Univerzita Hradec Králové
Přírodovědecká fakulta
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Studium významných mykotoxinů v biologickém materiálu a jejich možné dopady na zdraví člověka

Disertační práce

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Prohlášení

Prohlašuji, že jsem disertační práci vypracovala samostatně a že jsem v seznamu použité literatury uvedla všechny prameny, ze kterých jsem vycházela.

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Anotace

PICKOVÁ, D. *Studium významných mykotoxinů v biologickém materiálu a jejich možné dopady na zdraví člověka*. Hradec Králové, 2023. Disertační práce na Přírodovědecké fakultě Univerzity Hradec Králové. Vedoucí disertační práce doc. RNDr. František Malíř, Ph.D. 65 s.

Disertační práce je koncipována jako komentovaný soubor 8 publikací se zaměřením na významné mykotoxiny a jejich výskyt v biologickém materiálu. Práce je tak členěna tradičním způsobem na teoretickou část a komentovaný přehled publikovaných prací.

Teoretická část pojednává o problematice zemědělsky významných mykotoxinů, především o ochratoxinu A, druhém nejvýznamnějším mykotoxinu z pohledu toxicity a prevalence výskytu v potravinách, který je hlavním předmětem této práce.

Komentovaný přehled publikovaných prací sestává z komentářů doprovázejících dílčí vydané publikace zaměřené především na monitoring mykotoxinů, zejména ochratoxinu A, v biologickém materiálu, kterým se obecně rozumí jakýkoliv materiál rostlinného a živočišného původu produkovaný či odvozený od žijících organismů. Řešeny jsou jak potraviny rostlinného i živočišného původu, které z hlediska výskytu mykotoxinů dosud nejsou nebo toho času nebyly regulovány, tak i biologický materiál z člověka.

Klíčová slova

mykotoxiny, ochratoxin A, nefrotoxicita, biologický materiál, HPLC, imunoafinitní chromatografie

Annotation

PICKOVA, D. *Study of important mycotoxins in biological material and their possible effects on human health.* Hradec Kralove, 2023. Dissertation at Faculty of Science, University of Hradec Kralove. Supervisor Assoc. Prof. Frantisek Malir. 65 p.

The dissertation is designed as an annotated collection of 8 publications dealing with important mycotoxins and their occurrence in biological material. Therefore, the dissertation is divided in a traditional way into a theoretical part and an annotated overview of the published papers.

The theoretical part deals with the topic of agriculturally important mycotoxins, especially ochratoxin A, the second most important mycotoxin in terms of toxicity and frequency of occurrence in food, which is also the main topic of this dissertation.

The annotated overview of the published papers consists of comments on published papers mainly dealing with the monitoring of mycotoxins, especially ochratoxin A, in biological material. Biological material is generally defined as any material produced or derived from living organisms, including raw food materials and products. It refers to food of animal and plant origin that has not yet been regulated for mycotoxins or was not regulated at that time, as well as biological material of human origin.

Keywords

mycotoxins, ochratoxin A, nephrotoxicity, biological material, HPLC, immunoaffinity chromatography

Obsah

Seznam použitých zkratek

AFs	Aflatoxiny
ALT	Altenuen
AME	Alternariol monomethyl ether
AOH	Alternariol
$BMDL_{10}$	Spodní limitní hodnota spolehlivosti referenční dávky 10 % dodatečného rizika (Benchmark Dose Lower Confidence Limit for an Extra Risk of 10%)
CIT	Citrinin
DG SANTE	Generální ředitelství pro zdraví a bezpečnost potravin (Directorate General for Health and Food Safety)
DON	Deoxynivalenol
EFSA	Evropský úřad pro bezpečnost potravin (European Food Safety Authority)
EK	Evropská komise
ELISA	Enzymová imunoanalýza (Enzyme-Linked ImmunoSorbent Assay)
EU	Evropská unie
FUMs	Fumonisiny
HPLC-FLD	Vysokoúčinná kapalinová chromatografie s fluorescenčním detektorem (High-Performance Liquid Chromatography with Fluorescence Detector)
HT-2	HT-2 toxin
IAK	Imunoafinitní kolonky
IARC	Mezinárodní agentura pro výzkum rakoviny (International Agency for Research on Cancer)
LC	Kapalinová chromatografie (Liquid Chromatography)
MOE	Hraniční expozice (Margin of Exposure)
MPL	Maximální limit
OATs	Transportéry organických aniontů (Organic Anion Tranporters)
OTA	Ochratoxin A
RASFF	Systém rychlého varování pro potraviny a krmiva (Rapid Alert System for Food and Feed)
T-2	T-2 toxin
VMH	Vláknité mikroskopické houby
ZEN	Zearalenon

1 Úvod

Termín mykotoxiny pochází z řeckého výrazu „*mykes*“ znamenajícího houba a latinského výrazu „*toxicum*“ znamenajícího jed nebo také otrava [1]. Nejedná se však o synonymní název s „houbovými jedy“, toxickými metabolity hub s makrostélkou „makromycetů“. Termínem mykotoxiny jsou zpravidla označovány toxické sekundární metabolity vláknitých hub s mikrostélkou „mikromycetů“, nevědecky označovaných jako „plísně“. Vláknité mikroskopické houby (VMH) jsou schopny kontaminovat téměř jakýkoli substrát včetně potravin a krmiv. Jejich působením dochází ke snížení nutriční hodnoty substrátu, avšak nejzávažnější je právě jejich produkce mykotoxinů, které představují jedny z nejzávažnějších kontaminantů přírodního původu [2]. Mykotoxinům je třeba věnovat pozornost, a to nejen kvůli možným ekonomickým ztrátám, ke kterým dochází v důsledku kontaminace potravin a krmiv, ale především kvůli zdravotnímu riziku plynoucímu z jejich přívodu do organismu, který je u většiny populace zprostředkován zejména skrze dietární expozici [3].

Dietární expozici danému mykotoxinu lze hodnotit dvěma způsoby: 1) odhadem denního přívodu mykotoxinu z potravin, a to na základě znalosti: a) celkové spotřeby potravin konzumentem; b) známé koncentrace mykotoxinu v daných potravinách; a 2) stanovením biomarkeru mykotoxinu v lidském organismu: a) v tělních tekutinách (např. krev, sérum, plazma, moč); b) „*post mortem*“ ve tkáních (např. ledviny), případně ve tkáních odebraných při operaci [4].

Disertační práce je primárně zaměřena na druhý nejvýznamnější mykotoxin, ochratoxin A (OTA). Přirozený výskyt tohoto mykotoxinu je řešen ve vybraných potravinách, tj. v jednodruhových kořeních, bylinách a vepřových jelitech. Uvedené potraviny byly zvoleny s ohledem na jejich nedostatečné či toho času zcela chybějící regulace udávající maximální přípustný limit (MPL) pro OTA. Souběžně je v menší míře řešen přirozený výskyt dalších zemědělsky významných mykotoxinů ve vybraných komoditách. Vzhledem k nefrotoxickým účinkům byl OTA kromě potravin stanovován také v tělních tekutinách pacientů s nádorem ledvin, který je v České republice diagnostikován jako páté nejčastější nádorové onemocnění s celosvětově největší četností 30,9 incidencí na 100 000 obyvatel (k r. 2020) [5].

2 Cíle práce

Disertační práce „*Studium významných mykotoxinů v biologickém materiálu a jejich možné dopady na zdraví člověka*“ je koncipována jako soubor komentářů k publikovaným pracím, které se zabývají významnými mykotoxiny a jejich přirozeným výskytem v biologickém materiálu. Biologickým materiálem se obecně rozumí jakýkoliv materiál produkovaný či odvozený od žijících organismů [6], přičemž v této práci se pod takovým materiálem rozumí vedle materiálu lidského původu (lidské tkáně a tělní tekutiny) také materiál původu rostlinného (rostlinná pletiva, potravinové suroviny a potraviny) a živočišného (živočišné tkáně a odvozené produkty). Komentovaný přehled publikovaných prací je rozdělen dle charakteru publikací do dvou hlavních částí.

První část uvádí literární rešeršní studie zaměřené na významné mykotoxiny přirozeně se vyskytující v jednodruhových kořeních a doplňcích stravy na bázi ostropestřce mariánského. K příležitosti 60. výročí od objevení aflatoxinů (AFs) byly vydány dvě rešeršní publikace zaměřené na tuto nejvýznamnější skupinu karcinogenních mykotoxinů.

Druhá část uvádí vlastní výzkumné studie zaměřené na stanovení OTA v biologickém materiálu s využitím vhodné chromatografické analytické techniky. Výzkumné studie jsou dle charakteru biologického materiálu dále členěny na vyhledávání „nových“ dietárně expozičních zdrojů OTA v biologickém materiálu (a) rostlinného původu (jednodruhová koření a byliny) a (b) živočišného původu (vepřová jelita) a jeho stanovení v biologickém materiálu (c) lidského původu (moč pacientů s nádorem ledvin).

Jednotlivé publikace vycházejí z dílčích specifických výzkumů (č. 2112/2019, 2115/2020, 2010/2021 a 2106/2022) podpořených Přírodovědeckou fakultou Univerzity Hradec Králové.

3 Teoretická část

3.1 Zemědělsky významné mykotoxiny

Mykotoxiny jsou tzv. přírodní toxiny, což jsou chemické látky biologického původu produkované různými organismy či mikroorganismy. Producentem mykotoxinů jsou VMH, které je syntetizují jako své sekundární metabolity [2]. Jedná se zejména o zástupce z rodů *Aspergillus*, *Penicillium, Fusarium*, *Alternaria, Claviceps* a *Stachybotrys* [7–11], kteří se mohou rozvinout za vhodných teplotních a vlhkostních podmínek na zemědělských komoditách, a to již na poli, během sklizně, či v posklizňovém období při transportu a skladování [1].

Dosud bylo identifikováno více než 500 mykotoxinů, přičemž jejich počet i nadále narůstá, ale pouze některé z nich mají význam v potravním řetězci a představují zdravotní riziko pro člověka [12]. Mykotoxiny mohou u člověka i zvířat vyvolat intoxikaci, tzv. mykotoxikózu, s akutním (po jednorázovém přívodu velké dávky) či chronickým (po dlouhodobém přívodu nízkých dávek) průběhem [13].

Mykotoxiny mohou být členěny dle schopnosti destruktivního cíleného působení na buňky, orgány či soustavy např. na hepatotoxiny (cílovým orgánem jsou játra), nefrotoxiny (ledviny), kardiotoxiny (srdce), pulmotoxiny (plíce), neurotoxiny (nervová soustava), imunotoxiny (imunitní systém), gastroenterotoxiny (gastrointestinální trakt) či cytotoxiny (buňky). Pozdní účinky vznikající v důsledku chronické intoxikace jsou považovány za nejzávažnější. Jsou to zejména účinky karcinogenní, vyvolávající rakovinné bujení, mutagenní – zvyšující pravděpodobnost mutace, genotoxické – poškozující genetickou informaci, teratogenní – poškozující vývoj plodu, či imunosupresivní, které tlumí imunitní systém [2, 13].

Za jedny z nejdůležitějších mykotoxinů z pohledu toxicity a prevalence výskytu v zemědělských potravinách jsou považovány AFs – aflatoxiny B_1 , B_2, G_1 a G_2, M_1 – metabolit aflatoxinu B_1 a M_2 – metabolit aflatoxinu B_2, OTA, trichotheceny – zejména deoxynivalenol (DON), nivalenol, T-2 toxin (T-2) a HT-2 toxin (HT-2), dále pak fumonisiny (FUMs) – zejména fumonisin B_1, B_2 a B_3, zearalenon (ZEN), citrinin (CIT), patulin (PAT), kyselina cyklopiazonová, ergotové (námelové) alkaloidy a alternáriové mykotoxiny – zejména alternariol (AOH), alternariol monomethyl ether (AME), altenuen (ALT) a kyselina tenuazonová [14] – viz Tabulka 1.

Tabulka 1 Přehled významných mykotoxinů v zemědělských surovinách a potravinách

MT[a]	IARC[b]	Toxické účinky	Významní producenti v potravinách	Výskyt v potravinách
AFs[c]	1 [15]	cytotoxické, genotoxické, hepatotoxické, imunosupresivní, mutagenní, nefrotoxické, teratogenní, karcinogenní [16]	*Aspergillus. flavus, A. parasiticus, A. nomius, A. pseudotamarii, A. aflatoxiformans, A. agricola, A. arachidicola, A. austwickii, A. cerealis, A. luteovirescens, A. minisclerotigens, A. mottae, A. parasiticus, A. parvisclerotigenes, A. pipericola, A. pseudocaelatus, A. pseudonomius, A. sergi, A. texensis, A. togoensis, A. toxicus, A. transmontanensis, A. olivicola* [17]	obilniny (pšenice, ječmen, kukuřice, čirok, proso, rýže) a produkty z obilnin (mouka), ořechy (lískové, para, mandle, pistácie, kaštany, pekanové, vlašské, kešu), luštěniny (arašídy, sójové boby), koření (zázvor, chilli, pískavice řecké seno, kurkuma, koriandr, paprika, římský kmín, černý pepř, muškátový ořech, fenykl), ovoce (kokosový ořech, sušené fíky), semínka (slunečnicová, bavlněná, sezamová, melounová, hořčičná, meruňková), mléko a mléčné produkty (sýr), vejce, kakaové boby, káva, čaj, pivo [17–20]
OTA	2B [21]	genotoxické, hepatotoxické, imunotoxické, nefrotoxické, neurotoxické, embryotoxické, teratogenní, karcinogenní, kontroverzní mutagenní účinky [22, 23]	*Aspergillus carbonarius, A. westerdijkiae, A. steynii, A. lacticoffeatus, A. niger, A. sclerotioniger, A. tubingensis, A. foetidus, Penicillium verrucosum, P. nordicum* [23–25]	obilniny (ječmen, žito, pšenice, oves, rýže) a produkty z obilnin, hrozny a produkty z hroznů (hroznový džus, víno, sušené hrozny), vepřové a kuřecí maso, masné výrobky (salám, sušená šunka, klobásy, vepřová jelita), vepřové a kuřecí droby (játra, ledviny), vepřová krev, koření (paprika, muškátový květ, kurkuma, zázvor, pískavice řecké seno, kardamom, chilli, černý pepř, kmín kořenný, lékořice, koriandr, fenykl, česnek, kajenský pepř, vanilka, pomerančová kůra, římský kmín, muškátový ořech, hořčice bílá, bílý pepř, hřebíček, citronová kůra, sumah, růžový pepř), ovoce (fíky, olivy), zelenina, luštěniny (fazole, sójové boby), ořechy (pistácie, kaštany, kešu), mléko a mléčné výrobky (sýr), dýňová semínka, kakaové boby, čokoláda, čaj, káva, pivo [11, 18, 22, 23, 26, 27]
DON, NIV	3 [21]	cytotoxické, genotoxické, hepatotoxické, imunotoxické, hematotoxické, neurotoxické, účinek na reprodukci, teratogenní, embryotoxické, účinky na gastrointestinální trakt – vomitus, nauzea, diarea [28–31]	*Fusarium graminearum, F. culmorum, F. poae, F. equiseti, F. crookwellense, F. venenatum* [9, 25]	obilniny (pšenice, žito, ječmen, oves, kukuřice, pohanka, rýže, čirok) a produkty z obilnin (chléb, popcorn, nudle) paprika (koření), doplňky na bázi ostropestřce mariánského, slad, pivo [18, 30, 32, 33]
T-2, HT-2	3 [21]	imunotoxické, dermatotoxické, emetické, hepatotoxické, genotoxické, hematotoxické [31, 34, 35]	*Fusarium sporotrichoides, F. langsethiae* [9, 25]	obilniny (ječmen, kukuřice, oves, žito, pšence), snídaňové cereálie, slunečnicová semínka, slunečnicový olej, koriandr, doplňky na bázi ostropestřce mariánského [18, 32, 33, 34]
ZEN	3 [21]	estrogenní a anabolické účinky, účinky na reprodukci, imunotoxické, hepatotoxické, hematotoxické, genotoxické, cytotoxické, fetotoxické [36, 37]	*Fusarium graminearum, F. culmorum,* [9, 25]	obilniny (kukuřice, pšenice, ječmen, žito, oves, rýže, čirok), produkty z obilovin (snídaňové cereálie, chléb, těstoviny, mouka), koření (paprika), sezamová semena, luštěniny (fazole, sójové boby), slad, pivo, mléko, doplňky na bázi ostropestřce mariánského [18, 32, 36]

MT[a]	IARC[b]	Toxické účinky	Významní producenti v potravinách	Výskyt v potravinách
FUMs	2B [21, 38]	cytotoxické, embryotoxické, hepatotoxické, imunotoxické, kardiotoxické, nefrotoxické, neurotoxické, pulmotoxické (plicní edém), teratogenní (defekt neurální trubice), karcinogenní (rakovina jícnu), účinek na gastrointestinální trakt, účinek na reprodukci [39]	*Fusarium. verticillioides, F. proliferatum, F. anthophilum, F. dlamini, F. napiforme, F. nygamai, F. thapsinum* [9, 25]	obilniny (kukuřice, rýže, oves, žito, pšenice, ječmen) a produkty z obilnin (kukuřičná mouka, popcorn, polenta, snídaňové cereálie), koření (paprika, lékořice), pivo [18, 39]
CIT	3 [40]	cytotoxické, hepatotoxické, nefrotoxické, hematotoxické (účinky na kostní dřeň), teratogenní, kontroverzní genotoxické a mutagenní účinky [41, 42]	*Penicillium citrinum, P. expansum, P. verrucosum, P. radicicola, Monascus ruber, M. purpureus* [9, 25, 41, 43]	obilniny (ječmen, žito, pšenice, kukuřice, rýže) a produkty z obilnin (mouka, snídaňové cereálie, těstoviny), ovoce (jablka, hrušky, třešně, černý rybíz, olivy, hrozny, citrusy, fíky), ovocné a zeleninové džusy, koření (chilli, zázvor, koriandr, pískavice řecké seno), fermentované produkty (červená fermentovaná rýže, fermentované klobásy, sufu), fazole, byliny, sýry, pivo [18, 41, 42, 44, 45]
PAT	3 [40]	hepatotoxické, neurotoxické, imunotoxické, genotoxické, mutagenní, teratogenní, účinky na gastrointestinální trakt, nefrotoxické, kardiotoxické, embryotoxické, cytotoxické [46, 47]	*Penicillium expansum, P. griseofulvum, Aspergillus clavatus, Byssochlamys nivea* [9, 25]	ovoce (jablka a jablečné produkty, meruňky, citrusy, třešně, hrozny, hrušky, broskve, ananas, jahody, olivy), ovocné džusy (jablečný, hruškový, liči, ananasový, broskvový, granátové jablko), zelenina (rajčata), obilniny [11, 46, 47]
CPA	N	účinky na gastrointestinální trakt, nefrotoxické, neurotoxické, hepatotoxické, kardiotoxické, cytotoxické, imunotoxické [48]	*Penicillium camemberti, P. commune, P. dipodomyicola, P. griseofulvum, Aspergillus flavus, A. oryzae, A. tamarii* [48]	obilniny (kukuřice), maso a masné produkty, luštěniny (arašídy), sušené fíky, ořechy, olejnatá semena, mléko, sýr [48, 49]
EA	N	neurotoxické (konvulze, halucinace), vazokonstrikce, gangrenózní ztráta končetin, agalaktie, pocit pálení, účinek na gastrointestinální trakt (nauzea, vomitus), endokrinní funkci a kardiovaskulární systém [50, 51]	*Sphacelia segetum* (teleomorfa*: Claviceps purpurea*), *C. fusiformis, C. paspali* [25, 51]	obilniny (žito, ječmen, oves, pšenice, proso) a produkty z obilnin (mouka, chléb, těstoviny, pizza, snídaňové cereálie, sladké pečivo) [50, 51]
Alternáriové mykotoxiny	N	hepatotoxické, dermatotoxické, imunotoxické, účinky na reprodukci, účinky na estrogenní aktivitu, kardiotoxické (tachykardie), cytotoxické, účinky na gastrointestinální trakt (hemoragie), teratogenní, fetotoxické, mutagenní, genotoxické [10, 32, 52, 53]	*Alternaria alternata* [53]	obilniny (pšenice, čirok, ječmen, oves), ovoce (jablka, jablečné produkty, mandarinky, olivy, citrusy, japonské hrušky, švestky, maliny, rybíz), ovocné nápoje (jablečné, brusinkové a hroznové džusy, víno), zelenina (paprika, rajčata, rajčatové produkty, mrkev, meloun), olejnaté rostliny, jedlé oleje, slunečnicová semínka, koření (skořice, zázvor, chilli, paprika, kmín kořenný, koriandr, římský kmín, fenykl, česnek, majoránka, oregano, sumah, tymián, kurkuma), doplňky na bázi ostropestřce mariánského, luštěniny [18, 32, 53]

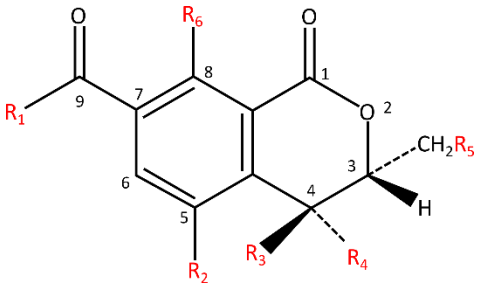
[a] MT – myktoxiny: AFs – aflatoxiny (AFB_1, AFB_2, AFG_1, AFG_2, metabolit AFM_1), OTA – ochratoxin A, DON – deoxynivalenol, NIV – nivalenol, T-2 – T-2 toxin, HT-2 – HT-2 toxin, ZEN – zearalenon, FUMs – fumonisiny ($FUMB_1$, $FUMB_2$, $FUMB_3$), CIT – citrinin, PAT – patulin, CPA – kyselina cyklopiazonová, EA – ergotové (námelové) alkaloidy (ergometrin, ergotamin, ergosin, ergokristin, egokryptin, ergokornin a odpovídající -inin epimery), Alternariové mykotoxiny (alternariol, alternariol monomethyl ether, altenuen, altertoxin I-III, kyselina tenuazonová); [b] Klasifikace dle Mezinárodní agentury pro výzkum rakoviny (IARC, International Agency for Research on Cancer): 1 – látky (směsi) karcinogenní pro člověka, 2B – látky (směsi) s možným karcinogenním účinkem pro člověka“, 3 – látky (směsi) jejichž karcinogenita pro člověka nelze klasifikovat“, N – není klasifikováno IARC

3.2 Ochratoxin A

OTA [PubChem CID: 442530] je nejrozšířenější a z toxikologického hlediska nejvýznamnější mykotoxin ze skupiny ochratoxinů – viz Tabulka 2, kapitola 3.2.1 [2, 54]. V současnosti je také považován za druhý nejvýznamnější mykotoxin – po AFs [7].

OTA byl objeven a chemicky charakterizován K. J. van der Merwem a jeho spolupracovníky v Jihoafrické republice v roce 1965, kdy byl poprvé izolován z VMH *Aspergillus ochraceus* (současný správný název *A. westerdijkiae*) rostoucí na kukuřičné moučce [55, 56]. V pozdějších letech byli objeveni další producenti OTA z rodu *Aspergillus* a *Penicillium* – viz kapitola 3.2.2. Pro člověka má největší význam dietární expozice OTA [3], tj. z kontaminovaných potravin rostlinného a v důsledku krmení hospodářských zvířat kontaminovaným krmivem také živočišného původu [57] – viz kapitola 3.2.4. Ke kontaminaci dochází zpravidla při nevhodném skladování či transportu [54]. Při běžném vaření je OTA pouze částečně degradován [21]. V lidském organismu působí toxicky, zejména na ledviny – viz podkapitola 3.2.3. V 90. letech byl OTA označen za hlavní etiologické agens balkánské endemické nefropatie, nádoru močových cest a chronické intersticiální nefropatie [58–60]. Vzhledem k negativním dopadům OTA na lidské zdraví je nutné zajišťovat bezpečnost potravin v souladu s Nařízením Komise 1881/2006 ve znění pozdějších předpisů uvádějícím regulace OTA v některých potravinách – viz kapitola 3.2.5.

