are

described in Table 1. Data analysis was carried out using Quan/Qual Browser Xcalibur v.3.1.66 (Thermo Fisher Scientific, Waltham, MA, USA).

Table S1. HPLC-Q-TOF HRMS parameters for the targeted mycotoxins.

Analyte	Retention time (min)	Elemental composition	Adduct ion	Theoretical mass (*m/z*)	Measured mass (*m/z*)	Accuracy (Δ ppm)
AFB1	7.33	$C_{17}H_{12}O_6$	$[M+H]^+$	313.0706	313.0707	-0.2
AFB2	5.88	$C_{17}H_{14}O_6$	$[M+H]^+$	315.0856	315.0863	-2.3
AFG1	5.15	$C_{17}H_{12}O_7$	$[M+H]^+$	329.0652	329.0656	-1.2
AFG2	4.23	$C_{17}H_{14}O_7$	$[M+H]^+$	331.0808	331.0812	-1.3
BEA	14.9	$C_{45}H_{57}N_3O_9$	$[M+NH_4]^+$	801.4433	801.4436	-0.4
ENNA	15.39	$C_{36}H_{63}N_3O_9$	$[M+NH_4]^+$	699.4923	699.4903	2.9
ENNA1	15.14	$C_{35}H_{61}N_3O_9$	$[M+NH_4]^+$	685.4773	685.4746	3.9
ENNB	14.64	$C_{33}H_{57}N_3O_9$	$[M+NH_4]^+$	657.4483	657.4463	3.0
ENNB1	14.92	$C_{34}H_{59}N_3O_9$	$[M+NH_4]^+$	671.4639	671.4609	4.5
T-2	12.78	$C_{24}H_{34}O_9$	$[M+NH_4]^+$	484.2530	484.2541	-2.3

For retrospective analysis, features were firstly extracted from total ion chromatograms through the software Agilent MassHunter Profinder (Agilent Technologies) using the batch recursive feature extraction algorithm for small molecules/peptides with the following setting: retention time filter, 1-19 min; ion intensity filter, 600 counts; retention time tolerance, ± 0.3 min; mass tolerance, ± 5 ppm; Q-score > 80. After peak deconvolution and alignment of chromatographic peaks, the extracted features were identified using the software MassHunter Mass Profiler and the spectral library Agilent Mycotoxins and Related Metabolites Personal Compound Database and Library (PCDL), that contains

spectral information of 455 compounds. Identification of features was conducted using the following parameters: mass score, 100; isotope abundance score, 60; isotope spacing score, 50; mass tolerance ± 5 ppm; positive ions including +H, +Na and $+NH_4$; negative ions including -H and +HCOO. Data processing was conducted using Agilent MassHunter Workstation software B.08.00 (Agilent Technologies).

2.5. Statistical Analysis

Statistical analysis of data was performed using IBM SPSS version 25 statistical software package (SPSS, Chicago, IL, USA). Non-parametric Kruskal-Wallis test was conducted for assessing significance in the concentration levels reported. Pearson chi-square test was applied for comparison of categorical data, meaning occurrence of mycotoxins related to age and gender. A p-value < 0.05 was considered as statistically significant.

3. Results and Discussion

3.1. Monitoring of Mycotoxins in Hair Samples

The methodology was in-house validated prior to monitoring study according to Commission Decision 2002/657/EC (EC, 2002). Calibration curves showed coefficients of correlation (r^2) above 0.990 for the selected range (100-0.4 ng/mL), with each level showing a deviation below 20%. Slopes from both calibration curves built in blank matrix and neat solvent were compared to assess the %SSE. Results showed a negligible matrix effect, with %SSE values comprehended within the range 80-120% except for T-2, whose quantification

was carried out using matrix-matched calibration curve. Analysis of blank samples (n = 10) confirmed the absence of co-elutants in the same retention time zones as the studied analytes, thus displaying proper selectivity. LOQs were established within the range 0.6-8.7 ng/g, showing high sensitivity for quantifying mycotoxins occurring in hair at low ng/g values. Spiking experiments at 100, 50 and 10 ng/g determined proper recoveries (70-120%) for the three fortification levels assayed, whereas intra-day and inter-day relative standard deviation values were below 20%, ensuring repeatable and reproducible measurements.

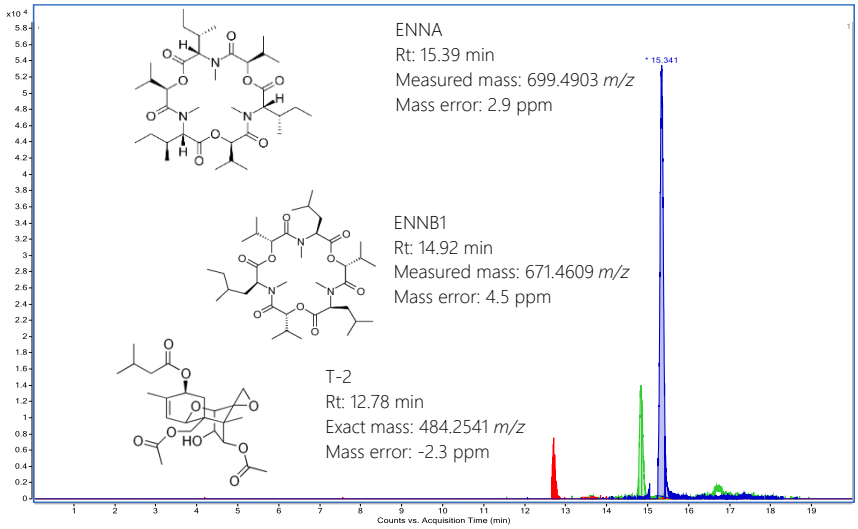

Figure 1. Chromatogram of a positive sample containing T-2 (33.14 ng/g), ENNB1 (14.46 ng/g) and ENNA (104.70 ng/g).

The validated analytical methodology was then applied to 100 human hair samples. Results are shown in Table 2 and only positive samples were considered for mean concentration calculation. At least one mycotoxin was detected in 43% of samples at concentration levels ranging from 2.7 to 106.1 ng/g. Emerging *Fusarium* toxins showed the highest prevalence, being ENNA1 (18%) > ENNA

(11%) > ENNB1 (10%), followed by AFB1 (7%). Quantitatively, the heaviest contamination was due to ENNA (mean: 46.4 ng/g) > T-2 (mean: 28.0 ng/g) > ENNB (mean: 21.4 ng/g), whereas AFB1 was quantified at a mean concentration of 11.0 ng/g. These results showed a cumulative dietary exposure to mycotoxins over a surveillance window of 5 months, according to the initial length of the sample (5 cm) (Wilkins *et al.*, 1998). Figure 1 shows a chromatogram from a sample contaminated with T-2, ENNB1 and ENNA. Chromatogram of a positive sample containing T-2 (33.14 ng/g), ENNB1 (14.46 ng/g) and ENNA (104.70 ng/g).

Table 2. Biomonitoring of mycotoxins in human hair samples.

Population group	Parameter	Mycotoxin										
		AFB1	AFB2	AFG2	AFG1	T-2	ENNB1	ENNA1	ENNA	BEA	ENNB	Total
Females *(n = 50)*	Prevalence (%)	10	6	2	2	4	10	24	14	8	6	56
	Range (ng/g)	8.5-24.0	2.7-5.0	7.2	10.9	29.1-40.1	9.5-42.8	3.4-37.5	4.4-81.8	15.1-24.0	12.1-60.8	2.7-81.1
	[a]Mean ± SD (ng/g)	12.6 ± 6.5	3.9 ± 1.2	7.2	10.9	34.6 ± 7.8	24.1 ± 14.1	19.5 ± 9.7	31.8 ± 26.8	18.6 ± 3.8	29.7 ± 27	21.0 ± 16.1
Males *(n = 50)*	Prevalence (%)	4	*nd*	*nd*	*nd*	6	10	12	8	2	6	30
	Range (ng/g)	5.0-13.3	*nd*	*nd*	*nd*	17.2-27.8	7.6-12.4	4.5-42.3	12.9-106.1	4.1	6.5-24.6	4.1-106.1
	[a]Mean ± SD (ng/g)	9.2 ± 5.8	*nd*	*nd*	*nd*	23.6 ± 5.6	8.8 ± 2.0	23.8 ± 12.1	71.9 ± 41.8	4.1	13.1 ± 10.0	25.3 ± 27.8
Total *(n = 100)*	Prevalence (%)	7	3	1	1	5	10	18	11	5	6	43
	Range (ng/g)	5.0-24.0	2.7-5.0	7.2	10.9	17.2-40.1	7.6-42.8	3.4-42.3	4.4-106.1	4.1-24.0	6.5-60.8	6.5-60.8
	[a]Mean ± SD (ng/g)	11.6 ± 6.0	3.9 ± 1.2	7.2	10.9	28.0 ± 8.2	16.5 ± 12.5	21.0 ± 10.4	46.4 ± 36.9	15.7 ± 7.3	21.4 ± 20.4	22.5 ± 20.9

[a] Only positive samples were considered for mean calculation; *nd* = not detected

By gender, mycotoxins prevalence in hair samples belonging to females (*n* = 50) rose up to 56% whereas prevalence in male samples (*n* = 50) reached 30% (Table 2). Figure 2 shows the relative prevalence of mycotoxins according to gender. Out of all positive samples, occurrence of *Fusarium* toxins showed a similar pattern across gender (female: 72%; male: 80%). On the contrary, AFs resulted to be more frequent in hair samples from females (female: 24%; male 8%). Statistical analysis confirmed a significant higher prevalence of AFs in hair samples from females (*p*-value = 0.04). In terms of concentration, similar mean values were observed in females (21.0 ng/g) and males (25.3 ng/g), showing no significant differences after statistical analysis.