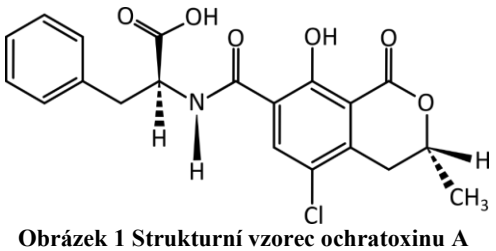
Tabulka 2 Významné metabolity ochratoxinu A

Název	Zkratka	Obecný strukturní vzorec derivátů OTA	R1	R2	R3	R4	R5	R6
Ochratoxin A	**OTA**		Phe	Cl	H	H	H	OH
Ochratoxin B	OTB		Phe	H	H	H	H	OH
Ochratoxin C	OTC		Phe-ethyl-ester	Cl	H	H	H	OH
Ochratoxin α	OTα		OH	Cl	H	H	H	OH
Ochratoxin β	OTβ		OH	H	H	H	H	OH
4R-hydroxy-OTA	4R-OH OTA		Phe	Cl	H	OH	H	OH
4S-hydroxy-OTA	4S-OH OTA		Phe	Cl	OH	H	H	OH
10-hydroxy-OTA	10-OH OTA		Phe	Cl	H	H	OH	OH
Otevřená laktonová forma OTA	OP-OTA		Phe	Cl	H	H	-	OH
Otevřená laktonová forma OTB	OP-OTB		Phe	H	H	H	-	OH
Otevřená laktonová forma OTα	OP-OTα		OH	Cl	H	H	-	OH
Otevřená laktonová forma OTβ	OP-OTβ		OH	H	H	H	-	OH
OTA chinon	OTQ		Phe	O	H	H	H	O
OTA hydrochinon	OTHQ		Phe	OH	H	H	H	OH
Dekarboxylovaný OTHQ	DC-OTHQ		Dekarboxylovaný Phe	OH	H	H	H	OH
Konjugát OTQ-glutathion	OTQ-Glutathion		Phe	O	H	H	H	O
Konjugát OTA-acyl hexóza	Acyl-hexóza-OTA		Phe acyl hexóza	Cl	H	H	H	OH
Konjugát OTA-acyl pentóza	Acyl-pentóza-OTA		Phe acyl pentóza	Cl	H	H	H	OH
OTA-methyl-ester	OTA-Me		Phe-methyl-ester	Cl	H	H	H	OH
OTB-methyl-ester	OTB-Me	$M(C_{20}H_{18}ClNO_6)$: 403,8 g/mol	Phe-methyl-ester	H	H	H	H	OH
OTB-ethyl-ester	OTB-Et		Phe-ethyl-ester	H	H	H	H	OH
4R-hydroxy-OTA-methyl-ester	4R-OH OTA-Me		Phe-methyl-ester	Cl	H	OH	H	OH
10-hydroxy-OTA-methyl-ester	10-OH OTA-Me		Phe-methyl-ester	Cl	H	H	OH	OH
Ethylamid-OTA	OE-OTA		Phe-ethyl-amid	Cl	H	H	H	OH
Dekarboxylovaný OTA	DC-OTA		Dekarboxylovaný Phe	Cl	H	H	H	OH
O-methyl-OTA	OM-OTA		Phe	Cl	H	H	H	OCH_3
d-OTA	d-OTA		d-Phe	Cl	H	H	H	OH
OTα-ester-methyl	M-OTα		OCH_3	Cl	H	H	H	OH
Tyrosin-OTA	OTA-Tyrosin		Tyrosin	Cl	H	H	H	OH

Strukturní vzorec zpracován v editoru vektorové grafiky Inkscape 0.92. Zpracováno dle Malir et al. [23] a El Khoury & Atoui [61].

3.2.1 Chemická a fyzikální charakteristika ochratoxinu A

OTA [$C_{20}H_{18}ClNO_6$, Phenylalanine-OTA, IUPAC: (2S)-2-[[(3R)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydroisochromene-7-carbonyl]amino]-3-phenylpropanoic acid] je bílý krystalický prášek, bez zápachu, v přítomnosti světla nestabilní, ale poměrně termostabilní s bodem tání 169 °C [62–65]. Chemická struktura je složena z dihydroizokumarinového kruhu spojeného s fenylalaninem přes amidovou vazbu – viz Obrázek 1 [66]. Molekula OTA může díky chirálním uhlíkům existovat ve 4 stereoizomerních formách, ovšem přirozeně se vyskytující forma je 3R14S-OTA [64, 67]. OTA vykazuje silnou přirozenou schopnost fluorescence v ultrafialovém světle [68] projevující se emitací zeleného světla v kyselých a modrého světla v alkalických roztocích [63]. Jako slabá kyselina je vysoce rozpustný v polárních organických rozpouštědlech např. chloroformu, methanolu, ethanolu, xylenu, slabě rozpustný ve vodě a rozpustný ve zředěných roztocích hydrogenuhličitanu [63–65].



Obrázek 1 Strukturní vzorec ochratoxinu A
Strukturní vzorec zpracován v editoru vektorové grafiky Inkscape 0.92. Zpracováno dle Köszegi & Poór [66].

3.2.2 Producenti ochratoxinu A

OTA je v potravinách rostlinného i živočišného původu produkován rody *Aspergillus* (viz podkapitola 3.2.2.1) a *Penicillium* (viz podkapitola 3.2.2.2), které se řadí mezi jedny z nejdominantnějších rodů světové houbové mikroflóry. Zatímco rod *Aspergillus* dominuje spíše v tropických oblastech, rod *Penicillium* je typický zejména pro oblasti s mírným podnebím. Rod *Aspergillus* je typický rychlejším růstem, ale také pomalejší sporulací, než je tomu v případě rodu *Penicillium*. Spory rodu *Aspergillus* bývají odolnější vůči světlu a chemikáliím. Taxonomické zařazení rodů *Aspergillus* a *Penicillium* uvádí Tabulka 3.

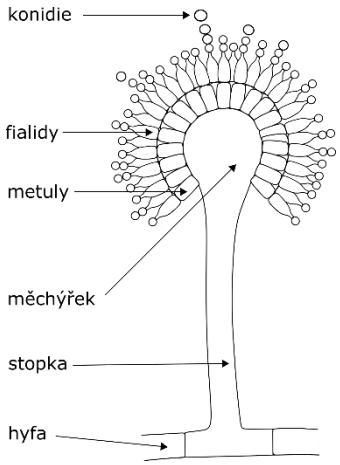
Tabulka 3 Taxonomické zařazení mikroskopických vláknitých hub rodu *Aspergillus* a *Penicillium*

Profil taxonu	Latinský název	Český název
Doména	*Eukaryota*	jaderní
Soustava	*Opisthoconta*	-
Říše	*Fungi*	houby
Oddělení	*Ascomycota*	vřeckovýtrusé houby
Pododdělení	*Pezizomycotina*	-
Třída	*Eurotiomycetes*	-
Podtřída	*Eurotiomycetidae*	-
Řád	*Eurotiales*	plesnivkotvaré
Čeleď	*Trichocomaceae*	plísňovkovité
Rod	*Aspergillus/Penicillium*	kropidlák/štětičkovec

Zpracováno dle Pitt & Hocking [69].

3.2.2.1 *Producenti ochratoxinu A rodu* Aspergillus

Rod *Aspergillus* poprvé popsal italský kněz a botanik Pier Antonio Micheli v roce 1729. Název *Aspergillus,* česky kropidlák, vychází z lat. slova „*aspergillum*“ znamenajícího nádoba na kropení svěcenou vodou, tzv. „kropítko“, které tento rod svou morfologií připomíná [10, 70, 71]. *Aspergillus* je asexuální stádium, tzv. anamorfa, charakterizovaná tvorbou konidioforů, které se na vrcholu stopky rozšiřují v měchýřky, tzv. vezikuly. Vezikuly nesou metuly s lahvicovitými konidiogenními fialidami (biseriátní konidiofor), nebo méně často přímo fialidy (uniseriátní konidiofor). Z fialid bazipetálně pučí kulovité konidie, spojující se pomocí konektiv v řetízky zakončené nejstarší konidií, která se následně odškrcuje a dává vznik novému konidioforu – viz Obrázek 2 [10, 69].



Obrázek 2 Morfologie biseriátního konidioforu rodu *Aspergillus*
Vlastní grafické zpracování dle Ellis et al. [72] pomocí editoru vektorové grafiky Inkscape 0.92

Druhy rodu *Aspergillus*, které produkují OTA v potravinách, spadají do sekcí *Circumdati* a *Nigri*. Sekce *Circumdati* zahrnuje 27 druhů, z nichž za ochratoxinogenní v potravinách jsou považovány dva druhy. Sekce *Nigri* zahrnuje 25 druhů, z nichž šest je považováno za ochratoxinogenní v potravinách – viz Tabulka 4.

Tabulka 4 Druhy rodu *Aspergillus* produkující ochratoxin A a jejich výskyt v potravinách

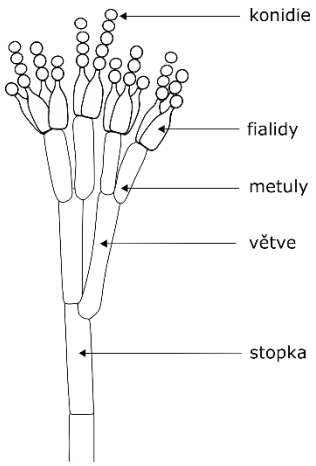
Sekce	Druh	Příklad potravin	Rok objevu
Circumdati	*A. steynii* a *A. westerdijkiae*[1]	sušené uskladněné potraviny, sójové boby, cizrna, koření, sušené ovoce, sušené ryby, sušené fazole, sezamová semínka, řepka, arašídy, ořechy (pekanové, lískové a vlašské ořechy, kešu, pistácie), obiloviny (rýže, pšenice, ječmen, kukuřice, čirok, mouka a otruby), maniok, sýry, zpracované maso, černé olivy	2004
Nigri	*A. carbonarius*	hrozny, kávová zrna, fíky, arašídy, vlašské ořechy, kukuřice, paprika	1996
	A. foetidus	hrozny	1996
	A. lacticoffeatus	kávová zrna	2004
	A. niger	produkty sušené na slunci, hrozny, solené sušené uzené ryby, sušené maso, kakaové boby, cizrna, arašídy, kokos, ořechy (pekanové a vlašské ořechy, kešu, pistácie, mandle), obiloviny (kukuřice, ječmen, čirok, rýže), sójové boby, slunečnicová semínka, řepka, koření, olivy, maso, sýry	1994
	A. sclerotioniger	kávová zrna	2004
	*A. tubingens*is	hrozny	2005

[1] Změna identifikace původního názvu druhu *A. ochraceus* na druh *A. westerdijkiae* s využitím molekulárně biologických metod. Zpracováno dle Malir et al. [23], Ostry et al. [57] a Pitt & Hocking [69].

3.2.2.2 Producenti ochratoxinu A z rodu Penicillium

Rod *Penicillium* objevil v roce 1809 německý přírodovědec a botanik Heinrich Friedrich Link. Název *Penicillium*, česky štětičkovec, získal tento rod na základě podobnosti fruktifikačních orgánů s malým štětečkem, latinsky „*penicillus*“ [69, 70]. *Penicillium* je anamorfa se třemi podrody *Aspergilloides*, *Furcatum* a *Penicillium*, přičemž z pohledu produkce OTA je významný pouze podrod *Penicillium*.

Fruktifikační orgány rodu *Penicillium* tvoří různě větvené struktury – jednoduchá nevětvená (monoverticiliátní konidiofor), jednostupňovitě větvená (biverticiliátní konidiofor), dvoustupňovitě větvená (terverticiliátní konidiofor) a třístupňovitě větvená (kvarterverticiliátní konidiofor). Pro podrod *Penicillium* jsou typické zejména terverticiliátní konidiofory, kde na stopku jsou navázány větve, metuly a konidiogenní fialidy – viz Obrázek 3 [69, 72].



Obrázek 3 Morfologie terverticiliátního konidioforu rodu *Penicillium*
Vlastní grafické zpracování dle Ellis et al. [72] pomocí editoru vektorové grafiky Inkscape 0.92

Ačkoliv je v literatuře možné se v souvislosti s produkcí OTA v potravinách setkat s různými „producenty“ rodu *Penicillium* [2, 73], uznávané jsou pouze dva druhy z podrodu *Penicillium,* série *Verrucosa* [24] – viz Tabulka 5.

Tabulka 5 Druhy rodu *Penicillium* produkující ochratoxin A a jejich výskyt v potravinách

Druh	Příklad potravin	Rok objevu
P. verrucosum[1]	obiloviny (pšenice, ječmen, žito, oves), sýry	1969
P. nordicum	substráty s vysokým obsahem proteinu, masné produkty, salám, sušená šunka, sýry	2001

[1] Změna v identifikaci druhu: dříve *A. viridicatum*. Zpracováno dle Malir et al. [23], Ostry et al. [57] a Pitt & Hocking [69].

3.2.3 Toxicita ochratoxinu A

Toxicita OTA je udávána jeho toxikokinetikou, tj. změnami jeho koncentrace, případně jeho struktury, v čase – viz kapitola 3.2.3.1 a toxikodynamikou, tj. interakcemi s biologickými cíli, mechanismy působení a jeho účinky na organismus – viz kapitola 3.2.3.2 [65].

3.2.3.1 Toxikokinetika ochratoxinu A

Toxikokinetika popisuje osud OTA v organismu, tj. jeho absorpci (proniknutí do krevního oběhu), distribuci (transport krví do cílových buněk, tkání či orgánů), biotransformaci (chemickou přeměnu) a exkreci (vylučování) [65].

Absorpce. Absorpce OTA je poměrně rychlá [22]. Dochází k ní na tzv. branách vstupu, kterými mohou být plíce, kůže či gastrointestinální trakt (GIT). Dermální a inhalační expozice má význam především u profesionálně exponovaných jedinců [74–77]. Pro většinu populace však představuje největší význam dietární expozice prostřednictvím konzumace kontaminovaných potravin [23].

Při dietární expozici OTA dochází v GIT k absorpci do krevního řečiště [65]. V GIT se může OTA vyskytovat v neionizované formě (OTA^0) či v ionizovaných formách jako monoanion (OTA^-) a dianion (OTA^{2-}). Zatímco neionizovaná forma OTA je rozpustná v tucích a má dominantní zastoupení v kyselých podmínkách žaludku (pH < 3), ionizované formy OTA jsou v tucích méně rozpustné a jsou přítomné ve dvanáctníku (pH ~ 7) [65, 78]. Formy OTA^0 a OTA^- jsou absorbovány do krve pasivním transportem ze žaludku a střední části tenkého střeva – lačníku [22, 65, 66], přičemž v tenkém střevě obecně dochází díky velké bohatě prokrvené absorpční ploše k největší absorpci [2]. V lačníku může také kromě pasivního transportu docházet k aktivnímu transportu, který je zřejmě zprostředkován specifickými proteinovými transportéry organických aniontů (OATs) [22].

Biodostupnost, tj. podíl podané látky, který vstoupí do krevního oběhu v nezměněné formě, se u různých zvířecích druhů liší, např. biodostupnost OTA u kuřat je 40 %, u králíků 56 % a u prasat 66 % [79]. Biodostupnost závisí hlavně na konkrétním druhu, dávce, způsobu podání, ale také na náplni žaludku [22]. Ve studii prováděné na lidských dobrovolnících byla 8 hodin po orálním podání OTA v dávce 0,02 nmol/kg t.hm. při prázdném žaludku zjištěna biodostupnost 93 % [80]. Poté, co je OTA absorbován do krevního oběhu, je krví dále distribuován [65].

Distribuce. Distribuce je znesnadněna, neboť je OTA z více než 99 % vázán na lidské sérové proteiny, zejména albumin [65, 81] se saturační schopností v řádu stovek mikrogramů OTA na mililitr krevního séra [82]. OTA je také vázán na neznámý protein o molekulové hmotnosti 20 kDa, který se v porovnání s albuminem vyskytuje v mnohem nižších koncentracích, nicméně vazba OTA na tyto proteiny je až milionkrát vyšší [4, 66] se saturační schopností 10-20 ng OTA/ml krevního séra [83]. Vzhledem k možnosti volné filtrace přes glomeruly mohou být komplexy s těmito proteiny jedním z etiologických faktorů balkánské endemické nefropatie [66]. Jen méně než 1 % OTA zůstává ve volné, tzv. biodisponibilní formě [81].

Vzniklé komplexy představují mobilní depa, ze kterých může být OTA později uvolňován [2, 65], což spolu s opětovnou reabsorpcí OTA z enterohepatálního oběhu a ledvinových proximálních a distálních tubulů prodlužuje biologický poločas, tedy čas, za který koncentrace OTA v organismu klesne na polovinu [65, 84], a to na 35,5 dne [73]. Tento poločas je v porovnání s ostatními zvířecími druhy nejdelší a je důvodem, proč se OTA snadno kumuluje v lidském organismu, jelikož v průběhu eliminace OTA může dojít k jeho dalšímu přívodu do organismu [66]. Kromě krve se OTA kumuluje v játrech a ledvinách, tj. hlavních orgánech biotransformace, ale také ve varlatech, střevech, svalech, tukových tkáních a v menším množstvím také v mozku [4, 65].

Biotransformace. Biotransformace zahrnuje sérii enzymatických procesů, které přeměňují toxické látky na produkty, které jsou méně lipofilní a snadněji se vylučují z organismu, především močí [2]. Výsledný produkt je obvykle méně toxický až netoxický a jedná se tedy o detoxikační proces, nicméně může dojít také k bioaktivaci, při které má výsledný produkt vyšší toxicitu než původní molekula [2, 85]. Biotransformace OTA se odehrává ve dvou fázích označovaných jako fáze I a fáze II.

Fáze I zahrnuje hydrolytické, oxidační a redukční procesy [2, 86] vedoucí ke vzniku metabolitů, jako jsou např. ochratoxin α (OTα), otevřená laktonová forma OTA (OP-OTA),

4R-hydroxy-OTA (4R-OH OTA), 4S-hydroxy-OTA (4S-OH OTA), 10-hydroxy-OTA (10-OH OTA), ochratoxin B (OTB), OTA chinon (OTQ), OTA hydrochinon (OTHQ) [65, 66, 73, 87]. Na těchto procesech se podílí řada enzymů, přičemž nejdůležitější je cytochrom P450 [88].

Hlavním mechanismem detoxikace OTA je hydrolýza na OTα [22, 65]. OTα vzniká štěpením amidové vazby spojující L-β-fenylalaninovou část s dihydroizokumarinovým základem, tj. OTα, prostřednictvím hydrolytických enzymů karboxypeptidázy A, katepsinu C a α-chymotrypsinu [73, 89, 90]. OTα ani oddělená fenylalaninová část nejsou toxické a lze tedy tento proces označit za detoxikační [73]. Předpokládá se, že OTA je degradován na OTα také střevní mikroflórou, přičemž nejaktivnější jsou v tomto směru prvoci v trávícím traktu přežvýkavců, nicméně bakteriální frakce hraje také významnou roli [22, 91]. Tato degradace se však odehrává až v tlustém střevě, tedy po průchodu hlavním místem absorpce, a proto spíše nemá u nepřežvýkavců velký význam [91]. Hydrolýzou laktonového kruhu za alkalických podmínek vzniká metabolit OP-OTA, který byl detekován ve žluči potkanů, kterým byl podán OTA. OP-OTA vykazuje u bakterií nebo myší nižší toxicitu, ale při intravenózním podání potkanům byl dokonce více toxický, než OTA [92].

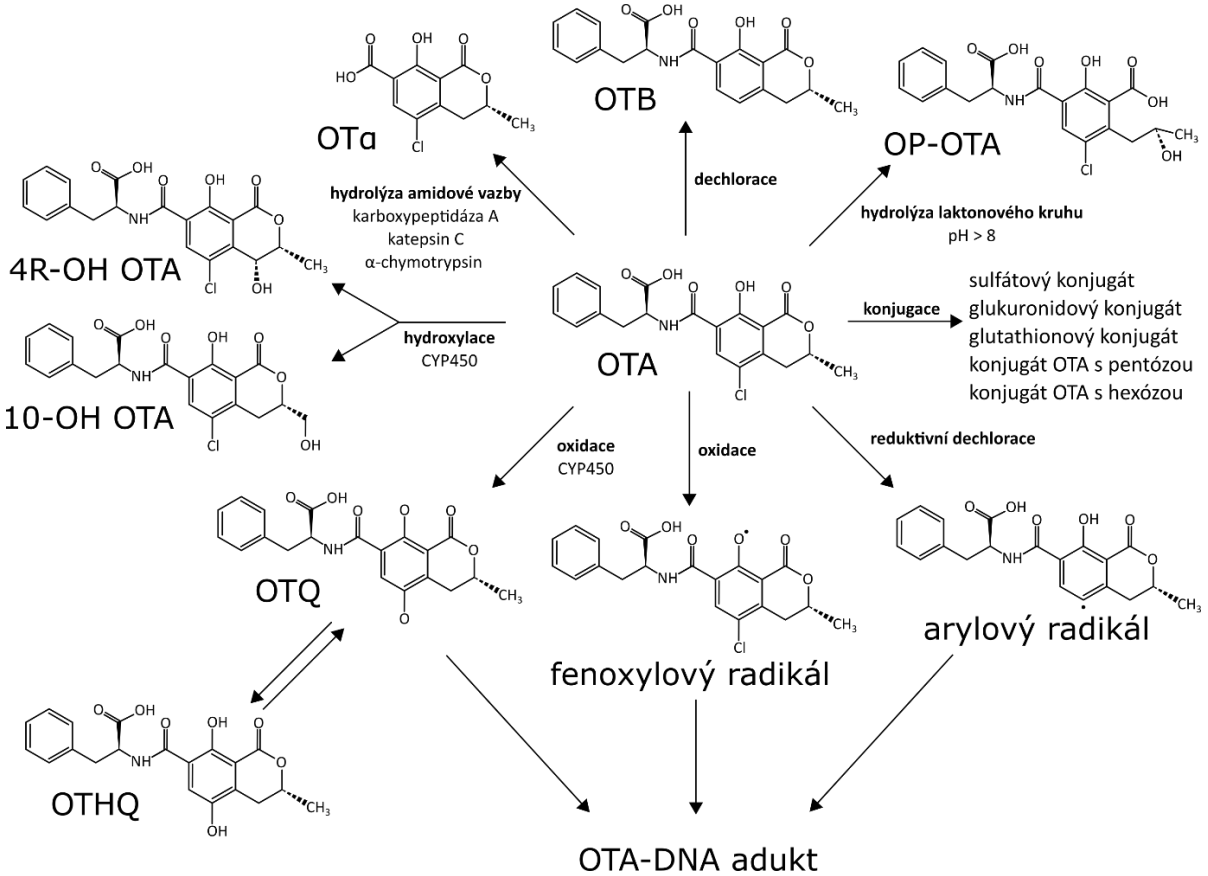
Hydroxylací zprostředkovanou rodinou enzymů cytochrom P450 mohou vznikat metabolity 4-OH OTA a 10-OH OTA. Metabolit 4-OH OTA vykazuje podobnou cytotoxicitu, imunosupresivitu [4] a inhibiční účinek na proteosyntézu jako OTA [21]. Vyskytuje se ve dvou epimerech, přičemž epimer 4R-OH OTA se tvoří v lidských a potkaních játrech a epimer 4S-OH OTA se tvoří v játrech prasete [22, 65, 93]. O něco méně toxický hydroxylovaný metabolit 10-OH OTA byl objeven *in vitro* v játrech králíka [94] a v bronchiálních epiteliálních buňkách člověka [95].

Oxidací zprostředkovanou cytochromem P450 mohou vznikat chinonové metabolity OTQ či OTHQ, které jsou údajně zodpovědné za tvorbu DNA aduktů [22, 96].

Dalšími cestami metabolizace OTA jsou reduktivní dechlorace za vzniku arylového radikálu, který je údajně zodpovědný za vznik uhlíkově vázaných DNA aduktů OTA-deoxyguanosin (C-C8dG-OTA), nebo oxidace na fenoxylový radikál, který je údajně zodpovědný za vznik kyslíkově vázaných DNA aduktů OTA-deoxyguanosin (O-C8dG-OTA) [22, 96, 97]. OTA může také projít dechlorací za vzniku méně genotoxického metabolitu OTB [22, 66].

Fáze II zahrnuje syntetické procesy, při kterých se na polární funkční skupinu, obvykle získanou ve fázi I [86], váže hydrofilní endogenní látka za vzniku nové sloučeniny, tzv. konjugátu, který je rychle vylučován z organismu [88].

OTA může podléhat tzv. glukuronidaci, při které se konjuguje s uridindifosfát glukurovonou kyselinou pomocí enzymu uridindifosfát glukuronosyltransférázy. Dále může podléhat sulfataci, při které dochází ke konjugaci OTA s 3'-fosfoadenosin-5'-fosfosulfátem, pomocí enzymu sulfatázy. Neméně důležitá je konjugace OTA s glutathionem pomocí enzymu glutathion S-transferázy za vzniku OTA-glutathionového konjugátu. Další možností je vznik esterových konjugátů OTA s pentózou či hexózou za vzniku konjugátů hex/pen-OTA [2, 4, 65, 98].