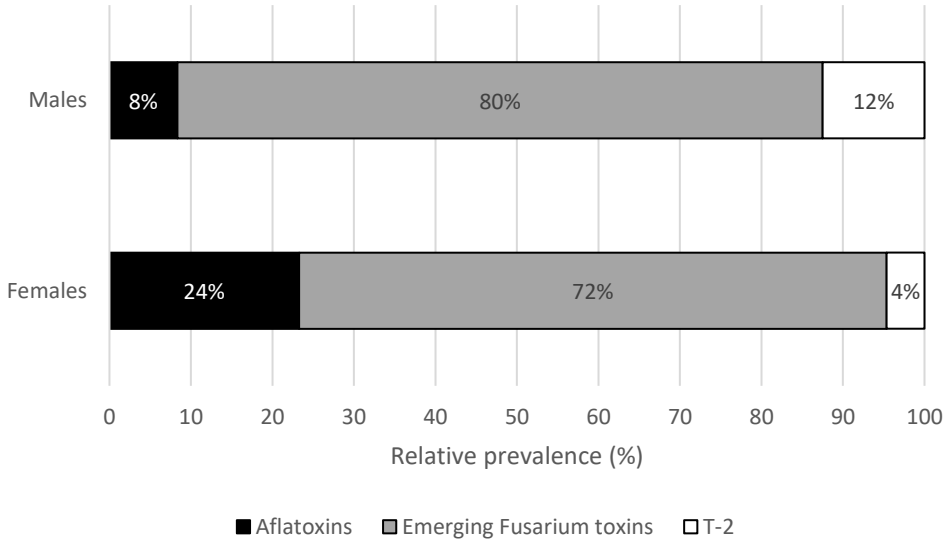

Figure 2. Relative prevalence of mycotoxins across gender

Accumulation of mycotoxins in human hair due to dietary exposure has also been reported by Sewram *et al.* (2003), who quantified FB1 in composite hair samples (n = 25) from 5 different regions of South Africa at mean levels ranging from 22.2 to 33.0 ng/g. Similarly, Bordin *et al.* (2015) detected FB1 in 7% of hair samples (n = 56) at a mean concentration of 21.3 ng/g, considering 7 cm long samples. The here-reported results pointed to similar accumulation as the previous studies, but some appreciations must be made when comparing chemically different mycotoxins occurring in hair samples.

Firstly, the mechanism of incorporation to the hair matrix has been suggested to be influenced by the chemical nature of the molecule. In this line, out of all the assayed mycotoxins, ENNs have the strongest lipophilic character (log *P* ranging from 3.81 to 4.79) compared to AFs (log *P* ranging from 0.5 to 1.45) (Arce-López *et al.*, 2020b). In addition, the amount of toxins occurring in hair is also dependent on the exposure. ENNs are still considered as "emerging *Fusarium*" toxins although they have been repeatedly found in foodstuffs worldwide and specially in the Mediterranean area (Gautier *et al.*, 2020; Gruber-Dorninger *et al.*, 2017; Rodríguez-Carrasco *et al.*, 2014a). According to the EFSA CONTAM Panel, acute exposure to ENNs does not represent a health concern, so regulatory authorities have not set any MLs for foods or foodstuffs (EFSA CONTAM Panel, 2014). Therefore, a more frequent contamination with ENNs in foodstuffs could be expected considering the lack of regulation. On the contrary, considering the well-known toxicity of AFs, a stringent legislation for AFB1 and the sum of AFs in cereals, nuts, raw milk and spices have been set (EC1881/2006, 2006).

Regarding the effect of gender, body composition has been suggested to play an important role in accumulation of chemicals in hair, considering that higher lipid levels could be responsible for slower metabolism and excretion processes (Iglesias-González *et al.*, 2020). Hence, accumulation could be preferably expected in women, as confirmed by the here-reported data, but the effect of gender is still uncertain in mycotoxin studies involving other biological matrices (Arce-López *et al.*, 2020b). Although scarce data about excretion or accumulation of ENNs is available, biomonitoring studies in urine have reported non-significant differences across gender (Rodríguez-Carrasco *et al.*, 2018; Rodríguez-Carrasco *et al.*, 2020), in accordance with the present results, whereas the effect of gender in AFs excretion or accumulation is yet inconclusive (Kang *et al.*, 2015; Seetha *et al.*, 2018).

Currently, biomarkers of chronic exposure have only been validated for AFs and OTA. Measurement of the complex AFB1-lysine in plasma samples allows an accurate estimation of AFB1 exposure, with the main drawback of the limited surveillance window of approximately 2-3 months considering the half-life of this complex (JECFA, 2017). Therefore, accumulation of AFs in hair could represent a complementary approach to evaluate chronic exposure since a wider surveillance window can be provided.

However, when no validated biomarkers are available, measurements of parent toxins can also provide an estimation. For current biomonitoring studies, urine is the preferred biological matrix implying an easy and non-invasive collection and providing a surveillance window up to 2-3 days before analysis

(Mupunga *et al.*, 2017; Turner and Snyder, 2021). In this line, biomonitoring studies including ENNs have only been carried out in urine, reporting mean concentration values of 0.012 ng/mL (Gerding *et al.*, 2015) and 0.065 ng/mL (Rodríguez-Carrasco *et al.*, 2018) both referring to ENNB and 0.016 ng/mL (Rodríguez-Carrasco *et al.*, 2020) referring to ENNB1. A toxicokinetic study of ENNB1 in pigs conducted by Devreese *et al.* (2014) reported clear systemic exposure, which could be favoring accumulation in hair rather than excretion through urine considering its lipophilic character. In this line, Rodríguez-Carrasco *et al.* (2016) highlighted a tendency of ENNB and BEA to bioaccumulate in lipophilic tissues after *in vivo* study with mice. Therefore, in this context where no biomarkers have been developed yet, monitoring throughout urine samples could be underestimating the exposure to ENNs.

Similarly, T-2 has been scarcely investigated in biological samples. A recent study carried out by De Ruyck *et al.* (2020) reported the presence of T-2 in serum (25%, n = 268) and urine samples (22%, n = 188), but no correlation was found between both matrices. On the contrary, no positive samples were observed neither in other urine-based studies conducted by Rodríguez-Carrasco *et al.* (2014b) (n = 54) and Gerding *et al.* (2015) (n = 287), nor in the plasma-based monitoring carried out by Arce-López *et al.* (2020a) (n = 438) despite including big sampling sizes. The rapid and extensive metabolism of T-2, including phase I and phase II products, hampers the detection of free parent toxin thus compromising the suitability of traditional matrices for assessing the exposure to T-2 as long as no biomarkers have been validated yet (Vidal *et al.*, 2018).

According to these results, hair could represent a suitable matrix for biomonitoring mycotoxin accumulation considering the high chemical stability provided by the hair matrix. Measurement of parent toxins in hair could represent a complementary approach to the current methodologies for performing chronic exposure assessment studies, even more considering that only scarce biomarkers are available in other matrices. However, the lack of data about toxicokinetics of mycotoxins in human hair precludes estimations of the exposure.

3.2. Co-Occurrence of Mycotoxins in Hair Samples

Co-occurrence of targeted mycotoxins has been observed in 42% of positive samples (n = 43) after quantification of two mycotoxins (28%) or three mycotoxins (14%) within the same sample. Results are shown in Table 3. Simultaneous presence of two emerging *Fusarium* toxins indistinctly combined was the main co-occurrence event (23%), with concentrations going up to 104.1 ng/g. Combination of two *Aspergillus* toxins was also detected in less frequency (2%) and at lower concentrations (9.9 ng/g). Combinations of three mycotoxins were more diverse, implying the presence of one or two toxins from both genera with no clear pattern, or only *Fusarium* toxins. Total concentration in samples containing only *Fusarium* toxins rose up to 144.3 ng/g, whereas combinations of genera reached a maximum of 51.2 ng/g.

Table 3. Co-occurrence of mycotoxins in the assayed samples

Co-occurrence	Number of samples (n, (% positive))	ΣC_{min} (ng/g)	ΣC_{max} (ng/g)
2 mycotoxins			
Fusarium toxins	10 (23)	20.7	104.1
Aspergillus toxins	1 (2)	9.9	9.9
Fusarium + *Aspergillus* toxins	1 (2)	29.4	29.4
3 mycotoxins			
Fusarium toxins	3 (7)	108	144.3
Fusarium + *Aspergillus* toxins	3 (7)	31	51.2

ΣC_{min} = minimum sum and ΣC_{max} = maximum sum of concentrations referring to co-occurring mycotoxins.

Simultaneous presence of mycotoxins could represent a health concern considering the toxicological outcomes resulting from their interactions. Several studies have reported that mixtures of AFs and its metabolites exerted stronger toxicity against macrophages (Alassane-Kpembi *et al.*, 2017) whereas the combination of AFB1 and AFB2 resulted in enhanced cytotoxicity in human ovarian cancer cell line A 2780 (Braicu *et al.*, 2010). Interactions between T-2 and AFB1 have shown higher mutagenic potential in the *Salmonella* prokaryote mutagenicity test (Smerak *et al.*, 2001). Combinations of two or three ENNs, the most common co-occurrence event reported in the present study, were able to

exert synergistic effects regarding their cytotoxicity in intestinal and ovarian cells (Lu *et al.*, 2013; Prosperini *et al.*, 2014).

Considering the high rate of co-occurrence events here reported, there might be a health concern highlighted by the combination of mycotoxins in hair samples. Regardless, a multi-analyte approach should be considered in biomonitoring studies in order to better understand simultaneous exposure to mycotoxins and its cumulative toxicological effects.

3.3. Untargeted Analysis

Retrospective analysis based on the spectral library Agilent Mycotoxins and Related Metabolites PCDL was performed after proper processing of total ion chromatograms in order to remove irrelevant information and provide reliable results. Results are shown in Tables 4 and 5. Up to 128 mycotoxins and related metabolites were tentatively identified. Although most of these compounds have been scarcely studied due to their low toxicological relevance, many of them have also been reported in food matrices, including agroclavine, apicidin, aurofusarin, brevianamide F, culmorin, cythochalasin J, elymoclavine, emodin, equisetin, nonactin or tryptophol (Abdallah *et al.*, 2017; Beccari *et al.*, 2018).

Table 4. Untargeted compounds tentatively identified through positive electrospray ionization and mass tolerance of ± 5 ppm.

Name	Formula	Detected mass	Q Score	Prevalence (%)
2-Amino-14,16-dimethyloctadecan-3-ol	$C_{20}H_{43}NO$	313.3337	98.9	15
10,11-Dehydrocurvularin	$C_{16}H_{18}O_5$	290.1174	94.6	5
5-Hydroxyculmorin	$C_{15}H_{26}O_3$	276.1719	99.1	30
16-Hydroxyroquefortine C	$C_{22}H_{23}N_5O_3$	400.2296	93.9	15
16-Keto-Aspergillimide	$C_{20}H_{27}N_3O_4$	390.2258	88.5	65
5-Methoxysterigmatocystin	$C_{19}H_{14}O_7$	376.0547	98.2	10
AAL Toxin TA1	$C_{25}H_{47}NO_{10}$	538.343	98.4	10
Aflatrem	$C_{32}H_{39}NO_4$	501.2932	96.7	25
Agistatin A	$C_{12}H_{18}O_5$	242.1149	99.5	10
Agistatin B	$C_{11}H_{18}O_4$	214.1218	84.5	15
Altenuene	$C_{15}H_{16}O_6$	292.0969	95.9	10
Andrastin A	$C_{28}H_{38}O_7$	486.2643	97.5	5
Andrastin D	$C_{26}H_{36}O_5$	445.2828	98.6	45
Antibiotic L696,474	$C_{30}H_{39}NO_4$	477.2853	100	40
AOH / Alternariol	$C_{14}H_{10}O_5$	275.081	98.3	10
Apicidin	$C_{34}H_{49}N_5O_6$	320.1985	99.2	35
Aphidicolin	$C_{20}H_{34}O_4$	360.2292	98.6	35
Aspinolide A	$C_{10}H_{16}O_3$	184.1096	99.1	20
Aspinolide C	$C_{14}H_{18}O_6$	304.0907	85.9	10
Aspinonene	$C_9H_{16}O_4$	205.131	99.1	30

Name	Formula	Detected mass	Q Score	Prevalence (%)
Aspochalasin D	$C_{24}H_{35}NO_4$	401.2569	98.1	20
Aurasperone D	$C_{31}H_{24}O_{10}$	556.1387	98.6	25
Aurofusarin	$C_{30}H_{18}O_{12}$	587.1032	98	5
Averantin	$C_{20}H_{20}O_7$	372.1202	96.9	10
Averufanin	$C_{20}H_{18}O_7$	387.1276	91.9	10
Bafilomycin A1	$C_{35}H_{58}O_9$	622.4043	96.6	70
Brevicompanine B	$C_{22}H_{29}N_3O_2$	389.2054	100	10
Brevianamid F	$C_{16}H_{17}N_3O_2$	283.1311	100	10
Calonectrin	$C_{19}H_{26}O_6$	372.1581	96.9	10
Cerulenin	$C_{12}H_{17}NO_3$	223.1203	100	75
Chaetomin	$C_{31}H_{30}N_6O_6S_4$	732.098	95.4	25
Culmorin	$C_{15}H_{26}O_2$	260.1774	98.5	35
Cycloaspeptide A	$C_{36}H_{43}N_5O_6$	641.3259	96.1	30
Cycloechinulin	$C_{20}H_{21}N_3O_3$	351.1577	98.7	35
Cyclopeptine	$C_{17}H_{16}N_2O_2$	297.1464	100	10
Cycloheximide	$C_{15}H_{23}NO_4$	303.1481	81.4	15
Cytochalasin H	$C_{30}H_{39}NO_5$	493.2805	92.7	15
Cytochalasin J	$C_{28}H_{37}NO_4$	451.2686	97.9	60
Decalonectrin	$C_{17}H_{24}O_5$	325.1896	98.4	15
Decarestrictine D	$C_{10}H_{16}O_5$	216.0987	97.7	30
Deepoxy deoxynivalenol	$C_{15}H_{20}O_5$	280.1276	97.7	45
Deoxyfusapyrone	$C_{34}H_{54}O_8$	612.3652	92	10

Name	Formula	Detected mass	Q Score	Prevalence (%)
Desferrioxamine E	$C_{27}H_{48}N_6O_9$	600.3473	98	50
Dihydrochlamydocin	$C_{28}H_{40}N_4O_6$	528.2955	86.9	25
Dihydroergocryptine	$C_{32}H_{43}N_5O_5$	577.3297	97.6	50
Dihydroergosine	$C_{30}H_{39}N_5O_5$	566.3221	95.5	45
Emodin	$C_{15}H_{10}O_5$	287.0825	94.2	10
Erginine	$C_{16}H_{17}N_3O$	267.137	93.6	10
Enniatin A2	$C_{36}H_{63}N_3O_9$	703.4346	89.4	10
Enniatin B2	$C_{32}H_{55}N_3O_9$	647.374	90.6	60
Equisetin	$C_{22}H_{31}NO_4$	373.226	100	45
Ergovalinine	$C_{29}H_{35}N_5O_5$	550.2963	96.5	10
FB3 / Fumonisin B3	$C_{34}H_{59}NO_{14}$	722.4255	92	40
FB4 / Fumonisin B4	$C_{34}H_{59}NO_{13}$	689.403	86.7	10
Festuclavine	$C_{16}H_{20}N_2$	262.1408	89.9	15
Fumagillin	$C_{26}H_{34}O_7$	458.2274	91.6	35
Fumigaclavine C	$C_{23}H_{30}N_2O_2$	366.2296	100	10
Fumiquinazoline F	$C_{21}H_{18}N_4O_2$	375.1686	80.7	10
Fumitremorgin C	$C_{22}H_{25}N_3O_3$	401.1727	80.9	10
Fusaproliferin	$C_{27}H_{40}O_5$	444.2871	97.9	55
Fusapyrone	$C_{34}H_{54}O_9$	623.4023	95.3	45
Fusaric acid	$C_{10}H_{13}NO_2$	179.0942	99.5	20
HC-Toxin	$C_{21}H_{32}N_4O_6$	458.2161	90.4	30
Helvolic acid	$C_{33}H_{44}O_8$	568.3068	100	65

Name	Formula	Detected mass	Q Score	Prevalence (%)
HFB1 / Hydrolysed Fumonisin B1	$C_{22}H_{47}NO_5$	405.3454	98.6	20
Isofusidienol A	$C_{16}H_{12}O_6$	317.0924	96.6	10
Josamycin	$C_{42}H_{69}NO_{15}$	844.4984	81.4	5
Ko 143	$C_{26}H_{35}N_3O_5$	486.2843	94.1	50
Lolitrem B	$C_{42}H_{55}NO_7$	685.4024	97.3	60
Lovastatin	$C_{24}H_{36}O_5$	404.2556	98.4	5
Marcfortine A	$C_{28}H_{35}N_3O_4$	494.2881	98.7	40
Marcfortine B	$C_{27}H_{33}N_3O_4$	480.2762	97.3	40
Marcfortine C	$C_{27}H_{33}N_3O_3$	447.2516	100	80
Monactin	$C_{41}H_{66}O_{12}$	750.4618	97.7	20
MPA / Mycophenolic acid	$C_{17}H_{20}O_6$	342.1085	96.8	10
Nigericin	$C_{40}H_{68}O_{11}$	702.4954	100	10
Nonactin	$C_{40}H_{64}O_{12}$	753.4639	93	40
Oligomycin A	$C_{45}H_{74}O_{11}$	812.506	80.6	55
Oligomycin B	$C_{45}H_{72}O_{12}$	826.4901	85.1	20
Ophiobolin B	$C_{25}H_{38}O_4$	402.2745	82	15
Palitantin	$C_{14}H_{22}O_4$	254.151	98.3	80
Paspalic acid	$C_{16}H_{16}N_2O_2$	285.1478	94.4	10
Paspaline	$C_{28}H_{39}NO_2$	438.3212	93.3	30
Paxilline	$C_{27}H_{33}NO_4$	452.2677	83.4	30
Pyrenophorol	$C_{16}H_{24}O_6$	334.1415	88.6	10
Roquefortine E	$C_{27}H_{31}N_5O_2$	474.2792	82.3	10