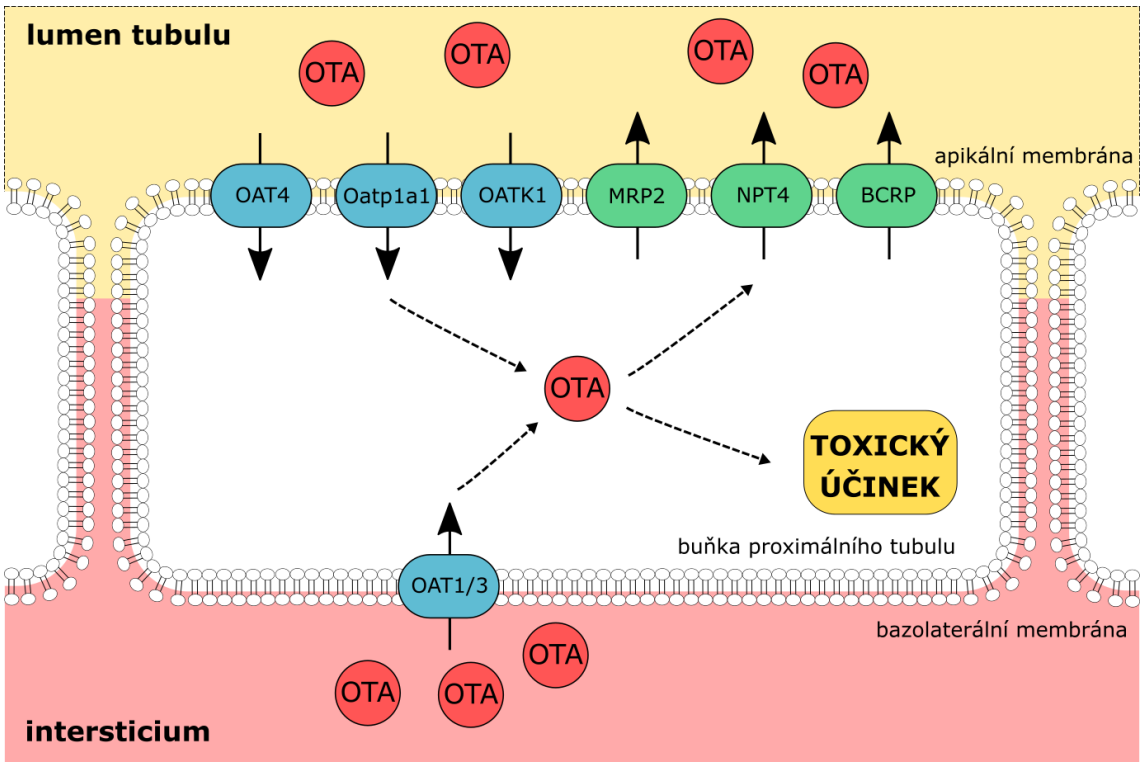
Obrázek 4 Biotransformace ochratoxinu A

Vlastní grafické zpracování dle Abrunhosa et al. [73], EFSA [22], Ringot et al. [65], Tran et al. [88], Kőszegi & Poór [66] pomocí editoru vektorové grafiky Inkscape 0.92.

Exkrece. Exkrece OTA je oproti absorpci pomalá zejména kvůli jeho vazbě na plazmatické proteiny [22, 99]. Nezávisle na dávce je denně vyloučeno 20–80 ng OTA [99].

OTA je z organismu vylučován převážně močí [65, 100]. Jelikož je glomerulární filtrace z krve do moče omezená molekulovou hmotností filtrované látky do 60 000 Da, není vzhledem k vysoké vazebné afinitě OTA k albuminu v případě OTA příliš účinná a podléhá jí pouze

biodisponibilní frakce [2, 65, 66, 84]. Větší význam má tzv. tubulární sekrece zprostředkovaná OATs v proximálních tubulech [65]. OAT1 a OAT3 na bazolaterální membráně jsou zodpovědné zejména za transport OTA z krve do buňky proximálního tubulu, ze které jsou pomocí efluxních transportérů na apikální membráně sekretovány do moči. Část sekretovaného OTA je prostřednictvím OAT4 a dalších transportérů na apikální membráně reabsorbována zpět do ledvinových buněk. Jelikož OAT1, 3 a 4 jsou účinnější než efluxní transportéry, dochází ke zpomalení vylučování OTA, jeho následné akumulaci a nefrotoxickému účinku [65, 66, 101–103] – viz Obrázek 5.



Obrázek 5 Transport ochratoxinu A pomocí transportérů organických iontů v buňkách proximálního tubulu. OTA, ochratoxin A; OATs, transportéry organických aiontů (OAT1, OAT3, OAT4, OATK1); OATp1a1, polypeptid transportující organické anionty; MRP2, protein multilékové rezistence 2; NPT4, Na+ dependentní fosfátový transportér; BCRP, protein rezistence rakoviny prsu. Vlastní grafické zpracování dle George et al. [102] pomocí editoru vektorové grafiky Inkscape 0.92.

Dalším způsobem eliminace OTA je fekální exkrece, která úzce souvisí s biliární exkrecí. OTA je v tenkém střevě vstřebáván do krve a následně odveden do jater, ze kterých je exkretován do žluči, ze které je ve střevech opětovně absorbován [2, 104]. Tato enterohepatální recirkulace vede k redistribuci toxinu do různých tkání, což má za následek pomalé vylučování a dlouhý biologický poločas [2, 65].

Exkrece mlékem je z kvantitativního hlediska téměř zanedbatelná [2], nicméně vzhledem k prokázané souvislosti mezi výskytem OTA ve stravě matky a koncentrací OTA v mateřském mléce [105] může tento způsob exkrece představovat značné riziko pro kojence [2, 65, 104].

3.2.3.2 Toxikodynamika ochratoxinu A

OTA je známý pro své nefrotoxické účinky. Dále vykazuje hepatotoxické, embryotoxické, teratogenní, imunosupresivní, neurotoxické a genotoxické účinky. Karcinogenní účinky OTA byly prokázány na zvířatech (např. myš, potkan a pstruh) [101], nicméně z hlediska karcinogenity pro člověka je OTA dle IARC od roku 1993 klasifikován do skupiny 2B „možný karcinogen“ [21].

V závislosti na zvířecím druhu se LD_{50}, tj. smrtná dávka, při které při perorálním podání OTA zahyne 50 % testovaných jedinců, pohybuje v rozmezí od 0,2 (pes) do 58 (myš) mg/kg t. hm. Toxicita se obecně liší nejen u různých druhů zvířat, ale je závislá také na jejich pohlaví, věku či jiných individuálních vlastnostech. Způsob podání je též důležitým faktorem určujícím míru toxicity [73, 101, 106, 107]. Přesný mechanismus působení OTA není dosud plně objasněn, nicméně jsou známy některé dílčí mechanismy podílející se na jeho toxickém účinku. Mezi tyto mechanismy patří např. narušení syntézy proteinů, DNA a RNA v důsledku podobnosti OTA s fenylalaninem; tvorba reaktivních forem kyslíku a následné oxidační poškození DNA a proteinů; lipidová peroxidace; narušení permeability plazmatické membrány s následným narušením intercelulární homeostázy Ca^{2+}; inhibice mitochondriální respirace a následný úbytek a nedostatek ATP a apoptóza různých buněk a tvorba DNA-aduktů [65, 81, 99, 108].

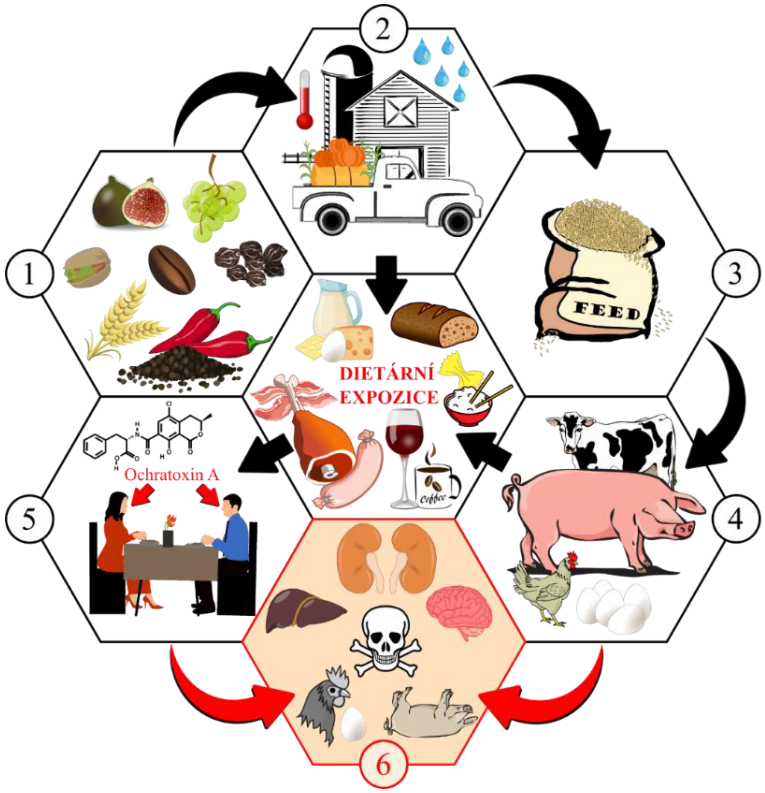
3.2.4 Dietární expozice ochratoxinu A

OTA se u běžné populace dostává do organismu majoritně především dietární cestou, tj. prostřednictvím kontaminované potravy [23] – viz Obrázek 6. Pro člověka má význam zejména přímá expozice OTA prostřednictvím kontaminovaných plodin a produktů z nich vyrobených [109]. Dále je možná také nepřímá expozice prostřednictvím živočišných produktů získaných z hospodářských zvířat (např. maso, vejce a mléko) krmených zaplísněným krmivem s obsahem OTA [109]. Obilniny představují z tohoto pohledu obzvláště vysoké riziko [109], nicméně dochází ke kontaminaci i dalších plodin, např. hroznů, pistácií, fíků, koření, kávy, rýže apod. [18; 23, 110, 111].

VMH produkující OTA napadají plodiny zejména v posklizňovém období, tj. spíše při nevhodném skladování a transportu než na poli, přičemž teplotní a vlhkostní podmínky mají na růst VMH zcela zásadní vliv [112]. Kontaminované krmivo narušuje metabolismus a zdraví hospodářských zvířat, přičemž monogastrické druhy, např. prasata a drůbež, jsou citlivější než polygastrické druhy, např. skot, ovce a kozy, kvůli méně efektivnímu odbourávání OTA v důsledku absence bachoru, ve kterém je OTA mikrobiálně degradován [109]. Důležitou roli

hraje též složení krmiv, neboť krmivo pro dobytek se, na rozdíl od krmiva pro prasata či drůbež, skládá převážně z pícnin a pouze částečně z obilovin, které jsou z pohledu kontaminace OTA vysoce rizikové [109]. Přenos OTA do živočišných produktů závisí na: 1) úrovni expozice hospodářských zvířat OTA z krmiva, 2) míře absorpce OTA do krevního řečiště, 3) úrovni perzistence OTA v krvi a jeho akumulace v tkáních a 4) míře přechodu OTA do konečných produktů jako jsou maso, mléko a vejce [109]. Z tohoto pohledu jsou nejvíce rizikové vepřové produkty, zejména produkty s vepřovou krví, droby – ledviny a játra a další masné produkty. Drůbeží produkty, tj. masné produkty a vejce, jsou méně rizikové než vepřové vzhledem k účinnější exkreci OTA u drůbeže, která může být zapříčiněna nižší afinitou OTA k sérovému albuminu drůbeže [79]. Ke kontaminaci hovězích, ovčích či kozích konečných produktů jako jsou masné produkty a mléko dochází až při velmi vysokých dávkách OTA, které se v krmivech běžně nevyskytují [109].

Dietární expozice OTA má za následek především poškození ledvin, méně jater či nervové soustavy. Krmení hospodářských zvířat kontaminovaným krmivem může způsobit ekonomické ztráty v důsledku snížení přívodu krmiva vedoucímu k úbytku na váze, celkovému zhoršení produktivity a zdravotního stavu zvířete či jeho úhynu [109].



Obrázek 6 Dietární expozice ochratoxinu A

1) Plodiny náchylné ke kontaminaci ochratoxinem A; 2) Nevhodné skladování a transport; 3) Kontaminované krmivo; 4) Zkrmování hospodářských zvířat kontaminovaným krmivem; 5) Konzumace kontaminovaných potravin rostlinného a živočišného původu lidmi; 6) Dopady na zdraví lidí/zvířat z konzumace kontaminovaných potravin/krmiv. Vlastní grafické zpracování pomocí editoru vektorové grafiky Inkscape 0.92.

3.2.5 Regulace ochratoxinu A v Evropské unii

Výskyt mykotoxinů v potravinách je vzhledem k jejich vážným dopadům na zdraví člověka zcela nežádoucí. Z tohoto důvodu byly v mnoha zemích stanoveny MPL regulující výskyt mykotoxinů v potravinách. Regulace mykotoxinů v Evropské unii (EU) je v celosvětovém srovnání nejkomplexnější a je závazná pro 27 členských států.

V současné době je OTA v určitých potravinách regulován dle Nařízení Komise 1881/2006 „*ze dne 19. prosince 2006, kterým se stanoví maximální limity některých kontaminujících látek v potravinách*“ ve znění pozdějších předpisů, oddílu 2 „*Mykotoxiny*“, položky 2.2 „*Ochratoxin A*“ – viz Tabulka 6. Kromě OTA jsou v oddílu 2 uvedeny také MPL pro další mykotoxiny jako jsou AFs, PAT, CIT, DON, ZEN, FUMs, T-2 a HT-2 a ergotové alkaloidy [113].

Tabulka 6 Regulace ochratoxinu A dle Nařízení Komise 1881/2006 ve znění pozdějších předpisů

Číslo	Potraviny	OTA[a] [µg/kg]	Reference
2.2.1	Nezpracované obiloviny	5	[113]
2.2.2	Všechny produkty pocházející z nezpracovaných obilovin, s výjimkou potravin uvedených v bodech 2.2.3-5, 2.2.12 a 2.2.13 Obiloviny uváděné na trh pro konečného spotřebitele	3	[114]
2.2.3	Pečivo, svačinky z obilovin a snídaňové cereálie		[114]
	Výrobky neobsahující olejnatá semena, ořechy nebo sušené ovoce	2	[114]
	Výrobky obsahující nejméně 20 % sušených hroznů révy vinné a/nebo sušených fíků	4	[114]
	Ostatní výrobky obsahující olejnatá semena, ořechy a/nebo sušené ovoce	3	[114]
2.2.4	Nealkoholické sladové nápoje	3	[114]
2.2.5	Pšeničný lepek neuváděný na trh pro konečného spotřebitele	8	[114]
2.2.6	*Sušené ovoce*		
	Sušené hrozny révy vinné (korintky, rozinky a sultánky) a sušené fíky	8	[114]
	Ostatní sušené ovoce	2	[114]
2.2.7	Datlový sirup	15	[114]
2.2.8	*Pražená káva*		
	Pražená kávová zrna a mletá pražená káva kromě rozpustné kávy	3	[114]
	Rozpustná káva (instantní káva)	5	[114]
2.2.9	Víno (včetně šumivého vína, s výjimkou likérového vína a vína s obsahem alkoholu nejméně 15 % objemových) a ovocné víno	2	[113]
2.2.10	Aromatizované víno, aromatizované vinné nápoje a aromatizované vinné koktejly	2	[113]
2.2.11	Hroznová šťáva, rekonstituovaná koncentrovaná hroznová šťáva, hroznový nektar, rekonstituovaný hroznový mošt a rekonstituovaný koncentrovaný hroznový mošt uváděné na trh pro konečného spotřebitele	2	[114]
2.2.12	Obilné příkrmy a ostatní příkrmy určené pro kojence a malé děti	0,5	[113]
2.2.13	Dietní potraviny pro zvláštní léčebné účely, určené speciálně pro kojence a malé děti	0,5	[114]
2.2.14	Koření, včetně sušeného koření, kromě *Capsicum* spp.	15	[114]
	Capsicum spp. (sušené plody, celé nebo mleté, včetně chilli, mletého chilli, kayenského pepře a papriky)	20	[115]
	Směsi koření	15	[114]
2.2.15	*Lékořice* (Glycyrrhiza glabra, Glycyrrhiza inflata *a další druhy*)		
	Kořen lékořice, mimo jiné jako složka bylinných čajů	20	[114]
	Výtažek z lékořice pro použití v potravinářských výrobcích, zejména v nápojích a cukrovinkách	80	[116]
	Cukrovinky z lékořice obsahující ≥ 97 % výtažku z lékořice v sušině	50	[114]

Číslo	Potraviny	OTA[a] [µg/kg]	Reference
	Ostatní cukrovinky z lékořice	10	[114]
2.2.16	Sušené byliny	10	[114]
2.2.17	Kořeny zázvoru pro použití v bylinných čajích	15	[114]
	Kořeny proskurníku lékařského, kořeny pampelišky (smetánky) a květy pomerančovníku pro použití v bylinných čajích nebo v náhražkách kávy	20	[114]
2.2.18	Slunečnicová semena, dýňová semena, semena melounu (vodního), konopná semena, sójové boby	5	[114]
2.2.19	Pistácie, které musí být před uvedením na trh pro konečného spotřebitele nebo před použitím jako složka potravin vytříděny nebo jinak fyzikálně ošetřeny	10	[114]
	Pistácie uváděné na trh pro konečného spotřebitele nebo jako složka potravin	5	[114]
2.2.20	Kakaový prášek	3	[114]

[a] maximální limit OTA.

Jelikož byla přítomnost OTA zjištěna také v potravinách, pro které nebyly stanoveny MPL, bylo vhodné je stanovit i pro tyto potraviny. Na základě diskuse Generálního ředitelství pro zdraví a bezpečnost potravin (DG SANTE) byly předloženy návrhy na nové MPL pro OTA v dosud neregulovaných potravinách a návrhy na změnu některých stávajících limitů [117]. Dne 1. ledna 2023 byly navrhované změny pro regulaci OTA uvedeny v platnost Nařízením Komise 2022/1370 „*ze dne 5. srpna 2022, kterým se mění nařízení (ES) č. 1881/2006, pokud jde o maximální limity ochratoxinu A v některých potravinách*“. Nové nařízení uvádí nové regulace OTA v dalších potravinách, konkrétně se jedná o nealkoholické sladové nápoje, sušené ovoce jiné než sušené hrozny révy vinné, datlový sirup, sušené byliny, některé složky bylinných čajů a náhražek kávy, některé výrobky z lékořice, všechna koření včetně jejich směsí, některá olejnatá semena, sójové boby, pistácie a kakaový prášek. Zároveň došlo k úpravě, resp. zpřísnění i některých stávajících limitů u potravin jako jsou pekárenské výrobky, sušené hrozny révy vinné, pražená káva a rozpustná káva. Potraviny, kterých se změna v regulaci OTA týká a byly uvedeny na trh před datem 1. ledna 2023 mohou zůstat na trhu do uplynutí data minimální trvanlivosti nebo data spotřeby. Pro stanovení MPL OTA v šunce a sýrech je doporučeno další monitorování [114].

Produkty, které obsahují nadlimitní množství daných kontaminujících látek, by neměly být uvedeny na trh, a to ani jako složky jiných potravin. Nemělo by docházet k mísení podílů splňujících limity s podíly, které limity nesplňují. Dekontaminace plodin chemickou cestou je v případě mykotoxinů rovněž zakázána. Pro snížení úrovně kontaminace potravin je povoleno pouze třídění či jiné fyzikální ošetření, kterým lze dosáhnout požadovaného efektu, a to za předpokladu, že se takto ošetřené potraviny nemísí s potravinami určenými k přímé lidské spotřebě nebo s potravinami určenými pro použití jako potravinová složka [113].

Pro potraviny, které nepodléhají právním předpisům EU je možné využít tzv. expoziční limity, které však slouží pouze pro zpětné vyhodnocení zdravotního rizika po dietární expozici. Ještě donedávna se v případě OTA uplatňoval přístup tzv. směrné hodnoty stanovené z hlediska ochrany zdraví (v angl. označováno jako health-based guidance value; HBGV), respektive tolerovatelný týdenní přívod 120 ng/kg t. hm. stanovený Evropským úřadem pro bezpečnost potravin (EFSA) v roce 2006 [118]. Ve stanovisku EFSA z roku 2020 zabývajícím se hodnocením rizika OTA v potravinách byl přístup HBGV v případě OTA označen za nevhodný. Vzhledem k nejnovějším poznatkům EFSA preferuje přístup tzv. hraniční expozice (MOE), která je dána poměrem spodní limitní hodnoty spolehlivosti referenční dávky 10% dodatečného rizika ($BMDL_{10}$) a dietární expozice. Pro charakterizaci nenádorových účinků na základě ledvinových lézí u prasat byla stanovena hodnota $BMDL_{10}$ 4,73 μg/kg t. hm./den, přičemž za hodnotu vzbuzující nízké obavy se považuje MOE ≥ 200. Pro charakterizaci nádorových účinků na základě nádorů ledvin u potkanů byla stanovena hodnota $BMDL_{10}$ 14,5 μg/kg t. hm./den, přičemž za hodnotu vzbuzující nízké obavy se považuje MOE ≥ 10 000. Tuto hodnotu stanovil Vědecký panel EFSA pro kontaminanty v potravinovém řetězci obecně pro látky, které jsou genotoxické a karcinogenní, nicméně dle vědeckého stanoviska EFSA může být tato hodnota v případě OTA zvláště obezřetná, jelikož nejsou důkazy pro přímou genotoxicitu a karcinogenitu OTA průkazné [22].

K efektivnímu snížení zdravotního rizika vyplývajícího z expozice mykotoxinům v potravinách je nezbytné pravidelné kontrolování míry kontaminace potravin mykotoxiny nebo monitorování jejich producentů či biomarkerů [119]. Analýza potravin za účelem posouzení míry kontaminace mykotoxiny je důležitou základní praxí pro zajištění bezpečnosti a kvality potravin [120].

Na národní úrovni je úřední kontrola bezpečnosti potravin zajišťována dozorovými orgány v gesci Ministerstva zdravotnictví, tj. Orgány ochrany veřejného zdraví; a Ministerstva zemědělství, tj. Státní zemědělská a potravinářská inspekce, Státní veterinární správa, Ústřední kontrolní a zkušební ústav zemědělský a Ústav pro státní kontrolu veterinárních biopreparátů a léčiv [119, 121].

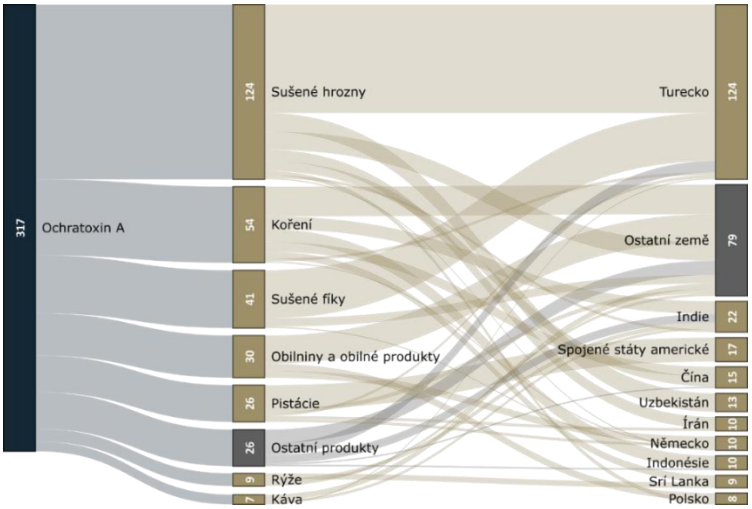
Mykotoxiny obecně představují poměrně častý důvod pro odmítnutí na hranicích, a tedy vstup na trh EU [110]. Na úrovni EU je klíčovým nástrojem pro bezpečnost potravin, ale i krmiv Systém rychlého varování pro potraviny a krmiva (RASFF), jehož účelem je co nejrychlejší šíření oznámení o nebezpečných potravinách a krmivech mezi členy systému, kterými jsou

EK, členské státy EU a EFTA (Island, Lichtenštejnsko a Norsko) a EFSA [122]. Státní zemědělská a potravinářská inspekce představuje pro Českou republiku tzv. národní kontaktní místo, které komunikuje na jedné straně s EK a na druhé s národními dozorovými orgány nad potravinami a krmivy. EK vyhodnocuje všechna hlášení a předává je všem členům systému prostřednictvím 4 typů oznámení: 1) varování (angl. alert notification); 2) informace (angl. information); 3) odmítnutí na hranicích (ang. border rejection) a 4) novinky (angl. news) [110].

V roce 2021 bylo systémem RASFF hlášeno 437 oznámení na mykotoxiny v potravinách, z toho 33 (7,5 %) oznámení na OTA. Česká republika byla ohlášena dne 10. 12. 2021 (alert notification 2021.6815) jako distributor sušených fíků pocházejících z Řecka s obsahem OTA 78,1 µg/kg (EU limit 8 µg/kg) [110].

V roce 2022 (ke dni 14. 12. 2022) bylo systémem RASFF hlášeno 465 oznámení pro mykotoxiny v potravinách, z toho 51 (11 %) oznámení na OTA. Česká republika se v roce 2022 objevila ve dvou oznámeních, a to dne 17. 6. 2022 (alert notification 2022.3578) jako země původu mouky s obsahem OTA 7,3 µg/kg (EU limit 3,0 µg/kg) a dne 17. 8. 2022 (alert notification 2022.477) jako oznamovatel výskytu OTA o koncentraci 2,94 µg/kg (EU limit 0,5 µg/kg) v cereálních produktech pro kojence pocházejících z Rakouska [110].

Souhrnná analýza dat za období 2019-2020 ukázala, že v tomto období bylo zaznamenáno 2 635 oznámení na mykotoxiny v potravinách; z toho 317 (12 %) na OTA. Více než 95 % oznámení pocházelo ze sekcí „*ovoce a zelenina*“, „*byliny a koření*“, „*obilniny a pekařské produkty*“, „*ořechy, ořechové produkty a semínka*“, a „*kakao a kakaové produkty, káva a čaj*“ [110]. Obrázek 7 znázorňuje souhrn oznámení pro OTA.

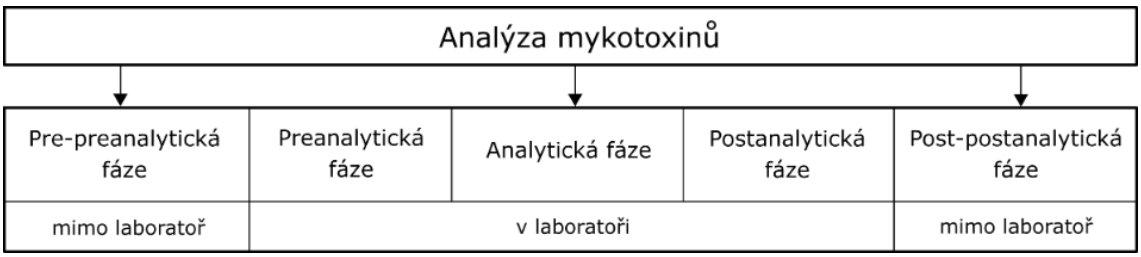


Obrázek 7 RASFF oznámení o výskytu ochratoxinu A v potravinách v letech 2016-2020

„Ostatní produkty“ zahrnují produkty hlášené méně než 5x. „Ostatní země“ zahrnují země, ze kterých pocházelo méně než 8 oznámení. Data byla zpracována dle RASFF databáze [110]. Vlastní grafické zpracování pomocí online generátoru aluviálních diagramů The Sankey Diagram Generator a editoru vektorové grafiky Inkscape 0.92

3.3 Metody pro stanovení mykotoxinů

Proces stanovení mykotoxinů může být ovlivněn řadou faktorů v různých fázích, tj. 1) před samotným stanovením v tzv. (pre-)preanalytické fázi zahrnující všechny kroky od odběru a transportu vzorku do laboratoře, přes skladování, až po jeho laboratorní přípravu k analýze; 2) během stanovení, v tzv. analytické fázi zahrnující vlastní stanovení analytu měřícím přístrojem; 3) po stanovení, v tzv. (post-)postanalytické fázi zahrnující vyhodnocení a interpretaci výsledků – viz Obrázek 8. Každá z uvedených fází ovlivňuje výsledek [123, 124].



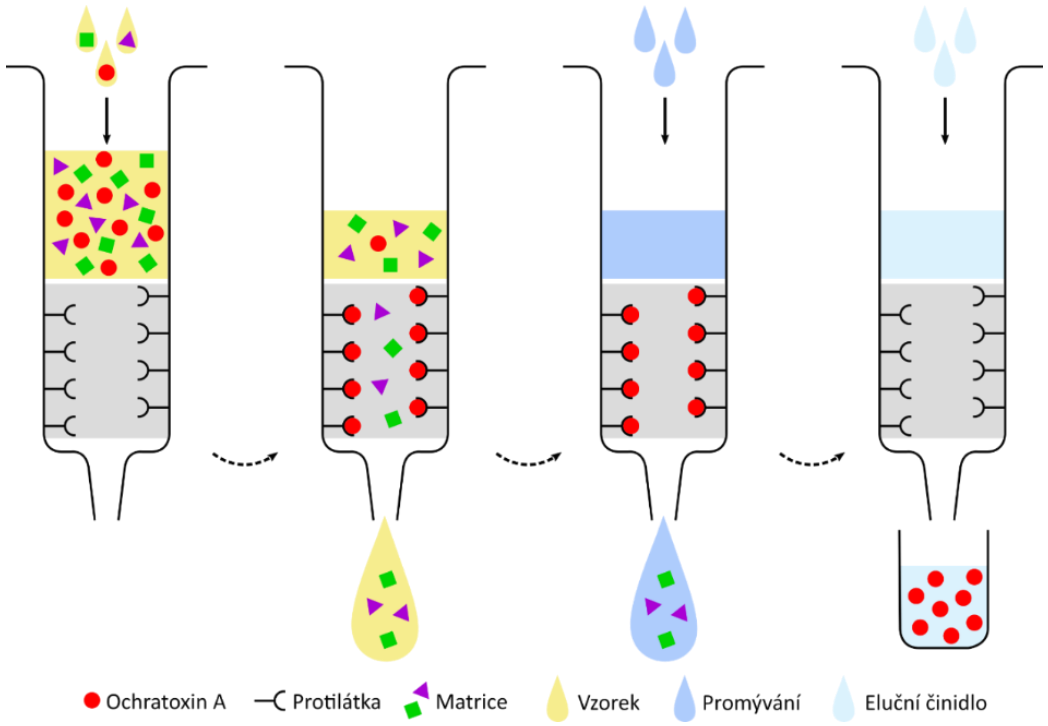
Obrázek 8 Obecné schéma procesu stanovení mykotoxinů

Stanovení mykotoxinů se obvykle provádí metodami, které zahrnují vzorkování, přípravu vzorků pro analýzu, extrakci hledaného analytu a přečištění extraktu, detekci a kvantifikaci [125]. S výjimkou tekutých nebo vysoce zpracovaných homogenních vzorků se mykotoxiny vyskytují v potravinách převážně nestejnoměrně. Aby bylo možné zaručit pravdivost výsledku, je nutné odebrat tzv. reprezentativní vzorek, který svým charakterem bude odpovídat celkovému vzorku [125].

Pro úspěšnou analýzu je extrakce hledaného analytu z komplexní matrice vzorku do kapalné fáze nezbytným krokem [125]. Pro extrakci mykotoxinů z matrice se volí extrakční rozpouštědla dle povahy hledaného mykotoxinu a s ohledem na matrici vzorku [125, 126]. Nejúčinnější jsou organická rozpouštědla jako např. chloroform, methanol, acetonitril, dichlormethan, aceton, ethylester kyseliny octové, diethylether, toluen, případně jejich směsi s vodou, která napomáhá penetraci organických rozpouštědel do matrice. Pro zvýšení účinnosti extrakce se k těmto rozpouštědlům často přidávají modifikátory ve formě kyselin nebo zásad, které mohou narušit silné vazby mezi analytem a složkami obsaženými v potravině, např. bílkovinami a cukry [125, 127].

Odstředění či filtrace má význam pro odstranění interferujících částic před následným přečištěním získaného extraktu [125]. Přečištění extraktu je důležité pro odstranění interferujících látek, které by mohly narušovat detekci hledaného mykotoxinu [125, 128]. Přečištění se také doporučuje pro ochranu chromatografické kolony [128].

Bylo vyvinuto několik metod pro přečištění extraktu, např. extrakce kapalina-kapalina či extrakce na tuhou fázi. Nejvíce využívané a oblíbené pro přečištění jsou imunoafinitní kolonky (IAK) fungující specificky na principu antigen-protilátka [129], které jsou využívány v této práci. Princip IAK spočívá v zachycení antigenu hledaného mykotoxinu a v jeho vazbě na specifické protilátky zakotvené na kolonce. Po aplikaci mycího roztoku jsou ostatní balastní látky odstraněny z kolonky. Zachycený mykotoxin je následně uvolněn elučním činidlem z komplexu antigen-protilátka. Zobrazení principu IAK např. pro OTA – viz Obrázek 9.



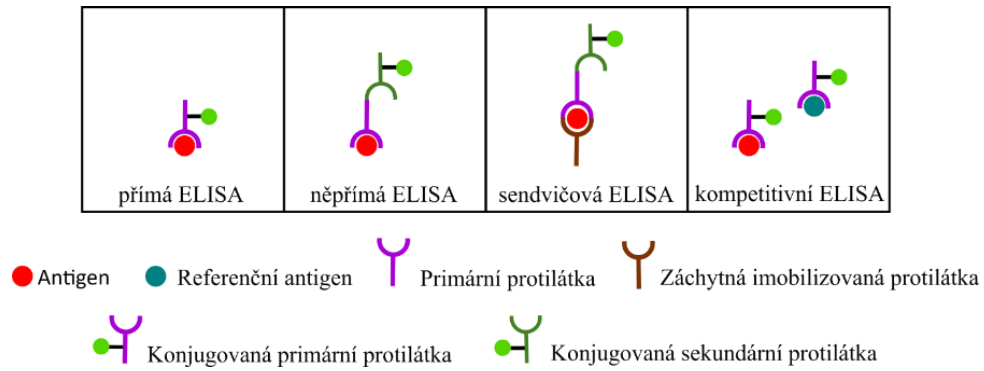
Obrázek 9 Princip separace ochratoxinu A na imunoafinitních kolonkách
Vlastní grafické zpracování pomocí editoru vektorové grafiky Inkscape 0.92.

Pro stanovení mykotoxinů bylo vyvinuto několik analytických metod. Nejvyužívanější jsou metody imunochemické a chromatografické [23]. Podrobněji uvedené principy metod jsou omezené pouze na ty, které byly využívány v průběhu doktorského studia: enzymová imunoanalýza (ELISA) – viz kapitola 3.3.1 a vysokoúčinná kapalinová chromatografie s fluorescenčním detektorem (HPLC-FLD) – viz kapitola 3.3.2.

3.3.1 Enzymová imunoanalýza

Metoda ELISA je uživatelsky příznivá a časově nenáročná metoda, neboť nevyžaduje žádný postup čištění. Tato metoda slouží k rychlému screeningu velkého množství vzorků v krátkém čase [130]. Princip metody je založen na vysoce specifické interakci mezi antigenem a protilátkou za tvorby imunokomplexu [130, 131]. Antigen či protilátka mohou v komplexu vystupovat jak v neznačené formě, tak v enzymaticky značené formě tzv. konjugátu [131].

ELISA může být využívána v různých uspořádáních – přímá, nepřímá, sendvičová, kompetitivní [130, 131] – viz Obrázek 10.



Obrázek 10 Základní typy uspořádání ELISA metody
Vlastní grafické zpracování pomocí editoru vektorové grafiky Inkscape 0.92.

Přímá metoda spočívá v přímém navázání konjugátu na hledaný antigen, který je imobilizován na pevné fázi – tj. na dně jamky mikrotitrační destičky. Po interakci vzniká komplex antigen-konjugát [131].

Nepřímá metoda je založena na vazbě antigenu s primární protilátkou, na kterou se následně naváže konjugát. Po interakci vzniká komplex antigen-protilátka-konjugát [131].

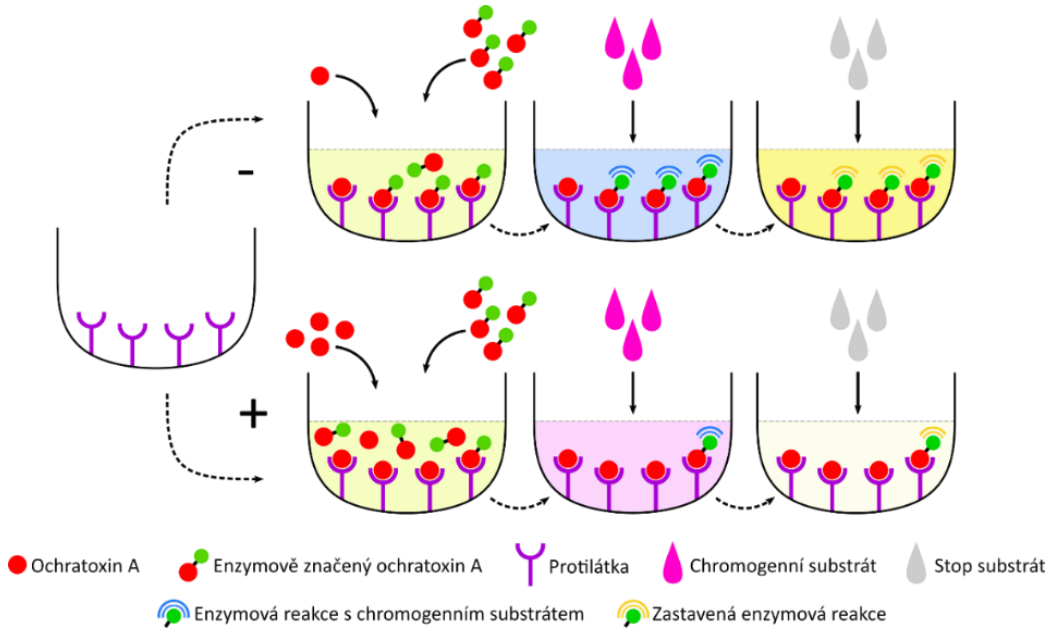
Sendvičová metoda je založena na interakci dvou odlišných protilátek – imobilizované (ukotvené) a signální (značené) protilátky se dvěma odlišnými epitopy, tj. vazebnými částmi stanovovaného antigenu. Po interakci vzniká komplex protilátka-antigen-konjugát (přímá sendvičová metoda), případně protilátka-antigen-protilátka-konjugát (nepřímá sendvičová metoda) [131].

Kompetitivní metoda spočívá v kompetici značených a neznačených protilátek o jeden epitop antigenu či značených a neznačených antigenů o protilátku. Na rozdíl od předchozích uspořádání je signál u této metody nepřímo úměrný koncentraci hledaného analytu [131].

Interakce mezi antigenem a protilátkou mohou být ovlivněny tzv. interferujícími látkami, což může způsobit falešnou pozitivitu či negativitu výsledku [131]. Příkladem falešné pozitivity je tzv. křížová reaktivita – tj. reakce protilátky se strukturně podobným, avšak necílovým antigenem, která může vést k nadhodnoceným výsledkům [61, 131]. Příkladem falešné negativity je tzv. „hook efekt“, tj. podhodnocení výsledků způsobené nadměrným množstvím antigenu v reakční směsi, což vede k omezené tvorbě „sendvičových“ komplexů protilátka-antigen-konjugát [131]. Podrobněji jsou uvedeny pouze principy ELISA metod využívaných v rámci doktorského studia, tj. pro OTA – viz kapitola 3.3.1.1 a CIT – viz podkapitola 3.3.1.2.

3.3.1.1 Princip stanovení ochratoxinu A pomocí ELISA metody

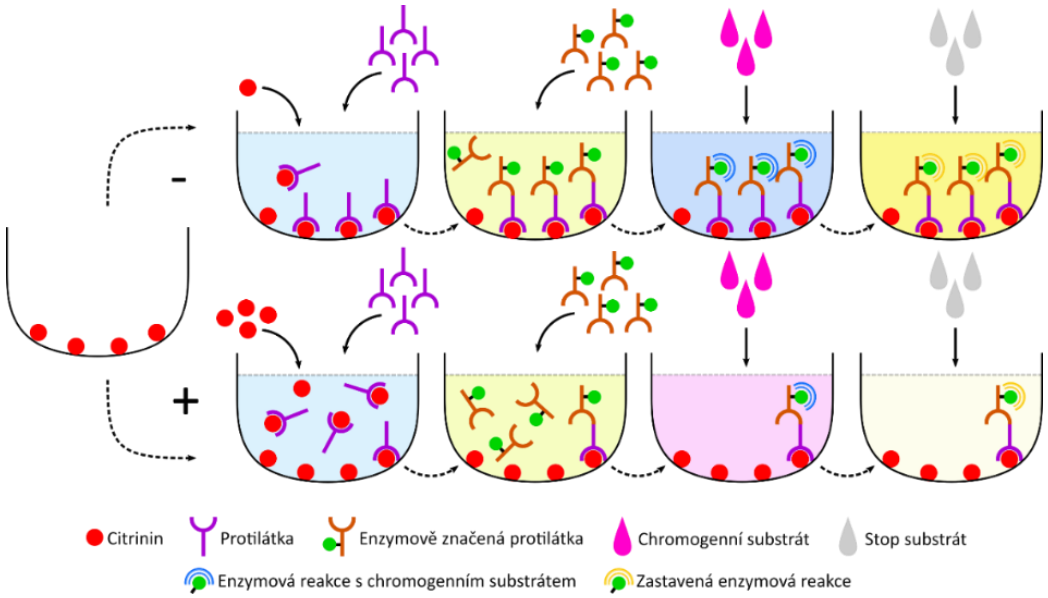
Pro stanovení OTA se využívá přímá kompetitivní ELISA, která je určená pro detekci hledaného antigenu, tj. v tomto případě OTA. Princip metody je tedy založen na reakci typu „antigen-protilátka“. Imobilizované protilátky jsou zakotvené na dně 96 jamek mikrotitrační destičky. Po aplikaci vzorku/standardu a následně konjugátu (OTA značený enzymem umožňující detekci) do jamky dochází ke kompetici mezi neznačenými (ze vzorku/standardu) a značenými (z konjugátu) antigeny. Konjugát, který se nenaváže na specifické protilátky, je při promývacím procesu odstraněn z jamky. Po aplikaci chromogen substrátu, který reaguje s konjugovaným enzymem, dochází ke vzniku modrého zbarvení, přičemž intenzita zbarvení je nepřímo úměrná s koncentrací antigenu ve vzorku/standardu. Následným použitím STOP substrátu dochází k zastavení reakce za vzniku žlutého zbarvení. Pro kvantitativní stanovení OTA je využíván ELISA reader – tj. spektrofotometr, který v jednotlivých jamkách odečítá absorbanci při vlnové délce 450 nm. Absorbance je v tomto případě nepřímo úměrná koncentraci OTA ve vzorku. Obrázek 11 znázorňuje princip stanovení OTA metodou ELISA [132].



Obrázek 11 Princip stanovení ochratoxinu A kompetetivní ELISA metodou
Vlastní grafické zpracování pomocí editoru vektorové grafiky Inkscape 0.92.

3.3.1.2 *Princip stanovení citrininu pomocí ELISA metody*

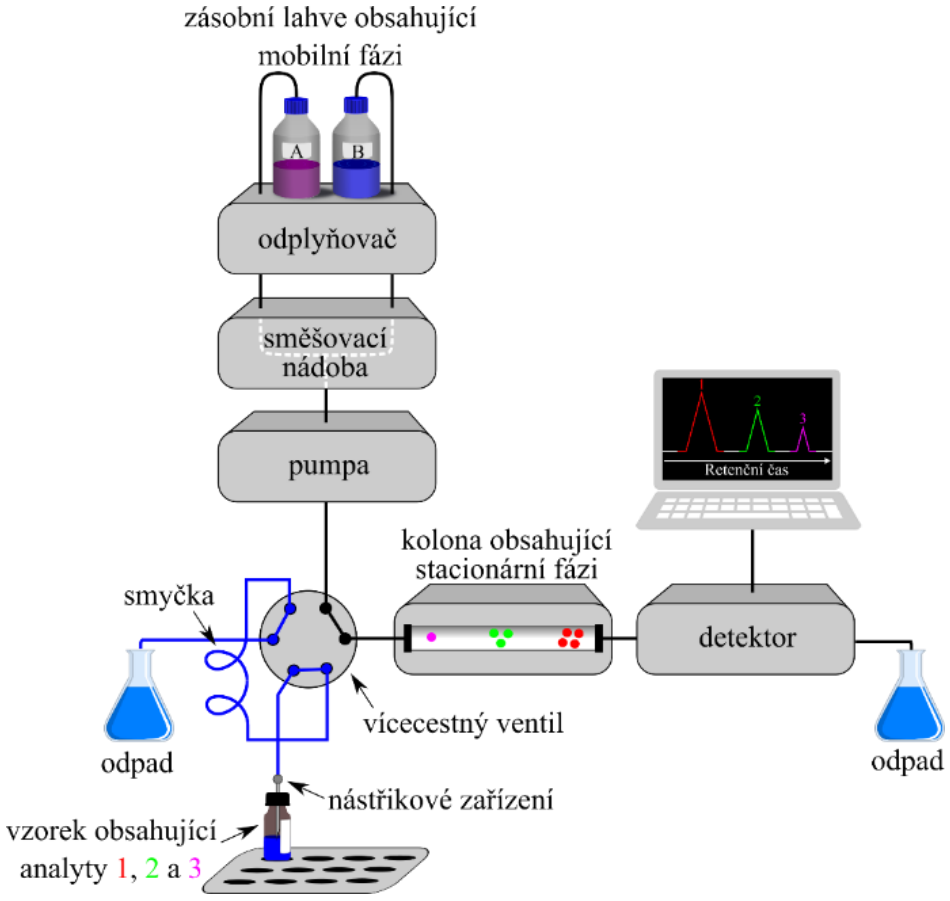
V rámci doktorské práce byl paralelně s OTA pomocí metody ELISA okrajově řešen také CIT. Pro stanovení CIT se využívá nepřímá kompetitivní ELISA. Princip metody je založen na reakci typu „antigen-protilátka“, kdy antigenem je v tomto případě CIT. Dno 48jamkové mikrotitrační destičky je pokryto imobilizovaným antigenem. Po aplikaci vzorku/standardu a následně primárních anti-CIT protilátek do jamky dochází ke kompetici mezi volným a imobilizovaným antigenem o epitopy, tj. vazebná místa, protilátek. Po promývacím procesu, při kterém jsou z jamek odstraněny volné primární protilátky, je aplikován konjugát obsahující sekundární protilátky značené enzymem peroxidázou, které se naváží na již navázané primární anti-CIT protilátky. Nenavázané sekundární protilátky jsou opět odstraněny v promývacím procesu. Po aplikaci chromogen substrátu dochází k reakci s konjugovaným enzymem na sekundárních protilátkách za vzniku modrého zbarvení. Po přidání STOP substrátu dochází k zastavení reakce za vzniku žlutého zbarvení. Pro kvantitativní stanovení CIT je využíván ELISA reader – tj. spektrofotometr, který z jamek odečítá absorbanci při vlnové délce 450 nm. Absorbance je v tomto případě nepřímo úměrná koncentraci CIT ve vzorku. Obrázek 12 znázorňuje princip stanovení CIT metodou ELISA [133].



Obrázek 12 Princip stanovení citrininu kompetetivní ELISA metodou
Vlastní grafické zpracování pomocí editoru vektorové grafiky Inkscape 0.92.

3.3.2 Kapalinová chromatografie s fluorescenčním detektorem

Chromatografie je moderní separační technika, která se používá k preparativním (separace jednotlivých složek směsi) i analytickým (kvalitativní a kvantitativní hodnocení jednotlivých složek směsi) účelům [134, 135]. Princip chromatografie spočívá v rozdílné interakci složek směsi se dvěma nemísitelnými fázemi – fází pohyblivou, tj. mobilní (MF) a nepohyblivou, tj. stacionární (SF). Technika využívající jako MF kapalinu se nazývá kapalinová chromatografie (LC) [135]. Kapalinový chromatograf sestává z několika modulů – viz Obrázek 13.



Obrázek 13 Schéma kapalinového chromatografu.
Vlastní grafické zpracování pomocí editoru vektorové grafiky Inkscape 0.92.