Name	Formula	Detected mass	Q Score	Prevalence (%)
Rubellin D	$C_{30}H_{22}O_{10}$	542.1265	86.1	5
Scirpentriol	$C_{15}H_{22}O_5$	282.1434	97.3	25
Sirolimus (Rapamycin)	$C_{51}H_{79}NO_{13}$	935.535	100	15
Spiramycin I	$C_{43}H_{74}N_2O_{14}$	864.4874	94.6	15
Tacrolimus	$C_{44}H_{69}NO_{12}$	803.4791	100	55
Tentoxin	$C_{22}H_{30}N_4O_4$	414.2256	94.3	25
Terphenyllin	$C_{20}H_{18}O_5$	355.1415	100	10
Terrein	$C_8H_{10}O_3$	176.0471	100	55
Trichodermin	$C_{17}H_{24}O_4$	292.1671	98.6	30
Tryprostatin A	$C_{22}H_{27}N_3O_3$	398.2307	90.3	30
Tryptophol	$C_{10}H_{11}NO$	172.0733	98.7	20
Tryptoquivaline F	$C_{22}H_{18}N_4O_4$	402.1317	96.7	15
Viridicatin	$C_{15}H_{11}NO_2$	254.105	97.3	10
ZEN / Zearalenone	$C_{18}H_{22}O_5$	318.1466	93.9	10

Table 5. Untargeted compounds tentatively identified through negative electrospray ionization mass tolerance of ± 5 ppm.

Name	Formula	Detected mass	Q Score	Prevalence (%)
6-Aminopenicillanic acid	$C_8H_{12}N_2O_3S$	262.0610	99.2	15
15-Hydroxyculmorone	$C_{15}H_{24}O_3$	298.1779	100.0	90
5-Methyl-mellein	$C_{11}H_{12}O_3$	252.0967	96.5	5
Agistatin D	$C_{11}H_{14}O_4$	210.0878	93.5	70
Agroclavine	$C_{16}H_{18}N_2$	238.1465	96.3	5
Andrastin B	$C_{28}H_{40}O_7$	534.2845	96.4	5
Andrastin D	$C_{26}H_{36}O_5$	474.2652	94.2	15
Aspinonene	$C_9H_{16}O_4$	248.1242	97.7	10
Aspochalasin D	$C_{24}H_{35}NO_4$	401.2564	96.7	20
Austdiol	$C_{12}H_{12}O_5$	236.0670	99.2	55
Calonectrin	$C_{19}H_{26}O_6$	396.1822	84.2	15
CT / Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	538.1389	100.0	15
Communesine B	$C_{32}H_{36}N_4O_2$	554.2981	95.6	5
Cyclopeptine	$C_{17}H_{16}N_2O_2$	280.1246	98.3	5
CPA / Cyclopiazonic acid	$C_{20}H_{20}N_2O_3$	336.1483	98.6	30
Deoxybrevianamide E	$C_{21}H_{25}N_3O_2$	411.2193	98.2	5
Deoxyfusapyrone	$C_{34}H_{54}O_8$	636.3828	97.4	5
Dehydrocyclopeptine	$C_{17}H_{14}N_2O_2$	338.1271	95.2	60
Dihydrochlamydocin	$C_{28}H_{40}N_4O_6$	550.2771	94.4	5

Name	Formula	Detected mass	Q Score	Prevalence (%)
Elymoclavine	$C_{16}H_{18}N_2O$	254.1434	99.4	5
FS-4	$C_{15}H_{22}O_3$	310.1779	100.0	15
Fulvic acid	$C_{14}H_{12}O_8$	308.0528	99.1	10
Fumagillin	$C_{26}H_{34}O_7$	458.2309	96.8	5
Fumigaclavine C	$C_{23}H_{30}N_2O_2$	366.2309	100.0	55
Fusidienol	$C_{16}H_{12}O_7$	316.0588	98.1	75
Elymoclavine	$C_{16}H_{18}N_2O$	254.1441	98.9	5
Mitomycine	$C_{15}H_{18}N_4O_5$	394.1477	85.4	25
NEO / Neosolaniol	$C_{19}H_{26}O_8$	442.1837	82.9	5
Oosporein	$C_{14}H_{10}O_8$	306.0395	96.5	5
Ophiobolin A	$C_{25}H_{36}O_4$	460.2853	83.4	10
Paspalic acid	$C_{16}H_{16}N_2O_2$	314.1257	84.1	25
PAT / Patulin	$C_7H_6O_4$	154.0242	100.0	85
Pyrenocine A	$C_{11}H_{12}O_4$	254.0815	96.3	5
Roquefortine A	$C_{18}H_{22}N_2O_2$	298.1670	100.0	30
Setosusin	$C_{29}H_{38}O_8$	560.2576	95.6	10
T-2 Tetraol	$C_{15}H_{22}O_6$	298.1442	98.9	25
TSA / Trichostatin A	$C_{17}H_{22}N_2O_3$	348.1661	98.3	5
Tryptoquivaline F	$C_{22}H_{18}N_4O_4$	402.1305	94.4	10
Verrucofortine	$C_{24}H_{31}N_3O_3$	455.2425	82	5

Among all the matches, it has to be highlighted the detection of several mycotoxins included in the classification released by International IARC. In this line, patulin (PAT) was tentatively identified in 85% of hair samples. This mycotoxin remains scarcely studied in biological samples, and only Ouhibi *et al.* (2020) have reported its presence in 20% and 30% of plasma samples belonging to individuals in a good health state (n = 50) and colorectal cancer patients (n = 50), respectively. PAT is preferably retained in erythrocytes and delivered to its main targets throughout the vascular system (Vidal *et al.*, 2019), so transference of PAT from blood into hair could be facilitated considering the extensive irrigation of the follicles. Hence, the high prevalence of PAT here-observed could be due to a potential accumulation as a consequence of dietary intakes over long periods of time. In fact, most of European countries are estimated to be under chronic exposure to PAT. Similarly, the tentative presence of ZEN has also been detected, although in a less proportion of samples (10%). Urine seems to be the most suitable matrix for detecting free ZEN and/or its phase I and phase II metabolites, attending to previous HBM studies that reported high incidence values (40-100%) (Al-Jaal *et al.*, 2019), whereas serum and plasma generally showed very low incidence (0-6%) (Arce-López *et al.*, 2020b). The rapid absorption and metabolization could explain the higher incidence of ZEN in urine (Carballo *et al.*, 2021), which is quickly excreted rather than accumulated in other matrices, as observed in the here-presented results.

Apart from parent toxins, metabolic products of relevant mycotoxins have also been tentatively identified in the assayed samples. FB3 and FB4 were observed in 40% and 10% of samples, respectively. These results are consistent

with a previous targeted study of FBs in human hair conducted by Sewram *et al.* (2003), that confirmed the accumulation of FB1, FB2 and FB3 in hair. T-2 metabolites T-2 tetraol and neosolaniol (NEO) were tentatively found in 25% and 5% of hair samples, respectively. In the context of exposure assessment, detection of metabolites is of crucial importance as shown by recent EFSA recommendations. Inclusion of FB1, FB2, FB3 and FB4 into the same TDI value has been proposed for properly assessing the exposure to FB1 (EFSA CONTAM Panel, 2018), whereas T-2 tetraol and NEO, alongside other modified forms, should also be considered for estimating the exposure to T-2 throughout a relative potency factor system (EFSA CONTAM Panel, 2017).

Alongside the above-mentioned, other mycotoxins not as toxic but also extensively found in foods and foodstuffs have been tentatively reported. Several emerging *Fusarium* toxins that were not initially targeted were reported after retrospective analysis. In order of incidence, ENNB2 (60%) > fusaproliferin (55%) > ENNA2 (10%). In addition, mycotoxins produced by *Alternaria* genus have also been tentatively detected, being tentoxin the most relevant compound (25%), whereas AAL toxin (10%), altenuene (10%) and alternariol (10%) showed lower incidence. Therefore, the accumulation of not only parent toxins but other relevant metabolites in hair could represent a useful feature for facing long-term exposure assessment studies.

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4. GENERAL DISCUSSION

4. General discussion

The scientific work carried out over the present thesis focused on evaluating human exposure to mycotoxins throughout the analysis of food and biological matrices.

Firstly, analytical methodologies for the quantification of multiple mycotoxins in food matrices were conveniently optimized and/or validated. Then, these methodologies were applied to real samples to quantify the occurrence of mycotoxins for ultimately understand the exposure to mycotoxins throughout food consumption.

Nonetheless, aiming for more accurate approximations within an exposure assessment context, a similar workflow was applied for the quantification of mycotoxins in human urine and hair. Although urine is a traditional matrix in the mycotoxin field, the lack of knowledge about hair was overcome through the development and optimization of a novel methodology for the quantification of multiple mycotoxins that was further applied to real samples. Additionally, non-targeted approaches revealed the presence of not only parent mycotoxins but also other several compounds derived from their human metabolism.

4.1. Optimization and validation of analytical methodologies

The studies conducted over the present thesis relied on either UHPLC-Q-Orbitrap or HPLC-Q-TOF HRMS. For each technique, a single method that included multiple mycotoxins was optimized once throughout the evaluation of analytical standards at a concentration of 1,000 ng/mL. Chromatographic

parameters (retention times) and MS parameters (ionization mode, adduct ion, products ion and mass accuracy) of the targeted mycotoxins were further used for analyzing different matrices.

However, each matrix required from a specific extraction methodology attending to their chemical composition or the mycotoxins of interest. Therefore, the main efforts for optimizing methodologies were put into the extraction stage. Every methodology was lastly validated according to the Commission Decision 2002/657/EC showing high sensitivity with LOQs below 1 ppb in many cases.

4.1.1. Extraction of mycotoxins from food and nutraceutical matrices

All the methodologies applied for the extraction of mycotoxins from the different food matrices here-analyzed share a common core, since they were all based on QuEChERS procedures using ACN as the solvent of extraction. ACN is considered an universal solvent for mycotoxins studies since many of them have high solubility in organic solvents. This fact makes ACN suitable for multi-mycotoxin methodologies. For the three matrices analyzed, water was previously added to the matrix in order to diminish the concentration of the sample and so the concentration of the potential interferents. In the case of the CBD-based capsules, water also helped to tear the soft gel capsules. Nevertheless, the ratio matrix:ACN (g/mL if solid matrix or mL/mL if liquid matrix) and the design of the clean-up stage were different for each food matrix.

The amount of ACN must be enough to extract the mycotoxins present within a sample without reaching a saturation point. This balance was optimized for the case of CBD-based nutraceuticals, since no previous methodologies were available. After evaluating four different ratios (1:2.5; 1:5; 1:7.5 and 1:10), a ratio 1:5 offered the best results in terms of recovery and matrix effect. In the case of ready-to-eat tree nuts and pear juices, ratios of 1:2 and 1:1 were respectively used.

The next step following extraction was the clean-up. An optimization stage was also conducted in the case of CBD-based nutraceuticals, evaluating the performance of four different sorbents: C18, PSA, GCB and the novel Z-Sep$^+$. Considering that the chemical composition of this matrix mainly consists in the oil contained inside the capsule, both C18 and Z-Sep$^+$ showed satisfactory results cleaning the extracts without affecting the burden of mycotoxins. Similarly, the use of C18 for the clean-up of tree nuts extracts provide a suitable performance in almonds, pistachios and walnuts. The design of the clean-up for extracts from pear juices incorporated a combination of C18 + PSA, since this last sorbent is also useful for removing some sugars and pigments.

4.1.2. Extraction of mycotoxins from biological matrices

The presence of mycotoxins in urine and hair was evaluated in several studies over the present thesis. The extraction methodologies were completely different from each other considering the nature of the assayed matrices, although ACN was the solvent of choice in both cases.

The extraction of mycotoxins from urine samples was based on a previous protocol optimized by Rodríguez-Carrasco et al. (2018) that consisted in a SALLE extraction with a clean-up step using C18. Nevertheless, this procedure had to be validated according to the present legislation for both studies regarding CIT + DH-CIT and T-2 + HT-2.

Hair samples required from a novel strategy for the extraction of multiple mycotoxins, since only one methodology has been successfully validated for only FB1 (Bordin et al., 2015). Therefore, a new procedure was developed using a previous protocol published by Míguez-Framil et al. (2013) that introduced a previous step consisting in the enzymatic digestion of hair samples using a protease. Hence, hair preparation process consisted in washing, enzymatic digestion and later extraction. Firstly, washing with a non-ionic detergent showed to be efficient enough to remove external contamination without affecting the total burden of mycotoxins. This was confirmed after recovery studies of mycotoxins in washed and non-washed samples alongside the analysis of several fractions of the rinses. Then, the enzymatic digestion, aimed for the release of mycotoxins bonded to the hair matrix, was also evaluated throughout spiking experiments of digested and non-digested samples. Results showed that enzymatic digestion is mandatory in order to achieve a complete release of mycotoxins. Additionally, as shown by the validation study, this process was innocuous for mycotoxins containing peptidic bonds such as ENNs or BEA. Lastly, a SALLE methodology was implemented for the extraction of mycotoxins from the digested aqueous samples. Extracts were finally subjected to clean-up with Z-Sep$^+$ providing satisfactory results. This sorbent was chosen considering the good

performance previously shown for the cleaning of CBD soft-gels capsules extracts. In this line, similarly to soft-gel capsules, the chemical composition of hair mainly based on proteins and fats. This methodology was conveniently validated according to Commission Decision 2002/657/EC. The tentative detection of up to 128 mycotoxins after retrospective analysis also proved the feasibility of the developed methodology for extracting a broad range of chemically different mycotoxins.

4.2. Evaluation of the content in mycotoxins

Throughout the present thesis, the content in mycotoxins has been evaluated in a total of 85 food samples and 400 biological samples:

- CBD-based nutraceuticals delivered as soft-gel capsules from different commercial brands (*n* = 10).
- Pear juices from different commercial brands and processing (conventional and organic) (*n* = 21).
- Ready-to-eat tree nuts from different commercial brands: almonds (*n* = 17), walnuts (*n* = 22) and pistachios (*n* = 15).
- Human urine samples collected from volunteers in South Italy (*n* = 300).
- Human hair samples collected from volunteers in South and South East Spain (*n* = 100).

4.2.1. Mycotoxins occurring in food matrices

Overall results showed a frequent contamination with mycotoxins, with 60 out of 85 food samples testing positive for at least one mycotoxin (70%).

CBD-based nutraceuticals were evaluated considering that there is no current legislation in terms of safety for these products. Therefore, several *Fusarium* (NEO, T-2, HT-2, ZEN, ZAN, α-ZEL, β-ZEL, α-ZAL, β-ZAL, ENNB, ENNB1, ENNA, ENNA1 and BEA) and *Aspergillus* (AFs) mycotoxins were targeted. Out of the ten analyzed samples, that corresponded to composite samples from different brands, seven showed contamination with at least one mycotoxin. Up to six different *Fusarium* mycotoxins were found, being ZEN (60%) and ENNB1 (30%), both found at a maximum level of 11.6 ng/g, the most prevalent compounds. Co-occurrence was observed in four samples, including one with ENNB1, ENNA and ENN A1.

The content of mycotoxins in pear juices available in markets was also evaluated. Since PAT is the only regulated toxin in this matrix, studies usually focus their methodology on the quantification of this single compound. Nevertheless, infection with *Fusarium* fungi has also been reported, so this study focused only on some of their mycotoxins. Out of the 21 analyzed samples, 14 tested positive (67%). ZEN was the most prevalent toxin (67%), followed by T-2 and HT-2 (33%) and ENNB and ENNA1 (19%). Nonetheless, occurrence of mycotoxins was determined at low levels, with a maximum concentration of 10.5 ng/mL corresponding to α-ZAL. Co-occurrence was observed in 14 out of the 20 positive

samples mainly due to the detection of ZEN alongside its metabolites or the combination of ENNs.

Ready-to-eat tree nut products were consciously evaluated in terms of their AFs content, according to the current legislation, and other *Fusarium* and *Alternaria* toxins. Out of the 54 samples subjected to analysis, 33 resulted to be positive for at least one mycotoxin (61%). The most frequent mycotoxin was β-ZEL (30%) followed by AOH (22%), whereas ENNs were also detected but with a lower incidence. Other ZEN derived were also detected in the samples, being responsible for most of the co-occurrence events (45% of positive samples). At a quantitative level, low concentrations were observed with most of the positive samples determined below 10 ng/g.

These results highlighted the extensive presence of mycotoxins in different food products even though they occurred at low concentration levels. Therefore, more evidence about the ubiquitous character of mycotoxins is provided, suggesting, once again, that the figure provided by FAO about contamination in the 25% of crops worldwide must be left apart and updated.

Of special concern is the fact that many mycotoxins that are not overseen in specific food matrices can frequently occur. In this line, ready-to-eat tree nuts products are often monitored to ensure that AFs are under legal requirements, but an extensive presence of other mycotoxins such as AOH or ZEN and derived forms has been reported. Similarly, PAT is the only toxin when evaluating pear (or fruit) juices, whereas a high prevalence of other toxins such as ZEN, T-2 and HT-2 has been here reported. This has crucial implications when it comes to exposure

assessments since food products could be contaminated with unexpected mycotoxins, so their consumption could be contributing to the exposure to those unexpected mycotoxins without even knowing.

4.2.2. Mycotoxins occurring in biological matrices

The biomonitoring of mycotoxins in urine (*n* = 300) and hair (*n* = 100) samples was conducted using two different approaches: quantification of targeted mycotoxins/metabolites and evaluation of the tentative presence of non-targeted mycotoxins/metabolites.

4.2.2.1. Targeted quantification

A target-driven strategy was selected for the quantification of CIT + DH-CIT and T-2 + HT-2 in urine samples. On the other hand, AFB1, AFB2, AFG1, AFG2, T-2, ENNA, ENNA1, ENNB, ENNB1 and BEA were assessed in hair samples.

A considerable amount of human urine samples tested positive for CIT (47%) whereas its metabolite DH-CIT was less frequent (21%), at a maximum concentration of 4.0 and 2.5 ng/mg Crea, respectively, but both at similar average values around 0.3 ng/mg Crea. At a quantitatively level, the lowest aged group of volunteers presented higher ratios DH-CIT:CIT compared to older volunteers, whereas gender did not seem to be a significant factor in the excretion pattern.

Regarding T-2 and HT-2, their prevalence in urine samples was of 21% and 30%, respectively, whereas maximum concentrations were detected at 6.54 ng/mg Crea for T-2 and 2.75 ng/mg Crea for HT-2. Nonetheless, concentrations

averaged similar values near 1.3 ng/mg Crea. No differences in the excretion patterns across gender or age were observed.