MF skládající se z jednoho či více roztoků je čerpána ze zásobních lahví pumpou do odplyňovače a následně do nástřikového zařízení. Složení MF, resp. poměr roztoků, může být po celu dobu měření konstantní – tzv. izokratická eluce či proměnlivé v čase – tzv. gradientová eluce. Za pomocí smyčky a vícecestného ventilu je analyzovaný vzorek vnášen do MF a unášen do termostatované analytické kolony, která bývá často vybavena ještě předkolonou. V analytické koloně naplněné SF následně probíhá vlastní separační proces. Látky s vyšší afinitou k SF jsou v koloně zadržovány – hovoří se o tzv. retenci. Naopak látky

s nižší afinitou k SF jsou kolonou unášeny rychleji. Jednotlivé analyty jsou zaznamenány detektorem v rozdílném čase. Záznam z detektoru je vyhodnocen počítačovým softwarem, přičemž výstupem je tzv. chromatogram znázorňující jednotlivé analyty v podobě píků v daných tzv. retenčních časech, které udávají dobu, za kterou byl daný analyt detekován od jeho nástřiku, přičemž platí, že čím je afinita k SF vyšší, tím je retenční čas delší [134, 135].

LC technika může být doplněna různými typy detektorů, např. fluorescenčním detektorem [136–139], UV/VIS detektorem [140], hmotnostním spektrometrem [141] či tandemovým hmotnostním spektrometrem [142–144]. Vzhledem k fluorescenční povaze OTA, ale také CIT, je hojně využívána metoda HPLC-FLD [23, 145], přičemž fluorescenční detektor je v posledních letech považován za třetí nejvyužívanější techniku v LC [134].

4 Publikované práce

V této části je uveden přehled 8 vydaných publikací zabývajících se významnými mykotoxiny, z nichž 4 jsou rešeršního charakteru a 4 výzkumného charakteru. Všechny publikace byly uveřejněny v impaktovaných časopisech s IF v rozsahu 3,531–6,475, z toho 7x v prvním kvartilu (Q1) a 1x ve druhém kvartilu (Q2).

Výzkumná činnost probíhala na pracovišti Univerzity Hradec Králové, Přírodovědecké fakulty, katedry biologie. Dílčí studie vycházejí ze specifických výzkumů č. 2112/2019, 2115/2020, 2010/2021 a 2106/2022 podpořených Přírodovědeckou fakultou Univerzity Hradec Králové.

Každá publikace je okomentována komentáři 1–8 – viz kapitoly 4.1–4.8. Komentář 9 je věnován připravované publikaci výzkumného charakteru – viz kapitola 4.9.

I. Rešeršní práce

Presence of mycotoxins in milk thistle (*Silybum marianum*) food supplements: A review
Pickova, D., Ostry, V., Toman, J. & Malir, F.
Toxins (2020), 12(12):782. 1–21
https://doi.org/10.3390/toxins12120782
Food Science & Technology; Toxicology
Impact factor $(IF)_{2019}$: 3,531; 34/139 (Q1); 21/92 (Q1)
Article influence score $(AIS)_{2019}$: 0,756 (Q1/Q2)
Podíl autorky: konceptualizace, metodologie, výzkum, správa dat, psaní – původní draft, psaní – revize a úpravy, vizualizace, administrace projektu, získávání finančních prostředků

A review on mycotoxins and microfungi in spices in the light of the last five years
Pickova, D., Ostry, V., Malir, J., Toman, J. & Malir, F.
Toxins (2020), 12(12):789. 1–33
https://doi.org/10.3390/toxins12120789
Food Science & Technology; Toxicology
Impact factor $(IF)_{2019}$: 3,531; 34/139 (Q1); 21/92 (Q1)
Article influence score $(AIS)_{2019}$: 0,756 (Q1/Q2)
Podíl autorky: konceptualizace, metodologie, výzkum, správa dat, psaní – původní draft, psaní – revize a úpravy, vizualizace, administrace projektu, získávání finančních prostředků

A recent overview of producers and important dietary sources of aflatoxins

Pickova, D., Ostry, V. & Malir, F.

Toxins (2021), 13(3):186. 1–15

https://doi.org/10.3390/toxins13030186

Food Science & Technology; Toxicology

Impact factor $(IF)_{2020}$: 4,546; 32/143 (Q1); 21/93 (Q1)

Article influence score $(AIS)_{2020}$: 0,838 (Q1/Q2)

Podíl autorky: konceptualizace, metodologie, výzkum, správa dat, psaní – původní draft, psaní – revize a úpravy, vizualizace, administrace projektu, získávání finančních prostředků

Aflatoxins: History, significant milestones, recent data on their toxicity and ways to mitigation

Pickova, D., Ostry, V., Toman, J. & Malir, F.

Toxins (2021), 13(6):399. 1–23

https://doi.org/10.3390/toxins13060399

Food Science & Technology; Toxicology

Impact factor $(IF)_{2020}$: 4,546; 32/143 (Q1); 21/93 (Q1)

Article influence score $(AIS)_{2020}$: 0,838 (Q1/Q2)

Podíl autorky: formální analýza, výzkum, správa dat, psaní – revize a úpravy, získávání finančních prostředků

II. Výzkumné práce

II.a Stanovení ochratoxinu A v biologickém materiálu rostlinného původu

Natural occurrence of ochratoxin a in spices marketed in the Czech Republic during 2019–2020

Pickova, D., Toman, J., Ostry, V. & Malir, F.

Foods. (2021), 10(12):2984. 1–18

https://doi.org/10.3390/foods10122984

Food Science & Technology

Impact factor $(IF)_{2020}$: 4,350; 37/143 (Q2)

Article influence score AIS_{2020}: 0,642 (Q2)

Podíl autorky: konceptualizace, metodologie, software, validace, formální analýza, výzkum, zdroje, správa dat, psaní – původní draft, psaní – revize a úpravy, vizualizace, administrace projektu, získávání finančních prostředků

II.b Stanovení ochratoxinu A v biologickém materiálu živočišného původu

Investigation of ochratoxin a in blood sausages in the Czech Republic: Comparison with data over Europe

Pickova, D., Toman, J., Mikyskova, P., Ostry, V. & Malir, F.

Food Research International (2022), 157(2022):111473. 1–8

https://doi.org/10.1016/j.foodres.2022.111473

Food Science & Technology

Impact factor $(IF)_{2021}$: 7,425; 13/144 (Q1)

Article influence score $(AIS)_{2021}$: 0,919 (Q1)

Podíl autorky konceptualizace, metodologie, software, validace, formální analýza, výzkum, zdroje, správa dat, psaní – původní draft, psaní – revize a úpravy, vizualizace, administrace projektu, získávání finančních prostředků

II.c Stanovení ochratoxinu A v biologickém materiálu lidského původu

Analyses of biomarkers of exposure to nephrotoxic mycotoxins in a cohort of patients with renal tumours

Malir, F., Louda, M., Ostry, V., Toman, J., Ali, N., Grosse, Y., Malirova, E., Pacovsky, J., **Pickova, D.**, Brodak, M. & Pfohl-Leszkowicz, A.

Mycotoxin research (2019), 35(4):391–403.

https://doi.org/10.1007/s12550-019-00365-9

Toxicology; Mycology

Impact factor $(IF)_{2018}$: 3,741; 19/93 (Q1); 7/29 (Q1)

Article influence score $(AIS)_{2018}$: 0,615 (Q2/Q3)

Podíl autorky: výzkum, formální analýza, psaní – revize a úpravy

Investigation of ochratoxin A biomarkers in biological materials obtained from patients suffering from renal cell carcinoma

Malir, F., Louda, M., Toman, J., Ostry, V., **Pickova, D.**, Pacovsky, J., Brodak, M. & Pfohl-Leszkowicz, A.

Food and Chemical Toxicology. (2021), 158(2021):112669. 1–13

https://doi.org/10.1016/j.fct.2021.112669

Food Science & Technology; Toxicology

Impact factor $(IF)_{2020}$: 6,025; 14/143 (Q1); 9/93 (Q1)

Article influence score $(AIS)_{2020}$: 0,868 (Q1/Q2)

Podíl autorky: formální analýza, vizualizace, psaní – revize a úpravy, získávání finančních prostředků

4.1 Komentář 1: Výskyt mykotoxinů v doplňcích stravy na bázi ostropestřce mariánského (*Silybum marianum*): Review

Rešeršní studie „*Presence of mycotoxins in milk thistle (*Silybum marianum*) food Supplements: A review*“ byla publikována 8. prosince 2020 v časopise Toxins ve speciálním vydání „*Occurrence and risk assessment of mycotoxins*“ – viz Příloha 1.

Publikace pojednává o doplňcích stravy na bázi byliny ostropestřce mariánského (*Silybum marianum* (L.) Gaertn). Shrnuje benefitní účinky silymarinového komplexu, tj. obsahových látek, ze kterých je silybin považován za nejvýznamnější, neboť je pro své hepatoprotektivní účinky celosvětově používán především lidmi s onemocněním jater. Zároveň ale poukazuje na náchylnost těchto doplňků ke kontaminaci VMH a jejich mykotoxiny.

V tomto přezkumu jsou uceleny výsledky z 9 relevantních původních studií vydaných v letech 2009–2019 zabývajících se výskytem VMH a/nebo mykotoxinů v 5 formách doplňků stravy na bázi ostropestřce mariánského – semínka, kapsle, tablety, granule a extrakty. Napříč studiemi bylo zohledněno 57 mykotoxinů. Na základě dostupných studií se nejkritičtější zdá být 12 mykotoxinů: AME, AOH, tentoxin produkované rodem *Alternaria* a DON, HT-2, T-2, ZEN, beauvericin a enniatiny A, A_1, B, B_1 produkované rodem *Fusarium* – Tabulka 7.

Tabulka 7 Kontaminace doplňků stravy na bázi *Silybum marianum* mykotoxiny

Mykotoxin[1]	n+/n	n+ (%)	Mykotoxin[1]	n+/n	n+ (%)
AME	57/58	98	**ENNB**	54/58	93
AOH	54/58	93	**ENNB$_1$**	55/58	95
BEA	54/58	93	**HT-2**	48/65	74
DON	37/67	55	**T-2**	52/67	78
ENNA	51/58	88	**TEN**	50/58	86
ENNA$_1$	52/58	90	**ZEN**	49/67	73

[1] AME, alternariol monomethyl ether; AOH, alternariol; BEA, beauvericin; DON, deoxynivalenol; ENNA, enniatin A, ENNA1, enniatin A1, ENNB, enniatin B, ENNB1, enniatin B1; T-2, T-2 toxin; TEN, tentoxin; ZEN, zearalenon.

Tento přehled poukazuje na možnost znehodnocení hepatoprotektivního účinku, jsou-li tyto doplňky současně kontaminovány mykotoxiny, mimo jiné s hepatotoxickým účinkem jako např. AOH, AME, TEN, DON, T-2 a ZEN. V závislosti na množství těchto mykotoxinů může hepatotoxický účinek mnohdy i převažovat nad benefitními účinky, a proto je používání takto kontaminovaného ostropestřce nevhodné, a to zejména u uživatelů s jaterními obtížemi.

Přehled poukazuje na potřebu striktního sledování výskytu hepatotoxických mykotoxinů v komerčně prodávaných doplňcích stravy na bázi ostropestřce mariánského, a také na potřebu zavedení regulačních limitů pro mykotoxiny v doplňcích stravy na rostlinné bázi, neboť v právních předpisech EU nejsou dosud zahrnuty.

4.2 Komentář 2: Přehled mykotoxinů a mikromycetů v koření za posledních 5 let

Rešeršní studie „*A review on mycotoxins and microfungi in spices in the light of the last five years*“ byla publikována 11. prosince 2020 v časopise Toxins ve speciálním vydání „*Exposure to mycotoxins via food chain*“ – viz Příloha 2.

Koření je malou, avšak nedílnou součástí stravy. Jeho produkce probíhá převážně v rozvojových zemích s tropickým a/nebo subtropickým klimatem, kde příznivé podmínky usnadňují růst VMH a produkci mykotoxinů. Kontaminace je navíc v těchto zemích mnohdy podpořena nedostatečnou zemědělskou, hygienickou a výrobní praxí. Ačkoliv koření nepředstavuje hlavní přívod mykotoxinů do organismu, jeho častá konzumace může vést k nepřetržitému přívodu.

Rešerše poskytuje komplexní souhrn výsledků z 56 relevantních původních studií zabývajících se kontaminací koření VMH a/nebo mykotoxiny v posledních 5 letech, tj. od r. 2015. V přezkumu je zahrnuto 38 druhů koření, 17 mykotoxinů a 14 VMH. Paprika, chilli, černý a bílý pepř, zázvor a kurkuma jsou nejčastěji kontaminované AFs a OTA, přičemž OTA je typický také v lékořici. Tato koření jsou v souvislosti s AFs a OTA součástí legislativy EU, nicméně z přehledu je patrné, že dochází ke kontaminaci mnoha dalších druhů koření různými mykotoxiny, jejichž regulace v legislativě chybí – viz Tabulka 8.

Tabulka 8 Významný výskyt mykotoxinů v koření nepodléhající regulacím EU

Mykotoxiny	Koření
Aflatoxiny	pískavice řecké seno, koriandr, římský kmín, fenykl
Ochratoxin A	muškátový květ, pískavice řecké seno, kardamom, kmín kořenný, koriandr, fenykl
Citrinin	chilli, zázvor, koriandr, pískavice řecké seno
Fumonisiny	paprika, lékořice
Trichotheceny	paprika
Zearalenon	paprika
Alternáriové mykotoxiny	paprika

Studie také poskytuje přehled o celosvětové produkci koření na základě statistické databáze Organizace pro výživu a zemědělství FAOSTAT a oznámení souvisejících s výskytem AFs a OTA v koření dle databáze RASFF. Velká pozornost je věnována regulačním limitům pro AFs a OTA v koření, a to v celosvětovém kontextu. Především se ale zaměřuje na legislativu EU, která je celosvětově považována za nejkomplexnější a je závazná pro 27 členských států.

Tento přehled poukazuje na nedostatečné regulace mykotoxinů v koření a zdůrazňuje důležitost sledování různých mykotoxinů, nejen AFs a OTA, v různých i dosud neregulovaných druzích koření. Také zdůrazňuje potřebu zvýšení informovanosti spotřebitelů o zdravotním riziku plynoucím z expozice mykotoxinům prostřednictvím koření.

4.3 Komentář 3: Recentní přehled producentů a zdrojů dietární expozice aflatoxinů

Rešeršní studie „*A recent overview of producers and important dietary sources of aflatoxins*“ byla publikována 3. března 2021 v časopise Toxins ve speciálním vydání „*Occurrence and risk assessment of mycotoxins*“ – viz Příloha 3.

V roce 2020 si vědecká komunita připomněla 60. výročí objevení AFs, tj. mykotoxinů s nejsilnějším prokázaným karcinogenním účinkem na člověka. AFs si však i nadále zasluhují pozornost, a to nejen kvůli jejich toxicitě, a tedy možným škodlivým účinkům na zdraví člověka, ale také kvůli ekonomickým ztrátám vznikajícím v důsledku kontaminace plodin. O tom svědčí několik vědeckých prací a řada oznámení hlášených klíčovými nástroji pro bezpečnost potravin pro sledování AFs v potravinách, případně krmivech. K příležitosti 60. výročí byl vydán přehled současně známých producentů a významných dietárních zdrojů AFs.

Rešerše poskytuje přehled 28 známých aflatoxinogenních potravinových producentů rodu *Aspergillus* ze sekcí *Flavi* (22 producentů), *Nidulantes* (4) a *Ochraceorosei* (2). Druhy *A. flavus, A. parasiticus* a *A. nomius* jsou považovány za nejvýznamnější producenty ze sekce *Flavi*. V roce 2020 byli konfirmováni tři noví producenti *A. agricola*, *A. toxicus* a *A. texensis*.

Každodenní dietární expozice AFs je celosvětový problém. AFs kontaminují řadu potravin, především arašídy, pistáciové oříšky, sušené fíky, lískové oříšky, koření, mandle, rýži, melounová semínka, para ořechy a kukuřici. Potraviny živočišného původu (např. mléko či živočišné tkáně) se podílejí na přívodu mykotoxinů do organismu méně.

Rešerše dále poskytuje přehled problematických komodit z pohledu kontaminace AFs v závislosti na kontinentu na základě vědeckých studií. Z celosvětového hlediska jsou též řešeny právní předpisy stanovující MPL pro AFs v potravinách. Největší pozornost je věnována regulačním limitům platným v EU. Dále je uveden přehled nejnovějších oznámení souvisejících s výskytem AFs v potravinách v databázi RASFF za roky 2015–2020 a v Mezinárodní síti úřadů bezpečnosti potravin INFOSAN za roky 2016–2020. Prostřednictvím infografiky jsou znázorněny potraviny kontaminované AFs včetně jejich původu dle RASFF za rok 2020.

Navzdory snahám o zmírnění koncentrací AFs v potravinách, a tím zajištění bezpečnosti potravin, jsou AFs i nadále přítomny, dokonce i ve vysokých koncentracích překračujících 1 000 ng/g, což je koncentrace spojovaná s rozvojem aflatoxikózy. Z tohoto důvodu zůstávají i nadále řešeným problémem ve světě.

4.4 Komentář 4: Aflatoxiny: Historie, významné milníky, recentní data o jejich toxicitě a způsoby zmírňování kontaminace

Rešeršní studie „*Aflatoxins: History, significant milestones, recent data on their toxicity and ways to mitigation*“ byla publikována 3. června 2021 v časopise Toxins ve speciálním vydání „*Occurrence, toxicity and mitigation od aflatoxins*“ – viz Příloha 4.

Rešeršní studie je pomyslným pokračováním publikace „*A Recent Overview of Producers and Important Dietary Sources of Aflatoxins*“. Zabývá se AFs ze tří aspektů – tj. historie, recentní toxikologie a novodobé strategie pro zmírňování úrovní AFs v potravinách.

První část přehledu poskytuje historický vývoj s významnými milníky od roku 1960 po současnost. První milník zaznamenává vypuknutí aflatoxikózy přezdívané krůtí „X“ onemocnění, při které na londýnské farmě zahynulo více než 100 000 krůťat po konzumaci brazilské arašídové moučky napadené VMH *Aspergillus flavus*. Toxin objevený v kontaminované moučce dostal název „***A****spergillus* ***fla****vus* ***toxin***“ – aflatoxin. Od té doby byl zahájen rozsáhlý výzkum AFs a pokračuje dodnes.

Druhá část se zbývá toxikologickými daty z nenovějších studií prováděných na zvířatech *in vivo* a různých buněčných liniích *in vitro*. Pozornost je dále věnována toxikologickým interakcím mezi: 1) AFs a dalšími mykotoxiny, tj. DON, OTA a ZEN; 2) AFs a dalšími kontaminanty, tj. těžkými kovy, pesticidy a toxiny sinic; 3) AFs a viry hepatitidy B a C.

Třetí část pojednává o předsklizňových a posklizňových strategiích pro prevenci a zmírnění kontaminace plodin AFs, které jsou klíčové pro zajištění bezpečnosti potravin a snížení zdravotního rizika. Některé fyzikální (např. třídění, tepelné ošetření, ozařování), chemické (např. adsorbenty, kyseliny, zásady) a biologické metody (bakterie, kvasinky) a metody genetického inženýrství byly prokázány jako účinné při eliminaci AFs v potravinách i krmivech.

4.5 Komentář 5: Přirozený výskyt ochratoxinu A v koření zakoupeném na trhu v České republice v letech 2019–2020

Experimentální studie „*Natural occurrence of ochratoxin A in spices marketed in the Czech Republic during 2019–2020*“ byla publikována 3. prosince 2021 v časopise Foods ve speciálním vydání „*Application of chromatography to food analysis*“ – viz Příloha 5.

Koření je celosvětově oblíbenou ingrediencí, a to především pro zlepšení chutě a vůně pokrmů. Pro tyto účely jsou využívány různé části rostlin – např. listy, semena, kořeny, plody, kůra, pupeny nebo stonky. Koření je dováženo zejména z rozvojových zemí, ve kterých klimatické podmínky v kombinaci s nedostatečnou zemědělskou, hygienickou a výrobní praxí představují důvod ke zvýšeným obavám z kontaminace koření VMH a mykotoxiny. Za účelem ochrany zdraví spotřebitele byly v legislativě EU zavedeny limity pro regulaci OTA v koření, ovšem v době vydání publikace existovaly jen pro omezený počet druhů – tj. pepř, muškátový oříšek, zázvor, kurkuma, chilli a lékořice. Současná legislativa již souhrnně pokrývá všechna ostatní koření včetně směsí, což bylo toho času v EK pouze předmětem diskuse.

Studie je zaměřena na výskyt OTA v 54 druzích jednodruhových koření zakoupených v 6 šaržích v letech 2019–2020 na českém trhu. OTA byl stanoven pomocí metody HPLC-FLD za předchozího přečištění na IAK. Získané výsledky prokázaly, že je česká populace exponovaná OTA z koření, neboť celkem u 19 (35 %) druhů byl shledán vždy alespoň jeden vzorek převyšující koncentrací OTA limit kvantifikace (0,1 ng/g). Naměřené koncentrace OTA byly v rozmezí 0,11–38,46 ng/g (kurkuma) – Tabulka 9.

Tabulka 9 Průměrné koncentrace ochratoxinu A v 19 jednodruhových kořeních zakoupených na českém trhu

Koření	Průměr ± SD (ng/g)	Koření	Průměr ± SD (ng/g)	Koření	Průměr ± SD (ng/g)
kurkuma	19,82 ± 11,93	chilli drcené	1,43 ± 0,48	pepř černý	0,31 ± 0,20
lékořice kořen	11,94 ± 3,27	vanilka	1,42 ± 0,33	hřebíček	0,29 ± 0,18
chilli mleté	7,50 ± 1,34	pomerančová kůra	1,04 ± 0,30	citronová kůra	0,18 ± 0,12
muškátový květ	5,27 ± 0,83	kmín římský	0,46 ± 0,27	sumah	0,14 ± 0,08
zázvor	3,40 ± 0,48	muškátový ořech	0,43 ± 0,30	pepř růžový	0,11*
kajenský pepř	2,59 ± 0,61	hořčice bílá	0,38 ± 0,30		
paprika sladká	2,26 ± 0,60	pepř bílý	0,36 ± 0,23		

* jediný pozitivní výsledek

Jedinečnost studie spočívá především ve velkém počtu studovaných druhů koření na českém trhu. Kromě nejvíce probádaných koření, jako je chilli a pepř, byly zahrnuty také druhy, které nebyly z hlediska mykotoxinů v legislativě EU regulovány, včetně 20 „nových“ druhů koření, které toho času nebyly nikdy nebo alespoň v recentní době studovány na přítomnost OTA.

4.6 Komentář 6: Výzkum ochratoxinu A v jelitech: Srovnání s daty v Evropě

Experimentální studie „*Investigation of ochratoxin A in blood sausages in the Czech Republic: Comparison with data over Europe*“ byla publikována 7. června 2022 v časopise Food research international – viz Příloha 6.

Jelito je masný výrobek, který dle české receptury obsahuje vepřové maso, ječné kroupy, případně kousky pečiva, vepřovou krev, vnitřnosti, tuk, koření a vepřové střívko. Všechny tyto ingredience jsou potenciálním zdrojem OTA. Mezi produkty živočišného původu jsou vepřové produkty považovány z hlediska kontaminace OTA za nejrizikovější. Je to dáno citlivostí vepřů na OTA, který se již při subchronických dávkách kumuluje, a to zejména v jejich krvi, kterou je dále distribuován především do ledvin, jater, svalových a tukových tkání. Vysoká vazebnost OTA k sérovým proteinům, zejména albuminu, významně ovlivňuje biologický poločas, který se u prasat pohybuje v rozmezí 72–120 hodin a je mezi běžnými hospodářskými zvířaty nejdelší. Přídavek vepřové krve tak může významně zvýšit obsah OTA ve finálním produktu. Kromě živočišné složky, včetně sádla, které u prasat tvoří také významné depo OTA, mohou k celkové kontaminaci přispívat i složky rostlinného původu. Z těchto důvodů může být konzumace jelit, ale i jiných vepřových produktů významným zdrojem OTA pro člověka.

V obchodní síti České republiky bylo v průběhu let 2020–2021 zakoupeno celkem 200 vzorků jelit, které byly analyzovány na OTA s využitím metody HPLC-FLD za předchozího přečištění na IAK. Tato studie prokázala kontaminaci všech 200 vzorků jelit OTA. Koncentrace OTA ve všech případech překročily limit kvantifikace 0,1 ng/g – viz Tabulka 10. Jelikož legislativa EU nezahrnuje regulační limity pro OTA v živočišných produktech, byly výsledky porovnány pouze s italským limitem 1 ng/g pro „vepřové maso a odvozené produkty“. Celkem 66 % vzorků obsahovalo OTA v koncentraci překračující tento limit.

Tabulka 10 Statistické zpracování – ochratoxin A v jelitech

n/n^+	n^+ (%)	Medián (ng/g)	90. percentil (ng/g)	Rozsah (ng/g)
200/200	100	1,26	2,77	0,15–5,68

Z výsledků je zřejmé, že u české populace dochází k expozici OTA prostřednictvím konzumace jelit. Z toho důvodu je třeba sledovat OTA v živočišných produktech a zavést příslušné regulační limity v EU. Počet studií zabývajících se výskytem OTA v jelitech je však velmi omezený a existující studie se zabývají relativně nízkým počtem vzorků. Tato studie je jedinečná nejen svým zaměřením, ale i rozsáhlým počtem vzorků. Velký význam mají především výsledky studie, které prokázaly nejen 100% pozitivitu, ale vzhledem k nalezeným koncentracím OTA a známé spotřebě jelit také potenciální riziko pro nádorový efekt.

4.7 Komentář 7: Analýza biomarkerů expozice nefrotoxickým mykotoxinům u kohorty pacientů s nádory ledvin

Experimentální studie „*Analyses of biomarkers of exposure to nephrotoxic mycotoxins in a cohort of patients with renal tumours*“ byla publikována 29. června 2019 v časopise Mycotoxin research – viz Příloha 7.

Česká republika zaujímá s četností 30 incidencí nádorů ledvin a vývodných močových cest/100 000 obyvatel první místo nejen v Evropě, ale i ve světě, ovšem etiologie tohoto onemocnění je nejasná a předpokládá se současné působení více faktorů, přičemž jedním z nich by mohlo být působení nefrotoxických mykotoxinů OTA a CIT. Byla položena hypotéza, zda se zmíněné nefrotoxické mykotoxiny mohou na tomto onemocnění významně podílet.

V letech 2015–2017 byla na Urologické klinice ve Fakultní nemocnici Hradec Králové odebrána moč a krev 50 pacientům s diagnózou C64 (zhoubný nádor ledvin mimo pánvičku), C675 (zhoubný nádor hrdla močového měchýře) nebo N131 (hydronefróza se strikturou ureteru). Byla provedena analýza mykotoxinů metodou HPLC-FLD pro OTA a HPLC-MS/MS pro CIT za předchozího přečištění na IAK – viz Tabulka 11.

Tabulka 11 Koncentrace ochratoxinu A a citrininu v biologickém materiálu českých pacientů s nádorem ledvin

Mykotoxin	Materiál	n/n^+	n^+ (%)	Průměr ± SD (ng/g)	Medián (ng/g)	Rozsah (ng/g)
OTA	moč	31/50	62	5,9 ± 5,97	5,41	nd–27,8
	krev	24/50	48	145 ± 213,8	20	nd–830
CIT	moč	45/50	90	16 ± 20	8	nd–87
	krev	49/50	98	61 ± 35	51	nd–182

Výsledky OTA a CIT v moči a krvi byly porovnány s kontrolními skupinami s využitím nepárového dvouvýběrového t-testu na hladině významnosti $\alpha = 0,05$. V případě OTA nebyl prokázán statisticky významný rozdíl mezi naměřenými koncentracemi OTA v moči a krvi českých pacientů a českých dobrovolníků. V případě CIT v moči a krvi nebyla dostupná data od zdravé české populace, a proto byla pro porovnání využita relevantní data zdravé německé populace. Zatímco v krvi nebyl prokázán statisticky významný rozdíl, v moči byla prokázána statisticky významná nižší koncentrace CIT u českých pacientů než u zdravých německých dobrovolníků.

Ačkoliv přítomnost obou mykotoxinů v lidské krvi a moči je přímým ukazatelem expozice českých pacientů OTA a CIT, souvislost mezi expozicí těmto nefrotoxickým mykotoxinům a výskytem nádorových onemocnění ledvin se v této studii nepodařilo jednoznačně prokázat.

4.8 Komentář 8: Studium biomarkerů ochratoxinu A v biologických materiálech získaných od pacientů s karcinomem ledvin

Experimentální studie „*Investigation of ochratoxin A biomarkers in biological materials obtained from patients suffering from renal cell carcinoma"* byla publikována 12. listopadu 2021 v časopise Food and chemical toxicology – viz Příloha 8.

V návaznosti na studii „*Analyses of biomarkers of exposure to nephrotoxic mycotoxins in a cohort of patients with renal tumours*" byla provedena analýza OTA včetně jeho metabolitů a DNA aduktů v biologickém materiálu pacientů s nádorem ledvin. Celkem se této studie účastnilo 33 pacientů, konkrétně 26 mužů a 7 žen ve věku 39–80 let.

V letech 2015–2017 byly na Urologické klinice ve Fakultní nemocnici Hradec Králové během operací 25 pacientů s diagnostikovaným karcinomem ledvin odebrány vzorky ledvin a odpovídajících nádorových tkání. Analýza OTA byla provedena metodou HPLC-FLD za předchozího přečištění na IAK – viz Tabulka 12.

Tabulka 12 Koncentrace ochratoxinu A v biologickém materiálu českých pacientů s nádorem ledvin

Materiál	n/n^+	n^+ (%)	Průměr ± SD (ng/g)	Medián (ng/kg)	Rozsah (ng/kg)
Ledviny	25/25	100	160 ± 110	186	72-385
Nádorová tkáň	25/25	100	150 ± 100	191	54-431

U 20 pacientů byly v moči analyzovány následující metabolity OTA: OTB, ochratoxin C, OTα, OP-OTA, otevřená laktonová forma OTB, 4S-OH-OTA, 4R-OH-OTA, OTHQ, OTHQ-glutathionový konjugát, OTB-glutathionový konjugát, OTHQ-N-acetylcystein, OTB-N-acetylcystein, dekarboxlovaný OTHQ. Pouze u dvou pacientů nebyl detekován žádný z těchto metabolitů OTA.

Zároveň byly u 20 pacientů v ledvinách analyzovány následující OTA-DNA adukty: C-C8dG-OTA, O-C8dG-OTA a adukty odvozené od OTHQ. DNA adukty byly nalezeny u 15 z 20 pacientů, přičemž adukt C-C8dG-OTA byl detekován ve všech 15 případech.

Tato studie ukázala 100% přítomnost OTA v ledvinách a nádorové tkáni a také korelaci mezi výskytem OTA metabolitů v moči a DNA aduktů v ledvinách u pacientů s nádorem ledvin.

4.9 Komentář 9: Stanovení ochratoxinu A a citrininu ve farmaceuticky významných rostlinách

V roce 2022 byla provedena experimentální studie na výskyt OTA a CIT v bylinách metodou HPLC-FLD v návaznosti na předchozí screening metodou ELISA. Studie byla podpořena specifickým výzkumem č. 2106/2022. Získané výsledky budou publikovány v roce 2023.

Bylinné produkty jsou stále častěji využívány, neboť mnoho lidí věří, že jsou bezpečnější a zdravější než syntetická léčiva [146–148]. V důsledku nesprávné manipulace, skladování či transportu však mohou být byliny napadeny toxinogenními VMH a kontaminovány mykotoxiny, které se tak mohou vyskytovat ve finálních produktech [148–150]. Dosud bylo v bylinách nalezeno více než 40 různých mykotoxinů [151]. Lidé konzumující bylinné produkty za účelem posílení zdraví tak mohou být v důsledku přívodu mykotoxinů z kontaminovaných bylin vystaveni zdravotnímu riziku [32]. Legislativa EU nebyla toho času z pohledu regulací mykotoxinů v bylinách dostatečně propracovaná a zahrnovala pouze regulace pro AFs a/nebo OTA pro zázvor a lékořici – viz Nařízení Komise č. 1881/2006 ve znění pozdějších předpisů [113]. V roce 2022 došlo ke změnám v regulaci OTA a od 1. ledna 2023 vešly v platnost nové MPL týkající se obecně sušených bylin a některých jednotlivých druhů bylin včetně kořenů proskurníku lékařského či kořenů pampelišky (smetánky), které jsou součástí této studie. Vzhledem k možným zdravotním rizikům plynoucím z užívání bylin je monitoring mykotoxinů v různých druzích bylin nezbytný.

Tato studie je zaměřena na výskyt nefrotoxických mykotoxinů OTA a CIT, které se mohou díky společnému producentovi *Penicillium verrucosum* vyskytovat společně a působit synergicky [152]. Vzhledem ke komplikovaným matricím, které se liší v různých částech dané rostliny i napříč různými druhy, může být analýza mykotoxinů v bylinách obtížná [153].

Na českém trhu bylo v průběhu let 2019–2020 zakoupeno 60 vzorků sušených bylin určených k přípravě odvarů – viz Tabulka 14. Pro zjištění míry kontaminace vzorků OTA a CIT byl proveden screening s využitím ELISA metody. Výsledky byly konfirmovány na HPLC-FLD za předchozího přečištění na IAK – viz Tabulka 13. Procentuální přechody OTA a CIT do odvarů byly zjišťovány u jednotlivých typů rostlinných částí (tj. kořen, nať, list, květ a plod) s využitím nejvíce přirozeně kontaminovaného vzorku pro danou část rostliny.

Tabulka 13 Výsledky ze stanovení ochratoxinu A a citrininu v bylinách

	OTA	**CIT**
n	60	60
n+ (%)	24 (40)	16 (26,7)
Rozsah (ng/g)	<LOQ (0,1) - 826	<LOQ (0,25) - 473

Tabulka 14 Vzorky bylin pro stanovení nefrotoxických mykotoxinu ochratoxinu A a citrininu metodou ELISA a HPLC-FLD

č.	Bylina	Botanický název	č.	Bylina	Botanický název	č.	Bylina	Botanický název
1	řebříček květ	*Millefolii flos*	**21**	tužebník nať	*Filipendula ulmaria herba*	**41**	bedrník kořen	*Pimpinellae radix cons.*
2	řepík nať	*Agrimoniae herba*	**22**	jahodník list [b]	*Fragariae folium*	**42**	jitrocel list	*Plantaginis folium*
3	kontryhel nať	*Alchemillae herba*	**23**	mařinka vonná nať	*Asperula odorata herba*	**43**	mochna stříbrná nať	*Potentillae argentii herba cons.*
4	proskurník list	*Althaeae folium cons.*	**24**	svízel nať	*Galium verum herba*	**44**	mochna nátržník kořen	*Tormentillae radix cons.*
5	andělika kořen	*Angelicae radix*	**25**	hořec kořen	*Gentianae radix*	**45**	prvosenka květ [b]	*Primula veris flos*
6	pelyněk nať	*Absinthii herba*	**26**	jinan list Ginkgo	*Ginkgo biloba folium plv.*	**46**	plicník list	*Pulmonariae folium*
7	lopuch kořen [b]	*Bardanae radix*	**27**	chmel otáčivý šištice	*Strobilus lupuli*	**47**	šípek čaj drcený plod	*Cynosbati fructus*
8	sedmikráska květ	*Bellidis flos*	**28**	yzop nať	*Hyssopi herba*	**48**	ostružiník list	*Rubi fruticosi folium*
9	brutnák nať	*Boraginis herba*	**29**	vlaštovičník nať [b]	*Chelidonium herba*	**49**	maliník	*Rubi idaei folium*
10	měsíček lékařský	*Calendulae sine calice flos*	**30**	ořešák list	*Juglandis folium*	**50**	černý bez květ	*Sambuci flos*
11	pupava kořen	*Carlinae radix cons.*	**31**	hluchavka nať	*Lamii albi herba*	**51**	ostropestřec mariánský plod [b]	*Cardui mariae fructus*
12	zeměžluč nať	*Centaurii herba*	**32**	levandule květ	*Levandulae flos*	**52**	kostival kořen	*Symphiti radix*
13	čekanka nať	*Cichorii herba*	**33**	lnice nať	*Linariae herba cons.*	**53**	pampeliška kořen	*Taraxaci radix*
14	benedikt čubet lékařský nať	*Cardui benedicti herba*	**34**	slézový květ	*Malvae flos*	**54**	mateřídouška nať	*Serpylli herba*
15	hloh plod	*Crataegi fructus*	**35**	slézový list [a]	*Malvae folium*	**55**	lipový květ	*Tiliae flos*
16	echinacea květ	*Echinaceae flos*	**36**	maral nať	*Maral leurea herbal*	**56**	kopřiva nať	*Urtica herba*
17	echinacea kořen	*Echinaceae radix*	**37**	jablečník nať	*Marrubii herbal*	**57**	borůvka nať	*Myrtilli herba*
18	pýr plazivý kořen	*Graminis radix*	**38**	komonice nať	*Meliloti herba*	**58**	brusinka list	*Vitis idaeae folium*
19	eukalyptus list	*Eucalypti folium*	**39**	meduňka nať	*Melissae herbal*	**59**	kozlík kořen	*Valeriana radix*
20	pohanka nať	*Fagopyrum saggittatum herbal*	**40**	devětsil kořen	*Petasitidis cons.*	**60**	divizna květ	*Verbasci flos*

[a] Pro stanovení metodou HPLC-FLD byl místo slézového listu použit smilový květ (*Helichrysum arenarium*); [b] byliny vybrané pro stanovení přechodu mykotoxinů do odvaru

5 Závěr

Předložená disertační práce je souhrnem publikační činnosti v oblasti mykotoxinů v rámci doktorského studia v letech 2018-2023 a zahrnuje jak experimentální, tak rešeršní studie. Celkem bylo v průběhu doktorského studia publikováno 8 publikací v impaktovaných časopisech, z toho 7 v prvním kvartilu a 1 ve druhém kvartilu. Devátá publikace je v přípravě do časopisu Mycotoxin Research v prvním kvartilu, a je v současné době (k datu 9. 2. 2023) ve druhém recenzním řízení.

Mykotoxiny jsou z hlediska lidského zdraví velmi závažné kontaminanty, a proto je třeba je monitorovat. Dietární expozice mykotoxinům je považována za majoritní, a proto má její hodnocení největší význam. Monitoring mykotoxinů lze provádět jak v samotném lidském organismu, resp. jeho tkáních a tělních tekutinách, tak v potravinách. Disertační práce se věnuje oběma uvedeným způsobům monitoringu – tj. v potravinách živočišného a rostlinného původu, ale i v biologickém materiálu lidského původu.

Práce poukazuje na významnost monitoringu mykotoxinů i v takových potravinách či potravinových doplňcích, které představují oproti cereáliím a cereálním produktům menší, byť mnohdy poměrně významný zdroj mykotoxinů, neboť mohou přispívat k celkovému dennímu dietárnímu přívodu mykotoxinů. Pro studium „nových“ zdrojů mykotoxinů byly vybrány komodity, které v legislativě EU byly toho času řešeny převážně okrajově či nebyly řešeny vůbec. Těmito komoditami jsou různé druhy koření a bylin a vepřová jelita, ve kterých byl stanovován primárně OTA (koření, byliny a jelita), sekundárně CIT (pouze byliny). MPL pro CIT v potravinách nejsou dosud řešeny. Existuje pouze MPL pro CIT v doplňcích stravy na bázi rýže fermentované červenými kvasnicemi *Monascus purpureus* dle Nařízení Komise (EU) 2019/1901 [154]. Ačkoliv pro OTA, na rozdíl od CIT, existují MPL pro mnoho potravin, pro koření a byliny byly toho času značně omezené a týkaly se pouze k několika málo druhů.

V průběhu doktorského studia byl na portálu Informačního centra bezpečnosti potravin Odborem bezpečnosti potravin Ministerstva Zemědělství pravidelně vydáván přehled hlavních kontaminantů v potravinách, které jsou sledovány a diskutovány v EK DG SANTE. V době doktorského studia tak byly studovány i komodity jako jsou koření a byliny, jejichž regulační limity byly předmětem diskuse. Tyto diskuse byly ukončeny a nově navrhované změny byly uvedeny v platnost 1. ledna 2023 Nařízením Komise 2022/1370 ze dne 5. srpna 2022, kterým se mění nařízení (ES) č. 1881/2006, pokud jde o maximální limity OTA v některých potravinách. Bohužel, ani v nových regulacích není ve vztahu k OTA žádná zmínka

o živočišných surovinách, a proto je třeba komoditám tohoto typu i nadále věnovat pozornost. Dle DG SANTE by se měl navíc monitoring v blízké budoucnosti zaměřit také na stanovení OTA v šunkách a sýrech, což by mohl být námět na další výzkumné studie v této oblasti.

Práce je dále významná z hlediska monitoringu nefrotoxických mykotoxinů v lidském biologickém materiálu. Nefrotoxické mykotoxiny jako jsou OTA a CIT by mohly být jedním z faktorů podílejícím se na etiopatogenezi nádorového onemocnění ledvin, které svou četností řadí Českou republiku v celosvětovém měřítku na první místo. V této oblasti monitoringu je snaha pokračovat ve spolupráci s Urologickou klinikou Fakultní nemocnice Hradec Králové, nicméně je tento typ monitoringu v současné době stále předmětem diskuse.

V neposlední řadě je samozřejmě velkým přínosem zavedení a optimalizace metodiky pro stanovení OTA a CIT metodou HPLC-FLD na pracovišti katedry biologie Přírodovědecké fakulty Univerzity Hradec Králové. Získané výsledky ze všech studií rozšíří poznání o přirozeném výskytu zkoumaných nefrotoxických mykotoxinů a zdravotním riziku plynoucího z jejich přívodu do organismu. Dále poslouží jako podklad „nových zdrojů“ OTA i CIT pro další výzkum v této oblasti, a také jako podklad pro hodnocení rizika v případě, že budou dostupná data o spotřebě daných komodit, či pro zavedení nových či úpravu stávajících regulačních limitů stanovujících MPL.

Literatura

1. SABER, S, YOUSSEF, M, ARAFA, R & HASSANE, A. Mycotoxins production by Aspergillus ostianus Wehmer and using phytochemicals as control agent. *J sci eng res*. 2016. Vol. 3, no. 2, p. 198–213.

2. HRDINA, V, HRDINA, R, JAHODÁŘ, L, MARTINEC, Z & MĚRKA, V. *Přírodní toxiny a jedy*. Praha, 2004. ISBN 80-7262-256-0.

3. DOHNAL, V, DVORAK, V, MALIR, F, OSTRY, V & ROUBAL, T. A comparison of ELISA and HPLC methods for determination of ochratoxin A in human blood serum in the Czech Republic. *Food Chem Toxicol*. 2013. Vol. 62, p. 427–431. https://doi.org/10.1016/j.fct.2013.09.010.

4. MALÍŘ, F, ROUBAL, T, SEVERA, J, ČERNÁ, M & BRNDIAR, M. Stanovení ochratoxinu A (OTA) v lidských ledvinách. *Vojenské zdravotnické listy*. 2002. Vol. LXXI, no. 1, p. 33–36.

5. IARC/WHO – International Agency for Research on Cancer/World Health Organization. Global cancer observatory. *Cancer today, Data visualization tools for exploring the global cancer burden in 2020*. Online. 2021. [Accessed 27 September 2022]. Available from: https://gco.iarc.fr/today/home

6. ROTHSCHILD, LJ. Synthetic biology meets bioprinting: enabling technologies for humans on Mars (and Earth). *Biochem Soc Trans*. 2016. Vol. 44, no. 4, p. 1158–1164. https://doi.org/10.1042/BST20160067.

7. BHAT, R, RAI, RV & KARIM, AA. Mycotoxins in food and feed: Present status and future concerns. *Compr Rev Food Sci Food Saf*. 2010. Vol. 9, no. 1, p. 57–81. https://doi.org/10.1111/j.1541-4337.2009.00094.x.

8. JESWAL, P & KUMAR, D. Mycobiota and natural incidence of aflatoxins, ochratoxin A, and citrinin in Indian spices confirmed by LC-MS/MS. *Int J Microbiol*. 2015. Vol. 2015, no. 3, p. 1–8. https://doi.org/10.1155/2015/242486.

9. FRISVAD, JC, ANDERSEN, B & SAMSON, RA. Association of moulds to foods. In : DIJKSTERHUIS, J & SAMSON, RA*, Food Mycology: A Multifaceted Approach to Fungi and Food*. Volume 25. US, 2007. p. 199–239. ISBN 978-0-8493-9818-6.

10. MALÍŘ, F, OSTRÝ, V & KOL. *Vláknité mikromycety (plísně), mykotoxiny a zdraví člověka*. Brno, 2003. ISBN 80-7013-395-3.

11. KARLOVSKY, P, SUMAN, M, BERTHILLER, F, DE MEESTER, J, EISENBRAND, G, PERRIN, I, OSWALD, IP, SPEIJERS, G, CHIODINI, A & RECKER, T. Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotox Res*. 2016. Vol. 32, no. 4, p. 179–205. https://doi.org/10.1007/s12550-016-0257-7.

12. HAQUE, MA, WANG, Y, SHEN, Z, LI, X, SALEEMI, MK & HE, C. Mycotoxin contamination and control strategy in human, domestic animal and poultry: A review. *Microb Pathog*. 2020. Vol. 142, p. 104095. https://doi.org/10.1016/j.micpath.2020.104095.

13. SUCHÝ, P & HERZIG, I. *Vědecký výbor výživy zvířat. Plísně a mykotoxiny, prevence jejich vzniku a dekontaminace v krmivech* Online. Praha, 2005. [Accessed 27 September 2015]. Available from: http://www.vuzv.cz/sites/File/vybor/Hezig,%20Such%C3%BD-Plisne%20a%20mykotoxiny.pdf

14. SELVARAJ, JN, WANG, Y, ZHOU, L, ZHAO, Y, XING, F, DAI, X & LIU, Y. A review of recent mycotoxin survey data and advanced mycotoxin detection techniques reported from China. *Food Addit Contam Part A*. 2015. Vol. 32, no. 4, p. 440–452. https://doi.org/10.1080/19440049.2015.1010185. 25604871

15. IARC – International Agency for Research on Cancer. *Chemical agents and related occupations: A review of human carcinogens.* Online. Lyon, France, 2012. [Accessed 10 August 2022]. ISBN 978 92 832 1323 9. Available from: https://www.ncbi.nlm.nih.gov/books/NBK304416/

16. PICKOVA, D, OSTRY, V, TOMAN, J & MALIR, F. Aflatoxins: History, significant milestones, recent data on their toxicity and ways to mitigation. *Toxins*. 2021. Vol. 13, no. 6, p. 399. https://doi.org/10.3390/toxins13060399.

17. PICKOVA, D, OSTRY, V & MALIR, F. A recent overview of producers and important dietary sources of aflatoxins. *Toxins*. 2021. Vol. 13, no. 3, p. 186. https://doi.org/10.3390/toxins13030186.

18. PICKOVA, D, OSTRY, V, MALIR, J, TOMAN, J & MALIR, F. A Review on mycotoxins and microfungi in spices in the light of the last five years. *Toxins*. 2020. Vol. 12, no. 12, p. 789. https://doi.org/10.3390/toxins12120789.

19. KUMAR, A, PATHAK, H, BHADAURIA, S & SUDAN, J. Aflatoxin contamination in food crops: causes, detection, and management: a review. *J Food Process*. 2021. Vol. 3, no. 1, p. 17. https://doi.org/10.1186/s43014-021-00064-y.

20. JALLOW, A, XIE, H, TANG, X, QI, Z & LI, P. Worldwide aflatoxin contamination of agricultural products and foods: From occurrence to control. *Compr Rev Food Sci Food Saf*. 2021. Vol. 20, no. 3, p. 2332–2381. https://doi.org/10.1111/1541-4337.12734.

21. IARC – International Agency for Research on Cancer. *IARC Monographs on the evaluation of carcinogenic risks to humans: Some naturally occuring substances: Food items and costituents, heterocyclic aromatic amines and mycotoxins*. Online. Lyon, France, 1993. [Accessed 11 October 2022]. ISBN 92-832-1256-8. Available from: https://www.ncbi.nlm.nih.gov/books/NBK513574/

22. EFSA – European Food Safety Authority. Risk assessment of ochratoxin A in food. *EFSA J*. 2020. Vol. 18, no. 5, p. 1–150. https://doi.org/10.2903/j.efsa.2020.6113.

23. MALIR, F, OSTRY, V, PFOHL-LESZKOWICZ, A, MALIR, J & TOMAN, J. Ochratoxin A: 50 years of research. *Toxins*. 2016. Vol. 8, no. 7, p. 191. https://doi.org/10.3390/toxins8070191.

24. CABAÑES, FJ, BRAGULAT, MR & CASTELLÁ, G. Ochratoxin A Producing Species in the Genus Penicillium. *Toxins (Basel)*. 2010. Vol. 2, no. 5, p. 1111–1120. https://doi.org/10.3390/toxins2051111.