Analysis of hair samples was conducted throughout a multi-mycotoxin methodology. At least one mycotoxin was detected in 43% of samples at concentration values ranging from 2.7 to 106.1 ng/g. Although ENNs and AFB1 were the most prevalent compounds, all mycotoxins included within the validated methodology were detected in at least one sample. Interestingly, AFs were more prone to accumulate into female hair than male hair samples, but no significant differences across gender were observed at a quantitative level.

Several authors have performed biomonitoring studies including the mentioned mycotoxins but only results extracted from studies with low LOQs (< 1 ng/mL) and a high sampling (n > 100) are comparable. Therefore, the application of MS methodologies in HBM of mycotoxins should comply the requisite of providing high sensitivity. Additionally, a recent publication addressing reference values for the biomonitoring of pollutants in human samples stated the necessity of a sample size of, at least, between 72-120 in order to obtain statistically robust results (Vogel et al., 2019). Therefore, sensitivity and reproducibility size seemed to be critical factors for obtaining unbiased results.

In terms of age, little is known about its effect on excretion patterns of CIT. DH-CIT, T-2 and HT-2. The only HBM of CIT and DH-CIT that classified samples according to age also reported a higher proportion of DH-CIT related to CIT in lower-aged groups (Heyndrickx et al., 2015). On the contrary, T-2 showed a similar distribution across age groups. These results cannot be confronted yet since no

previous biomonitoring studies of T-2 in urine have reported their results according to age groups.

The here-obtained results also remarked that gender did not show to be a significant factor influencing the urinary excretion pattern of CIT and DH-CIT, as reviewed by Ali and Degen (2019), whereas similar outcomes have been observed in the excretion patterns of T-2 and HT-2 as reviewed by Wu et al. (2020). On the other hand, after analyzing hair samples, the prevalence of AFs was significantly higher in samples from female volunteers. Many authors have remarked the necessity of more evidence in order to clarify the effect of both gender and age in the biomonitoring context due to inconclusive data.

4.2.2.2. Non-targeted screening

Retrospective non-targeted screenings were conducted in order to detect the tentative presence of several T-2 metabolites, the masked form T-2-3-Glc and its corresponding metabolites in urine samples. The tentative accumulation of non-targeted mycotoxins in hair samples was assessed using a library containing spectral information.

Metabolites of T-2 and T-2-3-Glc have been previously characterized after *in vitro* studies and their spectral information was used for a suspect screening in urine samples. Results showed a predominance for phase I metabolism, being hydroxylation products 3'-OH-T-2 and T-2 triol, respectively found in 99.7% and 56% of samples, the most common metabolites. The low relevance of phase II

metabolites is line with previous findings that pointed to feces as their main excretion way.

The vast occurrence of metabolites in human urine samples indicated an extensive biotransformation of T-2. Although scarce information is available on *in vivo* metabolism, a similar pattern across species has been observed with a preferential metabolism through phase I reactions for T-2 and low-to-no relevance of conjugation reactions.

Non-targeted strategy revealed the tentative accumulation of up to 128 mycotoxins in human hair samples, going from toxicologically relevant compounds such as PAT (85%) or ZEN (10%). Many metabolic products of parent toxins were tentatively identified including FB3, FB4, T-2 metabolites or ENNB2, among others, whereas several *Alternaria* toxins were also detected. Therefore, hair also proved to be a suitable matrix for the biomonitoring of a wide spectrum of mycotoxins and metabolites.

4.3. Exposure assessment of mycotoxins

The exposure assessment of mycotoxins was performed through two different approaches: the combination of contamination and food consumption data or the direct measurement of mycotoxins and metabolites in biological samples. Then, TDI values proposed by the EFSA CONTAM Panel were used as reference for the characterization of the risk.

4.3.1. Approximation based on food contamination

Exposure assessment studies based on this approximation were conducted as long as consumption data were available and the reported mycotoxins had a TDI or a TTC value assigned by scientific committees. In this case, the National Food Consumption Survey INRAN-SCAI (Leclercq et al., 2009) was used as reference since the sampling of food products was carried out in Italy. Unfortunately, considering the novelty of CBD-based nutraceuticals, no data regarding its intake have been reported yet, so these analysis focused on pear juice and tree nuts consumption.

The combination of data allowed to estimate a daily intake of mycotoxins due to consumption of pear juice and tree nuts. The characterization of risk used these estimates, known as PDI, for later comparison with the TDI values. In case of obtaining a considerable rate of negative samples for mycotoxins with a TDI assigned, as in the case of tree nuts products, data treatment considered the left-censored data in a two-scenarios approach: the upper bound, where the negative-tested samples were given a contamination value equal to the LOQ; and the lower bound, where the negative samples were considered as 0. In addition, the calculations were conducted with mean and percentile 95 consumption data and organized in age groups.

The consumption of pear juices accounted for a maximum percentage of 14.65% of the TDI for ZEN + α-ZAL, 55.95% of the TDI set for T-2 + HT-2 and up to 15.90% for ENNA + ENNA1 + ENNB + ENNB1. Although ENNs have not been given a TDI value yet, the lowest TDI set for a *Fusarium* toxin was used (20 ng/kg

bw day corresponding to T-2 + HT-2). According to age groups, children are always the most exposed considering their lower body weight. On the other hand, the intake of mycotoxins throughout contaminated tree nuts accounted for 10-20% of the TDI for ZEN + α-ZEL + β-ZEL + α-ZAL + β-ZAL + ZAN, AOH and AME in the worst-case scenario for all age groups.

These results suggested that the exposure to mycotoxins due to consumption of contaminated juices and tree nuts might not represent a health concern by themselves. Nevertheless, these finding provided evidence about the concern of mycotoxins that are not usually studied in these products and that could exert a considerable contribution to the total exposure, with the potential risk of surpassing the TDI values if considering the whole diet.

4.3.2. Human biomonitoring

Exposure assessment studies based on the direct measurement of mycotoxins and/or their metabolites in biological samples required a deep knowledge regarding the toxicokinetics of each mycotoxin in order to properly correlate the concentrations found in samples with the ingested quantity. Nevertheless, out of all the mycotoxin analyzed in the present thesis, only CIT + DH-CIT have been conveniently validated as biomarkers of exposure, with the quantity measured in urine accounting for 40.2% of the ingested amount as elucidated by Degen et al. (2018).

Referring to CIT + DH-CIT, the resulting Italian average exposures represents a range between 8 and 40%, being children (< 18 years old) the most

exposed population group and four individuals surpassed the limit for nephrotoxicity suggested by the EFSA. These results revealed non-negligible exposure levels to CIT within the Italian population and comparable to previous European studies.

The presence of T-2 and/or their metabolites in urine samples could not be correlated to dietary intake since little is known about toxicokinetics of T-2 in human. Attending to the large number of samples testing positive for T-2 or its metabolites, frequent exposure to T-2 was suggested, although there is a considerable variability in the available HMB studies that have used urine samples.

Thus, more sensitive analytical techniques should be validated for their application in biological matrices in order to clarify the impact of CIT and T-2 in humans. In addition, considering the frequent exposure to several metabolites that can also occur in foodstuffs, such as T-2 triol, analytical methodologies in food analyses should incorporate them. This could help to elucidate whether the presence of these metabolites is due to dietary exposure or to human metabolism.

The use of hair as matrix for monitoring the accumulation of mycotoxins is still a novel approach so no information about how mycotoxins incorporate into the matrix or their kinetics are available. Despite that, the results confirmed the accumulation of 12 targeted and 128 untargeted mycotoxins in hair after 5 months of cumulative exposure, according to the length of the sample. This novel approach could complement HBM in other matrices due to the possibility to evaluate long-term exposure.

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5. CONCLUSIONS

1. Several QuEChERS-based methodologies have been developed and validated for the simultaneous determination of aflatoxins B1, B2, G1 and G2, T-2 toxin, HT-2 toxin, neosolaniol, enniatins A, A1, B, B1 beauvericin, zearalenone, alpha-zearalenol, beta-zearalenol, alpha-zearalanol, beta-zearalanol, zearalenone, alternariol and alternariol monomethyl ether in food matrices.

2. Several SALLE-based methodologies have been developed and validated for the detection of citrinin, dihydrocitrinone, T-2 toxin and HT-2 toxin in human urine and aflatoxins B1, B2, G1 and G2, enniatins A, A1, B and B1, T-2 toxin and beauvericin in human hair.

3. The 70% of food samples analyzed were positive for at least one mycotoxin at concentration levels < 50 ppb. Co-occurrence of mycotoxins was detected in 49% food samples. The most common co-occurrence events included zearalenone alongside its derived forms and combinations of enniatins.

4. Exposure assessment based on consumption and contamination data highlighted that daily intake of alternariol, alternariol monomethyl ether and zearalenone and its derived forms might not represent a health concern by themselves, but their contribution to the total exposure should be taken into consideration specially with susceptible cohorts like children.

5. Analysis of human urine revealed the presence of either citrinin or its metabolite dihydrocitrinone in 142 out of 300 samples (47%), whereas T-2 and HT-2 were detected in 21 and 30% of samples, respectively.

6. Exposure assessment based on the measurement of urinary citrinin biomarker revealed an exposure accounting for 8 to 40% of the tolerable daily intake, being children (< 18 years old) the most exposed population group.