25. FRISVAD, JC, THRANE, U & SAMSON, RA. Mycotoxin producers. In : DIJKSERHUIS, J & SAMSON, RA, *Food Mycology: A Multifaceted Approach to Fungi and Food*. Volume 25. Boca Raton, US, 2007. p. 135–159.

26. PICKOVA, D, TOMAN, J, MIKYSKOVA, P, OSTRY, V & MALIR, F. Investigation of ochratoxin a in blood sausages in the Czech Republic: Comparison with data over Europe. *Food Res Int*. 2022. Vol. 157, p. 111473. https://doi.org/10.1016/j.foodres.2022.111473.

27. PICKOVA, D, TOMAN, J, OSTRY, V & MALIR, F. Natural occurrence of ochratoxin A in spices marketed in the Czech Republic during 2019-2020. *Foods*. 2021. Vol. 10, no. 12, p. 2984. https://doi.org/10.3390/foods10122984.

28. SUN, L-H, LEI, M, ZHANG, N-Y, ZHAO, L, KRUMM, CS & QI, D-S. Hepatotoxic effects of mycotoxin combinations in mice. *Food Chem Toxicol*. 2014. Vol. 74, p. 289–293. https://doi.org/10.1016/j.fct.2014.10.020.

29. WANG, X, TANG, J, GENG, F, ZHU, L, CHU, X, ZHANG, Y, RAHMAN, SU, CHEN, X, JIANG, Y, ZHU, D, FENG, S, LI, Y & WU, JJ. Effects of deoxynivalenol exposure on cerebral lipid peroxidation, neurotransmitter and calcium homeostasis of chicks in vivo. *Toxicon*. 2018. Vol. 150, p. 60–65. https://doi.org/10.1016/j.toxicon.2018.05.010.

30. SOBROVA, P, ADAM, V, VASATKOVA, A, BEKLOVA, M, ZEMAN, L & KIZEK, R. Deoxynivalenol and its toxicity. *Interdiscip Toxicol*. 2010. Vol. 3, no. 3, p. 94–99. https://doi.org/10.2478/v10102-010-0019-x.

31. EGBUTA, M, MWANZA, M & BABALOLA, O. Health risks associated with exposure to filamentous fungi. *Int J Environ Res Public Health*. 2017. Vol. 14, no. 7, p. 719. https://doi.org/10.3390/ijerph14070719.

32. PICKOVA, D, OSTRY, V, TOMAN, J & MALIR, F. Presence of mycotoxins in milk thistle (Silybum marianum) food supplements: A review. *Toxins (Basel)*. 2020. Vol. 12, no. 12. https://doi.org/10.3390/toxins12120782.

33. KOSICKI, R, TWARUŻEK, M, DOPIERAŁA, P, RUDZKI, B & GRAJEWSKI, J. Occurrence of mycotoxins in winter rye varieties cultivated in Poland (2017–2019). *Toxins (Basel)*. 2020. Vol. 12, no. 6, p. 423. https://doi.org/10.3390/toxins12060423.

34. EFSA – European Food Safety Authority. Human and animal dietary exposure to T-2 and HT-2 toxin. *EFSA J*. 2017. Vol. 15, no. 8, p. 1–57. https://doi.org/10.2903/j.efsa.2017.4972.

35. EFSA – European Food Safety Authority. Scientific Opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. *EFSA J*. 2011. Vol. 9, no. 12, p. 1–187. https://doi.org/10.2903/j.efsa.2011.2481.

36. ROPEJKO, K & TWARUŻEK, M. Zearalenone and its metabolites-general overview, occurrence, and toxicity. *Toxins (Basel)*. 2021. Vol. 13, no. 1, p. 35. https://doi.org/10.3390/toxins13010035.

37. EFSA – European Food Safety Authority. Scientific Opinion on the risks for public health related to the presence of zearalenone in food. *EFSA J*. 2011. Vol. 9, no. 6, p. 2197. https://doi.org/10.2903/j.efsa.2011.2197.

38. IARC – International Agency for Research on Cancer. *IARC Monographs on the evaluation of carcinogenic risks to humans: Some traditional herbal medicine, some mycotoxins, naphthalene and strene*. Lyon, France, 2002. ISBN 92-832-1282-7.

39. CHEN, J, WEN, J, TANG, Y, SHI, J, MU, G, YAN, R, CAI, J & LONG, M. Research progress on fumonisin B1 contamination and toxicity: A review. *Molecules*. 2021. Vol. 26, no. 17, p. 5238. https://doi.org/10.3390/molecules26175238.

40. IARC. *IARC Monographs on the evaluation of the carcinogenic risk of cemicals to humans. Some naturally occurring nd ynthetic food components, Furocoumarins and ultravioet radiation. Volume 40*. Lyon (France), 1986. ISBN 92 832 1240 1.

41. SILVA, LJG, PEREIRA, AMPT, PENA, A & LINO, CM. Citrinin in foods and supplements: A review of occurrence and analytical methodologies. *Foods*. 2020. Vol. 10, no. 1, p. 14. https://doi.org/10.3390/foods10010014.

42. DE OLIVEIRA FILHO, JWG, ISLAM, MT, ALI, ES, UDDIN, SJ, SANTOS, JVO, DE ALENCAR, MVOB, JÚNIOR, ALG, PAZ, MFCJ, DE BRITO, MRM, E SOUSA, JMC, SHAW, S, DE MEDEIROS, MGF, DANTAS, SMMM, ROLIM, HML, FERREIRA, PMP, KAMAL, MA, PIECZYNSKA, MD, DAS, N, GUPTA, VK, MOCAN, A, DOS SANTOS ANDRADE, TJA, SINGH, BN, MISHRA, SK, ATANASOV, AG & MELO-CAVALCANTE, AAC. A comprehensive review on biological properties of citrinin. *Food Chem Toxicol*. 2017. Vol. 110, p. 130–141. https://doi.org/10.1016/j.fct.2017.10.002.

43. WANG, Y-Z, JU, X-L & ZHOU, Y-G. The variability of citrinin production in Monascus type cultures. *Food Microbiology*. 2005. Vol. 22, no. 1, p. 145–148. https://doi.org/10.1016/j.fm.2004.01.006.

44. ZHANG, H, AHIMA, J, YANG, Q, ZHAO, L, ZHANG, X & ZHENG, X. A review on citrinin: Its occurrence, risk implications, analytical techniques, biosynthesis, physiochemical properties and control. *Food Res Int*. 2021. Vol. 141, p. 110075. https://doi.org/10.1016/j.foodres.2020.110075.

45. EFSA – European Food Safety Authority. Scientific Opinion on the risks for public and animal health related to the presence of citrinin in food and feed. *EFSA J*. 2012. Vol. 10, no. 3, p. 1–82. https://doi.org/10.2903/j.efsa.2012.2605.

46. VIDAL, A, OUHIBI, S, GHALI, R, HEDHILI, A, DE SAEGER, S & DE BOEVRE, M. The mycotoxin patulin: An updated short review on occurrence, toxicity and analytical challenges. *Food Chem Toxicol*. 2019. Vol. 129, p. 249–256. https://doi.org/10.1016/j.fct.2019.04.048.

47. SALEH, I & GOKTEPE, I. The characteristics, occurrence, and toxicological effects of patulin. *Food Chem Toxicol*. 2019. Vol. 129, p. 301–311. https://doi.org/10.1016/j.fct.2019.04.036.

48. OSTRY, V, TOMAN, J, GROSSE, Y & MALIR, F. Cyclopiazonic acid: 50th anniversary of its discovery. *World Mycotoxin J*. 2018. Vol. 11, no. 1, p. 135–148. https://doi.org/10.3920/WMJ2017.2243.

49. BURDOCK, GA & FLAMM, WG. Review article: Safety assessment of the mycotoxin cyclopiazonic acid. *Int J Toxicol*. 2000. Vol. 19, no. 3, p. 195–218. https://doi.org/10.1080/10915810050074964.

50. AGRIOPOULOU, S. Ergot alkaloids mycotoxins in cereals and cereal-derived food products: Characteristics, toxicity, prevalence, and control strategies. *Agronomy*. 2021. Vol. 11, no. 5, p. 931. https://doi.org/10.3390/agronomy11050931.

51. EFSA – European Food Safety Authority. Scientific Opinion on Ergot alkaloids in food and feed. EFSA Panel on Contaminants in the Food Chain (CONTAM). *EFSA J*. 2012. Vol. 10, no. 7, p. 2798. https://doi.org/10.2903/j.efsa.2012.2798.

52. FRAEYMAN, S, CROUBELS, S, DEVREESE, M & ANTONISSEN, G. Emerging Fusarium and Alternaria mycotoxins: Occurrence, toxicity and toxicokinetics. *Toxins*. 2017. Vol. 9, no. 7, p. 228. https://doi.org/10.3390/toxins9070228.

53. OSTRY, V. Alternaria mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J*. 2008. Vol. 1, no. 2, p. 175–188. https://doi.org/10.3920/WMJ2008.x013.

54. HEUSSNER, AH & BINGLE, LEH. Comparative ochratoxin toxicity: a review of the available data. *Toxins*. 2015. Vol. 7, no. 10, p. 4253–4282. https://doi.org/10.3390/toxins7104253.

55. VAN DER MERWE, KJ, STEYN, PS, FOURIE, L, SCOTT, DB & THREON, JJ. Ochratoxin A, a toxic metabolite produced by Aspergillus ochraceus Wilh. *Nature*. 1965. Vol. 205, p. 1112–1113.

56. VAN DER MERWE, KJ, STEYN, PS & FOURIE, L. Mycotoxins Part II. The constitution of Ochratoxins A, B and C, metabolites of Aspergillus ochraceus Wilh. *J Chem Soc (Resumed)*. 1965. Vol. 204, p. 7083–7088.

57. OSTRY, V, MALIR, F & RUPRICH, J. Producers and important dietary sources of ochratoxin A and citrinin. *Toxins*. 2013. Vol. 5, no. 9, p. 1574–1586. https://doi.org/10.3390/toxins5091574.

58. VUKELIĆ, M, ŠOŠTARIĆ, B & BELICZA, M. Pathomorphology of Balkan endemic nephropathy. *Food Chem Toxicol*. 1992. Vol. 30, no. 3, p. 193–200. https://doi.org/10.1016/0278-6915(92)90033-H.

59. MAAROUFI, K, ACHOUR, A, BETBEDER, AM, HAMMAMI, M, ELLOUZ, F, CREPPY, EE & BACHA, H. Foodstuffs and human blood contamination by the mycotoxin ochratoxin A: correlation with chronic interstitial nephropathy in Tunisia. *Arch Toxicol*. 1995. Vol. 69, no. 8, p. 552–558. https://doi.org/10.1007/s002040050211.

60. WAFA, EW, YAHYA, RS, SOBH, MA, ERAKY, I, EL-BAZ, M, EL-GAYAR, HA, BETBEDER, AM & CREPPY, EE. Human ochratoxicosis and nephropathy in Egypt: A preliminary study. *Hum Exp Toxicol*. 1998. Vol. 17, no. 2, p. 124–129. https://doi.org/10.1177/096032719801700207.

61. EL KHOURY, A & ATOUI, A. Ochratoxin A: General overview and actual molecular status. *Toxins*. 2010. Vol. 2, no. 4, p. 461–493. https://doi.org/10.3390/toxins2040461.

62. POHLAND, AE, SCHULLER, PL, STEYN, PS & VAN EGMOND, HP. Physicochemical data for some selected mycotoxins. *Pure Appl Chem*. 1982. Vol. 54, no. 11, p. 2219–2284. https://doi.org/10.1351/pac198254112219.

63. IARC – International Agency for Research on Cancer. *IARC Monographs on the evaluation of the carcinogenic risk od chemicals to humans: Some food additives, feed additives and naturally occurring substances*. Lyon, France, 1983. ISBN 92-832-1231-2.

64. PUBCHEM. *PubChem*. Online. 2021. [Accessed 7 June 2021]. Available from: https://pubchem.ncbi.nlm.nih.gov/

65. RINGOT, D, CHANGO, A, SCHNEIDER, Y-J & LARONDELLE, Y. Toxicokinetics and toxicodynamics of ochratoxin A, an update. *Chem-Biol Interact*. 2006. Vol. 159, no. 1, p. 18–46. https://doi.org/10.1016/j.cbi.2005.10.106. 16293235

66. KŐSZEGI, T & POÓR, M. Ochratoxin A: molecular interactions, mechanisms of toxicity and prevention at the molecular level. *Toxins*. 2016. Vol. 8, no. 4, p. 111. https://doi.org/10.3390/toxins8040111.

67. CRAMER, B, HARRER, H, NAKAMURA, K, UEMURA, D & HUMPF, H-U. Total synthesis and cytotoxicity evaluation of all ochratoxin A stereoisomers. *Bioorg Med Chem*. 2010. Vol. 18, no. 1, p. 343–347. https://doi.org/10.1016/j.bmc.2009.10.050.

68. TURNER, NW, SUBRAHMANYAM, S & PILETSKY, SA. Analytical methods for determination of mycotoxins: A review. *Anal Chim Acta*. 2009. Vol. 632, no. 2, p. 168–180. https://doi.org/10.1016/j.aca.2008.11.010.

69. PITT, J & HOCKING, A. *Fungi and Food Spoilage*. 4th. 2022. ISBN 978-3-030-85638-0.

70. ŠIMŮNEK, J. *Plísně a mykotoxiny*. Online. 2004. [Accessed 27 January 2020]. Available from: http://www.med.muni.cz/dokumenty/pdf/plisne_a_mykotoxiny.pdf

71. PITT, JI & TAYLOR, JW. Aspergillus, its sexual states and the new International Code of Nomenclature. *Mycologia*. 2014. Vol. 106, no. 5, p. 1051–1062. https://doi.org/10.3852/14-060.

72. ELLIS, D, DAVIS, S, ALEXIOU, H, HANDKE, R & BARTLEY, R. *Descriptions of medical fungi*. Online. 2. Adelaide, South Australia, 2007. [Accessed 2 February 2017]. ISBN 978-0-9598512-6-7. Available from: http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.694.2167

73. ABRUNHOSA, L, PATERSON, RR & VENÂNCIO, A. Biodegradation of ochratoxin A for food and feed decontamination. *Toxins*. 2010. Vol. 2, no. 5, p. 1078–1099. https://doi.org/10.3390/toxins2051078.

74. DEGEN, GH, MAYER, S & BLASZKEWICZ, M. Biomonitoring of ochratoxin A in grain workers. *Mycotoxin Res*. 2007. Vol. 23, no. 2, p. 88–93. https://doi.org/10.1007/BF02946032.

75. IAVICOLI, I, BRERA, C, CARELLI, G, CAPUTI, R, MARINACCIO, A & MIRAGLIA, M. External and internal dose in subjects occupationally exposed to ochratoxin A. *Int Arch Occup Environ Health*. 2002. Vol. 75, no. 6, p. 381–386. https://doi.org/10.1007/s00420-002-0319-3.

76. HALSTENSEN, AS, NORDBY, K-C, ELEN, O & EDUARD, W. Ochratoxin A in grain dust--estimated exposure and relations to agricultural practices in grain production. *Ann Agric Environ Med*. 2004. Vol. 11, no. 2, p. 245–254.

77. VIEGAS, C, FARIA, T, CAETANO, LA, CAROLINO, E, QUINTAL-GOMES, A, TWARUŻEK, M, KOSICKI, R & VIEGAS, S. Characterization of occupational exposure to fungal burden in Portuguese bakeries. *Microorganisms*. 2019. Vol. 7, no. 8, p. 234. https://doi.org/10.3390/microorganisms7080234.

78. ESCRIVÁ, L, AGAHI, F, VILA-DONAT, P, MAÑES, J, MECA, G & MANYES, L. Bioaccessibility Study of Aflatoxin B1 and Ochratoxin A in bread enriched with fermented milk whey and/or pumpkin. *Toxins*. 2022. Vol. 14, no. 1, p. 6. https://doi.org/10.3390/toxins14010006.

79. GALTIER, P, ALVINERIE, M & CHARPENTEAU, JL. The pharmacokinetic profiles of ochratoxin A in pigs, rabbits and chickens. *Food Cosmet Toxicol*. 1981. Vol. 19, p. 735–738. https://doi.org/10.1016/0015-6264(81)90528-9.

80. STUDER-ROHR, I, SCHLATTER, J & DIETRICH, DR. Kinetic parameters and intraindividual fluctuations of ochratoxin A plasma levels in humans. *Arch Toxicol*. 2000. Vol. 74, no. 9, p. 499–510. https://doi.org/10.1007/s002040000157. 11131029

81. MALIR, F, OSTRY, V & NOVOTNA, E. Toxicity of the mycotoxin ochratoxin A in the light of recent data. *Toxin Rev*. 2013. Vol. 32, no. 2, p. 19–33. https://doi.org/10.3109/15569543.2013.782504.

82. SCHWERDT, G, FREUDINGER, R, SILBERNAGL, S & GEKLE, M. Ochratoxin A-binding proteins in rat organs and plasma and in different cell lines of the kidney. *Toxicology*. 1999. Vol. 135, no. 1, p. 1–10. https://doi.org/10.1016/S0300-483X(99)00028-1. 1045421

83. STOJKOVIĆ, R, HULT, K, GAMULIN, S & PLESTINA, R. High affinity binding of ochratoxin A to plasma constituents. *Biochem Int*. 1984. Vol. 9, no. 1, p. 33–38.

84. PAVLÍKOVÁ, D, PAVLÍK, M, MATĚJŮ, L & BALÍK, J. *Ekotoxikologie*. 2. Praha, 2008. ISBN 978-80-213-1843-4.

85. BEYERLE, J, FREI, E, STIBOROVA, M, HABERMANN, N & ULRICH, CM. Biotransformation of xenobiotics in the human colon and rectum and its association with colorectal cancer. *Drug Metab Rev*. 2015. Vol. 47, no. 2, p. 199–221. https://doi.org/10.3109/03602532.2014.996649.

86. BUHLER, DR & WILLIAMS, DE. The role of biotransformation in the toxicity of chemicals. *Aquat Toxicol*. 1988. Vol. 11, no. 1, p. 19–28. https://doi.org/10.1016/0166-445X(88)90004-5.

87. GAJĘCKA, M, OBREMSKI, K, JAKIMIUK, E, SKORSKA-WYSZYŃSKA, E, ZIELONKA, Ł & GAJĘCKI, M. The biotransformation of chosen mycotoxins. *Pol J Vet Sci*. 2009. Vol. 12, no. 2, p. 293–303.

88. TRAN, VN, VIKTOROVÁ, J & RUML, T. Mycotoxins: Biotransformation and bioavailability assessment using Caco-2 cell monolayer. *Toxins (Basel)*. 2020. Vol. 12, no. 10, p. 628. https://doi.org/10.3390/toxins12100628.

89. ZEPNIK, H, VÖLKEL, W & DEKANT, W. Toxicokinetics of the mycotoxin ochratoxin A in F 344 rats after oral administration. *Toxicol Appl Pharmacol*. 2003. Vol. 192, no. 1, p. 36–44. https://doi.org/10.1016/s0041-008x(03)00261-8.

90. PITOUT, MJ. The hydrolysis of ochratoxin A by some proteolytic enzymes. *Biochem Pharmacol*. 1969. Vol. 18, no. 2, p. 485–491. https://doi.org/10.1016/0006-2952(69)90224-x.

91. MOBASHAR, M, HUMMEL, J, BLANK, R & SÜDEKUM, K-H. Ochratoxin A in ruminants–a review on its degradation by gut microbes and effects on animals. *Toxins (Basel)*. 2010. Vol. 2, no. 4, p. 809–839. https://doi.org/10.3390/toxins204809.

92. XIAO, H, MADHYASTHA, S, MARQUARDT, RR, LI, S, VODELA, JK, FROHLICH, AA & KEMPPAINEN, BW. Toxicity of ochratoxin A, its opened lactone form and several of its analogs: structure-activity relationships. *Toxicol Appl Pharmacol*. 1996. Vol. 137, no. 2, p. 182–192. https://doi.org/10.1006/taap.1996.0071.

93. STØRMER, FC, HANSEN, CE, PEDERSEN, JI, HVISTENDAHL, G & AASEN, AJ. Formation of (4R)- and (4S)-4-hydroxyochratoxin A from ochratoxin A by liver microsomes from various species. *Appl Environ Microbiol*. 1981. Vol. 42, no. 6, p. 1051–1056. https://doi.org/10.1128/aem.42.6.1051-1056.1981.

94. STØRMER, FC, STØREN, O, HANSEN, CE, PEDERSEN, JI & AASEN, AJ. Formation of (4R)- and (4S)-4-hydroxyochratoxin A and 10-hydroxyochratoxin A from Ochratoxin A by rabbit liver microsomes. *Appl Environ Microbiol*. 1983. Vol. 45, no. 4, p. 1183–1187. https://doi.org/10.1128/aem.45.4.1183-1187.1983.

95. PINELLI, E, EL ADLOUNI, C, PIPY, B, QUARTULLI, F & PFOHL-LESZKOWICZ, A. Roles of cyclooxygenase and lipoxygenases in ochratoxin A genotoxicity in human epithelial lung cells. *Environ Toxicol Pharmacol*. 1999. Vol. 7, no. 2, p. 95–107. https://doi.org/10.1016/s1382-6689(99)00008-3.

96. PFOHL-LESZKOWICZ, A & MANDERVILLE, RA. An update on direct genotoxicity as a molecular mechanism of ochratoxin A carcinogenicity. *Chem Res Toxicol*. 2012. Vol. 25, no. 2, p. 252–262. https://doi.org/10.1021/tx200430f. 22054007

97. MALIR, F, LOUDA, M, TOMAN, J, OSTRY, V, PICKOVA, D, PACOVSKY, J, BRODAK, M & PFOHL-LESZKOWICZ, A. Investigation of ochratoxin A biomarkers in biological materials obtained from patients suffering from renal cell carcinoma. *Food Chem Toxicol*. 2021. Vol. 158, p. 112669. https://doi.org/10.1016/j.fct.2021.112669.

98. KANĎÁR, R. Stanovení glutathionu a glutathiondisulfidu v biologických vzorcích. *Chemické listy*. 2016. No. 110, p. 754–760.

99. PFOHL-LESZKOWICZ, A, TOZLOVANU, M, MANDERVILLE, R, PERAICA, M, CASTEGNARO, M & STEFANOVIC, M. New molecular and field evidences for the implication of mycotoxins but not aristolochic acid in human nephropathy and urinary tract tumor. *Mol Nutr Food Res*. 2007. Vol. 51, no. 9, p. 1131–1146. https://doi.org/10.1002/mnfr.200700045. 17729220

100. SEVERA, J, MALÍŘ, F & ROUBAL, T. Stanovení ochratoxinu A v moči metodou HPLC. *Sborník přednášek*. 2005. Vol. 16, no. 1, p. 116–119.

101. PFOHL-LESZKOWICZ, A & MANDERVILLE, R. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Mol Nutr Food Res*. 2007. Vol. 51, no. 1, p. 61–99. https://doi.org/10.1002/mnfr.200600137.

102. GEORGE, B, YOU, D, JOY, MS & ALEKSUNES, LM. Xenobiotic transporters and kidney injury. *Adv Drug Deliv Rev*. 2017. Vol. 116, p. 73–91. https://doi.org/10.1016/j.addr.2017.01.005. 28111348

103. JUTABHA, P, ANZAI, N, HAYASHI, K, DOMAE, M, UCHIDA, K, ENDOU, H & SAKURAI, H. A novel human organic anion transporter NPT4 mediates the transport of ochratoxin A. *J Pharmacol Sci*. 2011. Vol. 116, no. 4, p. 392–396. https://doi.org/10.1254/jphs.10227sc.

104. PHILP, RB. *Ecosystems and Human Health: Toxicology and Environmental Hazards*. 2. USA, 2001. ISBN 1-56670-568-1.

105. SKAUG, MA, HELLAND, I, SOLVOLL, K & SAUGSTAD, OD. Presence of ochratoxin A in human milk in relation to dietary intake. *Food Addit Contam*. 2001. Vol. 18, no. 4, p. 321–327. https://doi.org/10.1080/02652030117740.

106. MALLY, A & DEKANT, W. Mycotoxins and the kidney: Modes of action for renal tumor formation by ochratoxin A in rodents. *Mol Nutr Food Res*. 2009. Vol. 53, no. 4, p. 467–478. https://doi.org/10.1002/mnfr.200800149.

107. PURCHASE, IFH & THERON, JJ. The acute toxicity of ochratoxin A to rats. *Food Cosmet Toxicol*. 1968. Vol. 6, no. 4, p. 479IN5481-480IN12483.