7. Retrospective suspect analysis of urine samples revealed a high prevalence of the T-2 hydroxylated metabolites, especially 3-hydroxy-T-2 toxin, occurring in more than 99% of samples studied. This might represent a health concern considering that it does not exert significantly lower toxicity when compared to its parent toxin.

8. Hair samples showed frequent contamination with a 43% of positive samples for at least one mycotoxin, being enniatins and aflatoxin B1 the most prevalent compounds. Concentration values ranged from 2.7 to 106 ng/g and corresponded to a cumulative exposure over five months according to the length of the samples.

9. Retrospective non-targeted screening in hair samples revealed the presence of 128 mycotoxins, including some relevant compounds such as patulin (85%). Hair could stand as a novel matrix for the biomonitoring of mycotoxins in humans.

ANNEX II

Dissemination of research findings

toxins

MDPI

Article

Ultra-High-Performance Liquid Chromatography Coupled with Quadrupole Orbitrap High-Resolution Mass Spectrometry for Multi-Residue Analysis of Mycotoxins and Pesticides in Botanical Nutraceuticals

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Abstract: Cannabidiol (CBD) food supplements made of *Cannabis sativa* L. extracts have quickly become popular products due to their health-promoting effects. However, potential contaminants, such as mycotoxins and pesticides, can be coextracted during the manufacturing process and placed into the final product. Accordingly, a novel methodology using ultra-high-performance liquid chromatography coupled with quadrupole Orbitrap high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS) was developed to quantify 16 mycotoxins produced by major *C. sativa* fungi, followed by a post-target screening of 283 pesticides based on a comprehensive spectral library. The validated procedure was applied to ten CBD-based products. Up to six different *Fusarium* mycotoxins were found in seven samples, the most prevalent being zearalenone (60%) and enniatin B1 (30%), both found at a maximum level of 11.6 ng/g. Co-occurrence was observed in four samples, including one with enniatin B1, enniatin A and enniatin A1. On the other hand, 46 different pesticides were detected after retrospective analysis. Ethoxyquin (50%), piperonyl butoxide (40%), simazine (30%) and cyanazine (30%) were the major residues found. These results highlight the necessity of monitoring contaminants in food supplements in order to ensure a safe consumption, even more considering the increase trend in their use. Furthermore, the developed procedure is proposed as a powerful analytical tool to evaluate the potential mycotoxin profile of these particular products.

Keywords: mycotoxins; pesticides; Q-Exactive Orbitrap; CBD capsule; nutraceutical

Key Contribution: The first multi-class analysis of CBD-based supplements regarding mycotoxins and pesticide residues using high-resolution mass spectrometry techniques.

1. Introduction

Nutrition is known to be an essential component of the health state, so having an unbalanced diet can lead to several disorders and diseases [1]. Due to current lifestyles, new and fast ways to maintain proper dietary habits are required. Nutraceuticals have emerged as an alternative to increase the input of nutrients, contributing to an improvement in health. These products are bioactive compounds

foods

MDPI

Article

Target Quantification and Semi-Target Screening of Undesirable Substances in Pear Juices Using Ultra-High-Performance Liquid Chromatography-Quadrupole Orbitrap Mass Spectrometry

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Abstract: Fruit juices are common products in modern diets due to the supply of vegetal nutrients combined with its tastiness. Nevertheless, potential contaminants, such as mycotoxins and pesticides, can be present in commercial products due to a potential carry-over. Therefore, the aim of this study was to investigate for the first time the presence of 14 *Fusarium* mycotoxins using a quick, easy, cheap, effective, rugged, and safe (QuEChERS)-based extraction followed by an ultra-high-performance liquid chromatography-quadrupole Orbitrap high-resolution mass spectrometry in 21 pear juice samples from Italian markets. Up to nine different mycotoxins were detected, particularly an extensive presence of zearalenone (67%, $n = 21$, mean value = 0.88 ng/mL). Emerging *Fusarium* mycotoxins enniatins B, B1, A, and A1 were also detected. Additionally, 77 pesticide residues were tentatively identified through a retrospective analysis based on a mass spectral library. The prevalent presence of some non-approved pesticides, such as ethoxyquin (64%, $n = 21$) and triazophos (55%, $n = 21$), must be highlighted. The results obtained indicate an extensive contamination of marketed pear juice with undesirable compounds, and they should be taken into consideration when performing risk assessment studies.

Keywords: pear juice; mycotoxins; pesticides; *Fusarium*; Q-Exactive Orbitrap

1. Introduction

During the last years, diets have gravitated to higher intakes of fruits and vegetables, mainly due to its beneficial effects on health status and its protective role against chronic diseases [1]. In this line, fruit juices have become an appealing alternative, recommended as a good vitamin C source for children, and have been introduced as part of breakfast in conventional diets [2]. According to the European Fruit Juice Association (AIJN), juice consumption was 9.2 billion liters in 2017, with pear juice being one of the most consumed flavors in several countries, such as Italy [3]. The frequent intake of pear juice demands strict quality controls, especially when children become an important target group, in order to ensure safe consumption.

metabolites

MDPI

Article

Occurrence and Exposure Assessment of Mycotoxins in Ready-to-Eat Tree Nut Products through Ultra-High Performance Liquid Chromatography Coupled with High Resolution Q-Orbitrap Mass Spectrometry

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Abstract: Tree nuts have become popular snacks due to their attributed benefits in the health state. Nevertheless, their susceptibility to fungal contamination lead to the occurrence of potentially dangerous mycotoxins. Hence, the aim of this work was to evaluate the presence of mycotoxins in ready-to-eat almonds, walnuts, and pistachios from Italian markets. The most relevant mycotoxin found in almonds was α-zearalanol in 18% of samples (n = 17) ranging from 3.70 to 4.54 µg/kg. Walnut samples showed frequent contamination with alternariol, present in 53% of samples (n = 22) at levels from 0.29 to 1.65 µg/kg. Pistachios (n = 15) were the most contaminated commodity, with β-zearalenol as the most prevalent toxin present in 59% of samples ranging from 0.96 to 8.60 µg/kg. In the worst-case scenario, the exposure to zearalenone-derived forms accounted for 15.6% of the tolerable daily intake, whereas it meant 12.4% and 21.2% of the threshold of toxicological concern for alternariol and alternariol monomethyl-ether, respectively. The results highlighted the extensive presence of *Alternaria* toxins and zearalenone-derived forms, scarcely studied in ready-to-eat tree nut products, highlighting the necessity to include these mycotoxins in analytical methods to perform more realistic risk assessments.

Keywords: almonds; pistachios; walnuts; mycotoxins; Q-Exactive Orbitrap; risk characterization

1. Introduction

Tree nuts have become a popular alternative to unhealthy snacks due to their attributed benefits. The intake of tree nuts has been related to a lower risk of suffering from cardiovascular diseases through several mechanisms, and they can also act as antioxidant suppliers [1–4]. According to the International Nut and Dried Fruit Council (INC), the annual production of tree nut products has increased over the last ten years, especially forn almonds, walnuts and pistachios, reaching a maximum of 4.6 million metric tons in 2019 and highlighting a global trend in tree nut consumption [5].

Nevertheless, tree nuts are susceptible to fungal growth that can occur for several reasons related to environmental factors, such as moisture and temperature. In addition, improper post-harvest practices and storage conditions can also promote fungal contamination [6]. As a consequence of these mentioned factors, mycotoxins could also be expected in crops. These are secondary metabolites produced by several filamentous fungi, mainly *Alternaria*, *Aspergillus*, *Claviceps*, *Fusarium*, and *Penicillium* spp.,

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Article

Citrinin Dietary Exposure Assessment Approach through Human Biomonitoring High-Resolution Mass Spectrometry-Based Data

Alfonso Narváez,[‖] Luana Izzo,[‖] Yelko Rodríguez-Carrasco,[*] and Alberto Ritieni

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ABSTRACT: Citrinin (CIT) is a scarcely studied mycotoxin within foodstuffs, so the biomonitoring of this toxin and its metabolite dihydrocitrinone (DH-CIT) in biological samples represents the main alternative to estimate the exposure. Hence, this study aimed to evaluate the presence of CIT and DH-CIT in 300 urine samples from Italian individuals in order to assess the exposure. Quantification was performed through an ultrahigh-performance liquid chromatography high-resolution mass spectrometry (UHPLC-Q-Orbitrap HRMS)-based methodology. CIT was quantified in 47% of samples (n = 300) up to 4.0 ng/mg Crea (mean = 0.29 ng/mg Crea), whereas DH-CIT was quantified in 21% of samples up to 2.5 ng/mg Crea (mean = 0.39 ng/mg Crea). Considering different age groups, average exposure ranged from 8% to 40% of the provisional tolerable daily intake, whereas four individuals surpassed the limits suggested by the European Food Safety Authority. These results revealed non-negligible exposure levels to CIT, encouraging further investigation in foodstuffs monitoring studies.

KEYWORDS: *Orbitrap, biomarkers, exposure, citrinin, biomonitoring, urine*

INTRODUCTION

Mycotoxins are secondary metabolites produced by several fungi genera, primarily *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps*. These compounds can be found in cereal grains, food commodities, and animal feed under propitious environmental conditions or because of bad practices at any point from the preharvest interval to the storage.[1,2] Once ingested, mycotoxins can display a wide variety of adverse effects including immunosuppression, neurotoxicity, or carcinogenicity.[3,4] In consequence, regulatory authorities set maximum limits (MLs) in certain foodstuffs for several hazardous mycotoxins, in light of tolerable daily intake (TDIs) derived by Scientific Committees, e.g., the European Food Safety Authority or the Joint FAO/WHO Expert Committee on Food Additives.[5] Over the last years, citrinin (CIT) has become a relevant compound due to its occurrence in grains and grain products and its toxicity,[6,7] but the EFSA noted that occurrence data are insufficient to conduct dietary exposure assessments for humans.[8]

CIT is produced by several *Aspergillus*, *Penicillium*, and *Monascus* species, and it can be found in stored grain and other plant products like fruits, herbs, and spices, showing a wide distribution throughout different geographical areas around the world and occurring at concentration ranges from a few ng/g up to 1500 ng/g depending on the commodity.[7,9−13] This toxin has also been identified co-occurring with other toxins produced by these fungi, especially ochratoxin A (OTA).[7,14] Nonetheless, only the maximum level for citrinin in food supplements based on rice fermented with red yeast *Monascus purpureus* has been set to date.[15] CIT is a quinone with a planar and conjugated structure that targets primarily the kidney, resulting in necrosis of renal tubules.[8,16] Although the mechanism responsible for its toxicity is not fully understood, it could be related to the production of reactive oxygen species (ROS) linked to apoptotic processes.[17,18] Moreover, CIT has genotoxic properties and can induce micronuclei (mainly aneugenic) and chromosomal aberration in several animal and human cell lines.[8,19,20] The EFSA Contam Panel concluded that the combined effect of OTA and CIT is mainly additive.[8] In combination with OTA, a synergistic effect has been reported after *in vitro* assays, displaying a higher nephrotoxic[21] and genotoxicity[22] potential. Nevertheless, the limited toxicological data available is insufficient to evaluate its carcinogenicity potential, so CIT has been placed into group 3 within the classification released by the International Agency for Research on Cancer (IARC).[23]

Referring to the metabolism of CIT, data are scarce on the sites of its bioconversion and the enzymes involved. The main product of CIT metabolism is dihydrocitrinone (DH-CIT), first detected in rat urine by Dunn et al.[24] This compound showed a lower cytotoxic and genotoxic potential, so the conversion of CIT to DH-CIT could be considered as a detoxification process.[22] As regards the bioavailability, little is known in humans. The only toxicokinetic study carried out in humans determined a half-life of 6.7 and 8.9 h for CIT and DH-CIT, respectively, and a rapid absorption of CIT with at least a 40% of the initial dose being excreted in urine.[25] After

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toxins

MDPI

Article

Human Biomonitoring of T-2 Toxin, T-2 Toxin-3-Glucoside and Their Metabolites in Urine through High-Resolution Mass Spectrometry

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Abstract: The metabolic profile of T-2 toxin (T-2) and its modified form T-2-3-glucoside (T-2-3-Glc) remain unexplored in human samples. Therefore, the present study aimed to investigate the presence of T-2, T-2-3-Glc and their respective major metabolites in human urine samples ($n = 300$) collected in South Italy through an ultra-high performance liquid chromatography (UHPLC) coupled to Q-Orbitrap-HRMS methodology. T-2 was quantified in 21% of samples at a mean concentration of 1.34 ng/mg Crea (range: 0.22–6.54 ng/mg Crea). Almost all the major T-2 metabolites previously characterized in vitro were tentatively found, remarking the occurrence of 3′-OH-T-2 (99.7%), T-2 triol (56%) and HT-2 (30%). Regarding T-2-3-Glc, a low prevalence of the parent mycotoxin (1%) and its metabolites were observed, with HT-2-3-Glc (17%) being the most prevalent compound, although hydroxylated products were also detected. Attending to the large number of testing positive for T-2 or its metabolites, this study found a frequent exposure in Italian population.

Keywords: human biomonitoring; biomarkers; metabolites; high-resolution mass spectrometry; urine; T-2 toxin; exposure

Key Contribution: The metabolic profiles of mycotoxins T-2 and T-2-3-glucoside were assayed in human urine samples for the first time.

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1. Introduction

Mycotoxins are toxic metabolites resultant from the secondary metabolism of several species belonging to the *Fusarium*, *Aspergillus*, *Penicillium*, *Claviceps* and *Alternaria* genera. Under certain conditions, these fungi can colonize a broad variety of crops, eventually leading to the accumulation of mycotoxins. Among the mentioned fungal genera, the *Fusarium* species represents the major mycotoxin-producing pathogens of warm areas from America, Europe and Asia, especially affecting cereal grains and their derived products [1]. Throughout the consumption of contaminated materials, mycotoxins can cause severe adverse health effects in humans, such as immunotoxic, neurotoxic or even carcinogenic effects [2]. Therefore, regulatory authorities have established maximum limits (MLs) in susceptible foods and foodstuffs alongside tolerable daily intake (TDI) values for certain mycotoxins, setting a maximum level of dietary exposure to avoid the appearance of toxic effects.

The traditional methods for estimating dietary exposure are mainly based on the combination of consumption surveys and occurrence data throughout total diet studies or meta-analysis approaches [3,4]. Nevertheless, the outcomes can be considerably biased due

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Novel quadrupole-time of flight-based methodology for determination of multiple mycotoxins in human hair

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ABSTRACT

The potential of hair as matrix for assessing long-term exposure to mycotoxins remains scarcely explored. Therefore, this study aimed to develop and validate an analytical methodology for the simultaneous determination of aflatoxins, enniatins, beauvericin and T-2 toxin in human hair, based on a pretreatment stage prior to salt-assisted liquid–liquid extraction and followed by high performance liquid chromatography coupled to high resolution Q-TOF mass spectrometry for the first time. Washing with a non-ionic detergent was successfully applied, whereas enzymatic digestion with Pronase E was mandatory for releasing mycotoxins from the hair matrix. The methodology was validated according to Commission Decision 2002/657/EC, with limits of quantification ranging from 0.6 to 8.7 ng/g. The analysis of 10 samples showed at least one mycotoxin occurring in 67% of samples, including the carcinogenic aflatoxins. This is the first validated methodology for the quantification of multiple mycotoxins in human hair.

1. Introduction

Mycotoxins are toxic metabolites resulting from secondary metabolism of several fungi genera, mainly *Aspergillus*, *Fusarium*, *Penicillium*, *Alternaria* and *Claviceps*, that are estimated to occur in 50–90% of foods worldwide, depending on the food group, although at very low levels. Ingestion of contaminated foodstuffs represents a health concern due to the reported toxic effects derived from chronic exposure to mycotoxins, including immunosuppression, carcinogenicity, nephro- or hepatotoxicity [1]. Therefore, scientific committees have proposed tolerable daily intake (TDI) values for some mycotoxins in order to prevent from those adverse effects, so there is a crucial necessity for controlling exposure to mycotoxins within the provided framework. Currently, the most accurate approach for assessing dietary exposure to mycotoxins is human biomonitoring (HBM) through the direct measurement of either parent toxins or their metabolites in biological matrices such as urine, blood, plasma or serum [2]. However, these traditional matrices fail to provide information about exposure over long periods of time due to their rapid excretion rates and short half-lives [3].

In this context, human hair emerges as a novel matrix able to provide long-term information about exposure to mycotoxins and whose use is widely extended in exposure assessments of other dietary contaminants and drugs. Although the mechanisms remain unclear, these molecules seem to incorporate into the hair matrix throughout diffusion from either bloodstream to hair follicle or biological secretions, such as sweat, to developing or developed hair [4]. Hair provides a highly stable chemical environment based on the interactions that melanin and the keratin matrix establish with the incorporated contaminants, that could presumably include covalent bonds, among other [5]. Therefore, a wider surveillance window can be provided since contaminants remain stable when bounded to the hair matrix. In fact, considering a standard growth ratio of 1 cm/month, exposure can be estimated within a desired time frame depending on the length of the sample [6].

Accumulation of mycotoxins in human hair has been previously reported [7–9], but only Bordin et al. [8] provided a validated methodology following Commission Decision 2002/657/E [10] for the quantification of fumonisin B1 (FB1) in human hair samples. Although these previous methodologies have proven the feasibility of hair analysis in the mycotoxins field, the current HBM context is evolving towards more flexible methodologies able to extract and quantify multiple mycotoxins occurring in biological samples.

Extraction of multiple mycotoxins from biological samples requires from versatile procedures mainly based on liquid–liquid extraction (LLE) or solid-phase extraction (SPE) that usually incorporate a clean-up

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Human biomonitoring of multiple mycotoxins in hair: first large-scale pilot study

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Abstract. Human biomonitoring (HBM) represents the most accurate approach for assessing the exposure to mycotoxins, but traditional matrices fail to provide information about long-term exposure due to the rapid excretion rates and short half-lives of mycotoxins. Hair emerges as a promising matrix considering that contaminants can form stable links with hair components, such as keratins and melanin. Hence, the aim of the present study was to monitor the presence of up to ten mycotoxins (aflatoxins and *Fusarium* mycotoxins) in human hair samples (n = 100) through a high performance liquid chromatography coupled to Q-TOF high resolution mass spectrometry. A prevalence of 43% at concentrations ranging from 2.7 to 106.1 ng/g was observed, being enniatins and aflatoxin B1 the most prevalent compounds. Co-occurrence of up to three mycotoxins was observed in 42% of the positive samples. Retrospective untargeted analysis of hair samples tentatively identified up to 128 mycotoxins and related metabolites. These results confirm the accumulation of toxicologically relevant mycotoxins in hair matrix, thus standing as a suitable matrix for assessing long-term exposure.