108. DOMIJAN, A-M, RUDES, K & PERAICA, M. The effect of ochratoxin A on the concentration of protein carbonyls in rats. *Arh Hig Rada Toksikol* . 2005. Vol. 56, no. 4, p. 311–315.

109. BATTACONE, G, NUDDA, A & PULINA, G. Effects of ochratoxin A on livestock production. *Toxins*. 2010. Vol. 2, no. 7, p. 1796–1824. https://doi.org/10.3390/toxins2071796.

110. RASFF. - Rapid Alert System for Food and Feed portal database. Online. 2022. [Accessed 9 February 2020]. Available from: https://webgate.ec.europa.eu/rasff-window/portal/

111. TOMAN, J, MALÍŘ, F, OSTRÝ, V, GROSSE, Y, DVOŘÁK, V, ROUBAL, T & NEUCHLOVA, L. The occurrence of ochratoxin A in white and parboiled rice. *Czech J Food Sci*. 2016. Vol. 34, no. 1, p. 32–38. https://doi.org/10.17221/316/2015-CJFS.

112. MAGAN, N & ALDRED, D. Conditions of formation of ochratoxin A in drying, transport and in different commodities. *Food Addit Contam A*. 2005. Vol. 22, no. sup1, p. 10–16. https://doi.org/10.1080/02652030500412154.

113. EVROPSKÁ KOMISE. *Nařízení komise (ES) 1881/2006 ze dne 19. prosince 2006, kterým se stanoví maximální limity některých kontaminujících látek v potravinách*. Online. 2006. 1881/2006. [Accessed 22 August 2020]. Available from: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2006:364:0005:0024:CS:PDF

114. EVROPSKÁ KOMISE. *Nařízení Komise (EU) 2022/1370 ze dne 5. srpna 2022, kterým se mění nařízení (ES) č. 1881/2006, pokud jde o maximální limity ochratoxinu A v některých potravinách*. Online. 2022. 2022/1370. [Accessed 16 December 2022]. Available from: https://eur-lex.europa.eu/legal-content/CS/TXT/PDF/?uri=CELEX:32022R1370

115. EVROPSKÁ KOMISE. *Nařízení Komise (EU) 2015/1137 ze dne 13. července 2015, kterým se mění nařízení (ES) č. 1881/2006, pokud jde o maximální limit ochratoxinu A v koření Capsicum spp.* Online. 2015. 2015/1137. [Accessed 16 June 2022]. Available from: https://eur-lex.europa.eu/legal-content/CS/TXT/PDF/?uri=CELEX:32015R1137

116. EVROPSKÁ KOMISE. *Nařízení Komise (EU) č. 105/2010 ze dne 5. února 2010, kterým se mění nařízení (ES) č. 1881/2006, kterým se stanoví maximální limity některých kontaminujících látek v potravinách, pokud jde o ochratoxin A*. Online. 2010. 105/2010. [Accessed 21 September 2022]. Available from: https://eur-lex.europa.eu/legal-content/CS/TXT/PDF/?uri=CELEX:32010R0105

117. MINISTERSTVO ZEMĚDĚLSTVÍ ČR. Aktuální diskutovaná témata v oblasti kontaminantů v potravinách – leden 2022. *Informační centrum bezpečnosti potravin*. Online. 2022. [Accessed 20 February 2022]. Available from: http://www.bezpecnostpotravin.cz/kontaminanty-v-potravinach-prehled-hlavnich-temat-diskutovanych-v-ek.aspx

118. EFSA, EFS. Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to ochratoxin A in food. *EFSA J*. 2006. Vol. 4, no. 6, p. 1–56. https://doi.org/10.2903/j.efsa.2006.365.

119. MALIR, F, OSTRY, V, GROSSE, Y, ROUBAL, T, SKARKOVA, J & RUPRICH, J. Monitoring the mycotoxins in food and their biomarkers in the Czech Republic. *Mol Nutr Food Res*. 2006. Vol. 50, no. 6, p. 513–518. https://doi.org/10.1002/mnfr.200500175.

120. AZHAR, M, RAUF, S, HAYAT, A, CATANANTE, G, RAZA, R & MARTY, J. Determination of Mycotoxins in Food. In : WONG, Y & LEWIS RJ, *Analysis of Food Toxins and Toxicants*. 1st. Hoboken, New Jersey (USA), 2017. p. 137–168. ISBN 978-1-118-99272-2.

121. MINISTERSTVO ZEMĚDĚLSTVÍ ČR. *Strategie bezpečnosti potravin a výživy 2030*. 1. Praha, 2021. ISBN 978-80-7434-621-7.

122. PIGŁOWSKI, M. Food hazards on the European Union market: The data analysis of the Rapid alert system for food and feed. *Food Sci Nutr*. 2020. Vol. 8, no. 3, p. 1603–1627. https://doi.org/10.1002/fsn3.1448.

123. DURNER, J & WATTS, DC. Principles of Analytical Chemistry for Toxicology. In : REICHL, F-X & SCHWENK, M, *Regulatory Toxicology*. Online. 2nd. Berlin, Heidelberg, 2014. p. 321–357. [Accessed 13 March 2022]. ISBN 978-3-642-35373-4.

124. HAWKINS, R. Managing the pre- and post-analytical phases of the total testing process. *Ann Lab Med*. 2012. Vol. 32, no. 1, p. 5–16. https://doi.org/10.3343/alm.2012.32.1.5.

125. ALSHANNAQ, A & YU, J-H. Occurrence, toxicity, and analysis of major mycotoxins in food. *Int J Environ Res Public Health*. 2017. Vol. 14, no. 6, p. 632. https://doi.org/10.3390/ijerph14060632.

126. BEUCHAT, LR. *Food and beverage mycology*. 1987.

127. RAHMANI, A, JINAP, S & SOLEIMANY, F. Qualitative and quantitative analysis of mycotoxins. *Compr Rev Food Sci*. 2009. Vol. 8, no. 3, p. 202–251. https://doi.org/10.1111/j.1541-4337.2009.00079.x.

128. VALENTA, H. Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids. *J Chromatogr A*. 1998. Vol. 815, no. 1, p. 75–92. https://doi.org/10.1016/S0021-9673(98)00163-0.

129. LIU, X, LIU, X, HUANG, P, WEI, F, YING, G, LU, J, ZHOU, L & KONG, W. Regeneration and reuse of immunoaffinity column for highly efficient clean-up and economic detection of ochratoxin A

in malt and ginger. *Toxins (Basel)*. 2018. Vol. 10, no. 11, p. 462. https://doi.org/10.3390/toxins10110462.

130. CROWTHER, JR. *ELISA: Theory and practice*. 1995. ISBN 978-0-89603-279-8.

131. CIBIČEK, N & HEŘMANOVÁ, Z. Imunoanalýza. In : CIBIČEK, N, VACEK, J a kol*., Principy a využití vybraných analytických metod v laboratorní medicíně*. 1. Olomouc, 2014. p. 52–67. ISBN 978-80-244-3951-8.

132. R-BIOPHARM - RHÔNE LTD. *RIDASCREEN® Ochratoxin A 30/15 Art. No. R1311. Enzyme immunoassay for the quantitative analysis of ochratoxin A: In vitro Test*. 2018.

133. R-BIOPHARM - RHÔNE LTD. *RIDASCREEN®FAST Citrinin Art. No. R6302. Enzyme immunoassay for the quantitative determination of citrinin: In vitro Test*. 2014.

134. NOVÁKOVÁ, L & DOUŠA, M. *Moderní HPLC separace v teorii a praxi*. Europrint. Praha, 2013. ISBN 978-80-260-4243-3.

135. KOSINA, P. Kolonové separace. In : CIBEČEK, N, VACEK, J a kol*., Principy a využití vybraných analytických metod v laboratorní medicíně*. 1. Olomouc, 2014. p. 122–131. ISBN 978-80-244-3951-8.

136. SCHWEIGHARDT, H, SCHUH, M, ABDELHAMID, AM, BOHM, J & LEIBETSEDER, J. Method for quantitative determination of ochratoxin A in foods and feeds by means of high-pressure liquid chromatography (HPLC). *Z Lebensmittel Untersuch Forsch*. 1980. Vol. 170, no. 5, p. 355–359. https://doi.org/10.1007/BF01042973.

137. HUNT, DC, MCCONNIE, BR & CROSBY, NT. Confirmation of ochratoxin A by chemical derivatisation and high-performance liquid chromatography. *Analyst*. 1980. Vol. 105, no. 1246, p. 89–90. https://doi.org/10.1039/AN9800500089.

138. HOWELL, MV & TAYLOR, PW. Determination of aflatoxins, ochratoxin a, and zearalenone in mixed feeds, with detection by thin layer chromatography or high performance liquid chromatography. *J Assoc Off Anal Chem*. 1981. Vol. 64, no. 6, p. 1356–1363.

139. NESHEIM, S, STACK, ME, TRUCKSESS, MW, EPPLEY, RM & KROGH, P. Rapid solvent-efficient method for liquid chromatographic determination of ochratoxin A in corn, barley, and kidney: collaborative study. *J AOAC Int*. 1992. Vol. 75, no. 3, p. 481–487. https://doi.org/10.1093/jaoac/75.3.481.

140. JOSEFSSON, E & MÖLLER, T. High pressure liquid chromatographic determination of ochratoxin A and zearalenone in cereals. *J Assoc Off Anal Chem*. 1979. Vol. 62, no. 5, p. 1165–1168.

141. ABRAMSON, D. Measurement of ochratoxin A in barley extracts by liquid chromatography-mass spectrometry. *J Chromatogr A*. 1987. Vol. 391, p. 315–320. https://doi.org/10.1016/S0021-9673(01)94330-4.

142. AHN, J, KIM, D, KIM, H & JAHNG, K-Y. Quantitative determination of mycotoxins in urine by LC-MS/MS. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2010. Vol. 27, no. 12, p. 1674–1682. https://doi.org/10.1080/19440049.2010.505201.

143. EDIAGE, EN, DI MAVUNGU, JD, SONG, S, WU, A, VAN PETEGHEM, C & DE SAEGER, S. A direct assessment of mycotoxin biomarkers in human urine samples by liquid chromatography tandem mass spectrometry. *Anal Chim Acta*. 2012. Vol. 741, p. 58–69. https://doi.org/10.1016/j.aca.2012.06.038.

144. CRAMER, B, OSTERESCH, B, MUÑOZ, KA, HILLMANN, H, SIBROWSKI, W & HUMPF, H-U. Biomonitoring using dried blood spots: detection of ochratoxin A and its degradation product 2'R-ochratoxin A in blood from coffee drinkers. *Mol Nutr Food Res*. 2015. Vol. 59, no. 9, p. 1837–1843. https://doi.org/10.1002/mnfr.201500220.

145. KELLER, J, MOLDENHAUER, D, BYRNE, L, HAASE, H, RESCH-GENGER, U & KOCH, M. Complexes of the mycotoxins citrinin and ochratoxin A with aluminum ions and their spectroscopic properties. *Toxins*. 2018. Vol. 10, no. 12, p. 538. https://doi.org/10.3390/toxins10120538

146. ARROYO-MANZANARES, N, GARCÍA-CAMPAÑA, AM & GÁMIZ-GRACIA, L. Multiclass mycotoxin analysis in Silybum marianum by ultra high performance liquid chromatography–tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid–liquid microextraction. *J Chromatogr A*. 2013. Vol. 1282, p. 11–19. https://doi.org/10.1016/j.chroma.2013.01.072.

147. FENCLOVA, M, NOVAKOVA, A, VIKTOROVA, J, JONATOVA, P, DZUMAN, Z, RUML, T, KREN, V, HAJSLOVA, J, VITEK, L & STRANSKA-ZACHARIASOVA, M. Poor chemical and microbiological quality of the commercial milk thistle-based dietary supplements may account for their reported unsatisfactory and non-reproducible clinical outcomes. *Sci Rep*. 2019. Vol. 9, no. 1, p. 11118. https://doi.org/10.1038/s41598-019-47250-0.

148. FIBIGR, J, SATINSKY, D & SOLICH, P. Current trends in the analysis and quality control of food supplements based on plant extracts. *Anal Chim Acta*. 2018. Vol. 1036, p. 1–15. https://doi.org/10.1016/j.aca.2018.08.017.

149. ASHIQ, S, HUSSAIN, M & AHMAD, B. Natural occurrence of mycotoxins in medicinal plants: a review. *Fungal Genet Biol*. 2014. Vol. 66, p. 1–10. https://doi.org/10.1016/j.fgb.2014.02.005.

150. MAVUNGU, JDD, MONBALIU, S, SCIPPO, M-L, MAGHUIN-ROGISTER, G, SCHNEIDER, Y-J, LARONDELLE, Y, CALLEBAUT, A, ROBBENS, J, PETEGHEM, CV & SAEGER, SD. LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. *Food Addit Contam Part A*. 2009. Vol. 26, no. 6, p. 885–895. https://doi.org/10.1080/02652030902774649.

151. ZHANG, L, DOU, X-W, ZHANG, C, LOGRIECO, A & YANG, M-H. A review of current methods for analysis of mycotoxins in herbal medicines. *Toxins*. 2018. Vol. 10, no. 2, p. 1–39. https://doi.org/10.3390/toxins10020065.

152. WAWRZYNIAK, J & WAŚKIEWICZ, A. Ochratoxin A and citrinin production by Penicillium verrucosum on cereal solid substrates. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess*. 2014. Vol. 31, no. 1, p. 139–148. https://doi.org/10.1080/19440049.2013.861933.

153. CHO, H-D, SUH, JH, FENG, S, EOM, T, KIM, J, HYUN, SM, KIM, J, WANG, Y & HAN, SB. Comprehensive analysis of multi-class mycotoxins in twenty different species of functional and medicinal herbs using liquid chromatography–tandem mass spectrometry. *Food Control*. 2019. Vol. 96, p. 517–526. https://doi.org/10.1016/j.foodcont.2018.10.007.

154. EVROPSKÁ KOMISE. *Nařízení Komise (EU) č. 2019/1901 ze dne 7. listopadu 2019, kterým se mění nařízení (ES) č. 1881/2006, pokud jde o maximální limity citrininu v doplňcích stravy na bázi rýže fermentované červenými kvasnicemi Monascus purpureus*. Online. 2019. 2019/1901. [Accessed 13 March 2020]. Available from: https://eur-lex.europa.eu/legal-content/CS/TXT/PDF/?uri=CELEX:32019R1901&from=CS

Prezentace výsledků

29. 6. – 1. 7. 2022: 13th International conference „Mycotoxins and moulds – current trends“ (Bydgoszcz, Poland)

Plakátové sdělení

TOMAN, J., **PICKOVA, D**., MEĆAVA, M., BRANDOVA, K., MALIR, F. Occurrence of citrinin in pharmaceutical important herbs. In: 13th International conference Mycotoxins and moulds - current trends, Bydgoszcz, Poland, 2022, p. 91

30. 5. – 2. 6. 2021: 42nd Mycotoxin Workshop (Münster, Germany)

Online poster-style prezentace

PICKOVA, D., TOMAN, J., FRKOVA, V., OSTRY, V., MALIR, F. Screening determination of ochratoxin A in spices available on the Czech market using EIA method coupled with immunoaffinity columns. In: Conference abstracts from 42nd Mycotoxin Workshop, Münster, Germany, Society for Mycotoxin Research, 2021, p. 81.

PICKOVA, D., V., OSTRY, V., MALIR, F. Aflatoxin producers – a current information. In: Conference abstracts from 42nd Mycotoxin Workshop, Münster, Germany, Society for Mycotoxin Research, 2021, p. 128

6.– 8. 5. 2019: 41st Mycotoxin Workshop (Lisbon, Portugal)

Plakátové sdělení

TOMAN, J., **PICKOVA, D**., RATHGEB, A., MALIR, F., OSTRY, V., KARLOVSKY, P. *Astragalus propinquus* Schischkin root – A significant source of the mycotoxins? In: Conference abstracts from 41st Mycotoxin Workshop, Lisboa, Portugal, Society for Mycotoxin Research, 2019, p. 136

Účast na projektech

2022–2023: SV 2106/2022

Výskyt mykotoxinů ochratoxinu A a citrininu ve vybraných vzorcích farmaceuticky významných rostlin a stanovení jejich přechodu do nálevů a odvarů

Spoluřešitel

2021–2022: SV 2110/2021

Zavádění metodik pro stanovení mykotoxinů na HPLC-FLD

Hlavní řešitel

2020–2021: SV 2115/2020

Vyhledávání nových expozičních zdrojů vybraných mykotoxinů

Hlavní řešitel

2019–2021: SV 2112/2019

Výskyt mykotoxinů v koření, medicinálních rostlinách a doplňcích stravy

Hlavní řešitel

Studijní stáže

1. 10. – 30. 11. 2021: Doktorská odborná stáž (Bratislava, Slovensko), projekt ERASMUS+

Slovenská zdravotnická univerzita, Lekárska fakulta, Ústav mikrobiológie LF SZU, Oddelenie mikrobiológie

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Práce: Zapojení do experimentální a výzkumné činnosti při testování antimikrobiální účinnosti nových sloučenin na bakteriální kmeny pod vedením Ing. Renáty Lehotské, Ph.D. na oddělení mikrobiologie LF SZU. Absolvování série praktických cvičení, přednášek a exkurzí na oddělení mikrobiologie, oddělení toxických organických polutantů a oddělení toxikologie. Absolvování rámcových programů "školící místo" zaměřených na virologii, bakteriologii a mykologii.

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Práce: Příprava vzorků koření pomocí „rychlé, jednoduché, levné, efektivní, robustní a bezpečné metody" Quick, Easy, Cheap, Effective, Rugged and Safe (QuEChERS) pro stanovení OTA.

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Příloha 1

Presence of mycotoxins in milk thistle (*Silybum marianum*) food supplements: A review

toxins

MDPI

Review

Presence of Mycotoxins in Milk Thistle (*Silybum marianum*) Food Supplements: A Review

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Abstract: The consumption of herbal-based supplements, which are believed to have beneficial effects on human health with no side effects, has become popular around the world and this trend is still increasing. *Silybum marianum* (L.) Gaertn, commonly known as milk thistle (MT), is the most commonly studied herb associated with the treatment of liver diseases The hepatoprotective effects of active substances in silymarin, with silybin being the main compound, have been demonstrated in many studies. However, MT can be affected by toxigenic micro-fungi and contaminated by mycotoxins with adverse effects The beneficial effect of silymarin can thus be reduced or totally antagonized by mycotoxins. MT has proven to be affected by micro-fungi of the *Fusarium* and *Alternaria* genera, in particular, and their mycotoxins. Alternariol-methyl-ether (AME), alternariol (AOH), beauvericin (BEA), deoxynivalenol (DON), enniatin A (ENNA), enniatin A_1 ($ENNA_1$), enniatin B (ENNB), enniatin B_1 ($ENNB_1$), HT-2 toxin (HT-2), T-2 toxin (T-2), tentoxin (TEN), and zearalenone (ZEA) seem to be most significant in MT-based dietary supplements. This review focuses on summarizing cases of mycotoxins in MT to emphasize the need for strict monitoring and regulation, as mycotoxins in relation with MT-based dietary supplements are not covered by European Union legislation.

Keywords: milk thistle; food supplements; liver diseases; silymarin; mycotoxins

Key Contribution: Milk thistle-based supplements are mainly contaminated with *Alternaria* and *Fusarium* mycotoxins. Mycotoxins AME, AOH, TEN, DON, HT-2, T-2, ZEA, BEA, ENNA, $ENNA_1$, ENNB, $ENNB_1$ are the most significant in milk thistle-based dietary supplements. Capsules are the most contaminated form of milk thistle supplements by *Fusarium* mycotoxins The use of silymarin preparations contaminated with hepatotoxic mycotoxins may reduce or completely reverse its hepatoprotective effects.

1. Introduction

According to the definition set by Directive 2002/45/EC, "Food supplements means foodstuffs the purpose of which is to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional or physiological effect, alone or in combination, marketed in dose form, namely forms such as capsules, pastilles, tablets, pills and other similar forms, sachets of powder, ampoules of liquids, drop dispensing bottles, and other similar forms of liquids and powders designed to be taken in measured small unit quantities." [1] The consumption of herbal-based food (dietary) supplements, which the manufacturers claim to have beneficial effects on human health, has become popular and has significantly increased over the last decade [2–4]. These herbal-based supplements are

generally believed to be safer and healthier than synthetic drugs and free of side effects [4]. This may not always be the case, since herbal products can cause heavy liver damage leading to transplantation or even death [5]. One of the potential hazards lies in micro-fungi infestation of the plants, which can, as a result of inappropriate handling, storage and transport [6], lead to contamination with mycotoxins, which can persist in the final herbal supplementary products [4,7] The presence of mycotoxins and other adulterants impairs the quality of supplements and thus the safety of their consumption [4,6] The dishonesty of some manufacturers allows these reduced quality, and in the worst case potentially harmful, products to be marketed [4].

Milk thistle (MT) is a wild thorny herb considered a weed in many areas (see Section 2). Supplements based on this herb are among the top-selling herbal supplements in the US in the mainstream multioutlet channel. In 2018, it was the 20th best-selling herbal supplement with total sales of 16.6 million US dollars. However, compared to 2017, sales decreased by 1.6% [8]. MT is the most commonly researched herb associated with the treatment of liver disease [9], the cause of approximately two million deaths worldwide each year, accounting for 3.6% of all deaths worldwide [10]. However, its main biologically active compound, silymarin (see Section 3) has been proven to have many beneficial effects (see Section 4). Nevertheless, using modern analytical methods (see Section 5), infestation with various micro-fungi [3,11–14] and contamination with their mycotoxins [2,3,15–17] in MT-based supplements has been reported in several studies (see Sections 6 and 7) The highest multi-mycotoxin concentration found in MT-based supplements has reached up to 37.6 mg/kg in total [3]. This concentration slightly exceeds the value earlier determined in the study by Veprikova et al. [15].

Mycotoxins are produced by various micro-fungi as their secondary metabolites, with no biochemical significance in microfungal growth and development [18]. Although they are harmless to their producers, they can elicit adverse effects (carcinogenic, genotoxic, hepatotoxic, teratogenic, estrogenic, immunosuppressive, nephrotoxic, or neurotoxic) in other organisms, mainly in humans and/or animals upon the consumption of contaminated food/feed [18,19]. Some of the mycotoxins produced by *Alternaria* or *Fusarium* species have been shown to be significant in MT-based supplements (see Section 8). Although the occurrence of mycotoxins in herbal-based food supplements is not negligible, they are not yet regulated in EU legislation (see Section 9). This situation needs to be further monitored. Exposure assessment is also needed, but studies on this topic are scarce (see Section 10).

In this review, a total of nine relevant original papers [2,3,11–17] concerning mycotoxins and/or micro-fungi have been included. All these publications were published in the period 2009–2019.

2. Botanical Description

Silybum marianum (L.) Gaertn. (syn. *Carduus marianus* L.) is commonly known as milk thistle but is known by many other names such as blessed milk thistle, Blessed virgin thistle, Christ's crown, heal thistle, holy thistle, Marian thistle, Mary thistle, Saint Mary´s thistle, our lady´s thistle, sow thistle, variegated thistle, venue thistle, or wild artichoke [9,20]. It is a wild thorny annual or, rarely, biannual plant of the *Asteraceae* family [20–22], in many areas considered a weed due to its competitive and aggressive growth, usually reaching a height of 90-200 cm, but even up to 300 cm [23,24]. Purple flower heads and green leaves with milky white veins and strong spiny edges are typical features of the plant The fruits are black achenes with oily eliosome that has significance in myrmecochory–dispersal by ants [24] The plant originates in the Mediterranean basin, but it has spread to central Europe, America and South Australia [24] and nowadays is found worldwide [20,24].

3. Bioactive Compounds of Milk Thistle

The main bioactive complex of MT, collectively known as silymarin, consists mainly of flavonolignans (silybin A (PubChem Compound Identification Number /CID/: 31553), silybin B (PubChem CID: 1548994), isosilybin A (PubChem CID: 11059920), isosilybin B (PubChem CID: 10885340), silydianin (PubChem CID: 11982272), and silychristin (PubChem CID: 441764)), flavonoids (taxifolin (PubChem CID: 439533) and quercetin (PubChem CID: 5280343)), and polyphenolic

compounds [25–28]. However, silybin (syn. silibinin) is considered the main bioactive component [23,25] as it accounts approximately for 50%–60% of silymarin [9] The content of other components is approximately 5% for isosilibyn, 20% for silychristin, and 10% for silydianin and other compounds such as silimonin, isosilychristin, and isosilibinin [9]. Although silymarin is present throughout the whole plant, the highest concentration is found in the seeds [9,22] The chemical structures of the eight above-mentioned flavonolignans and flavonoids are depicted in Figure 1.

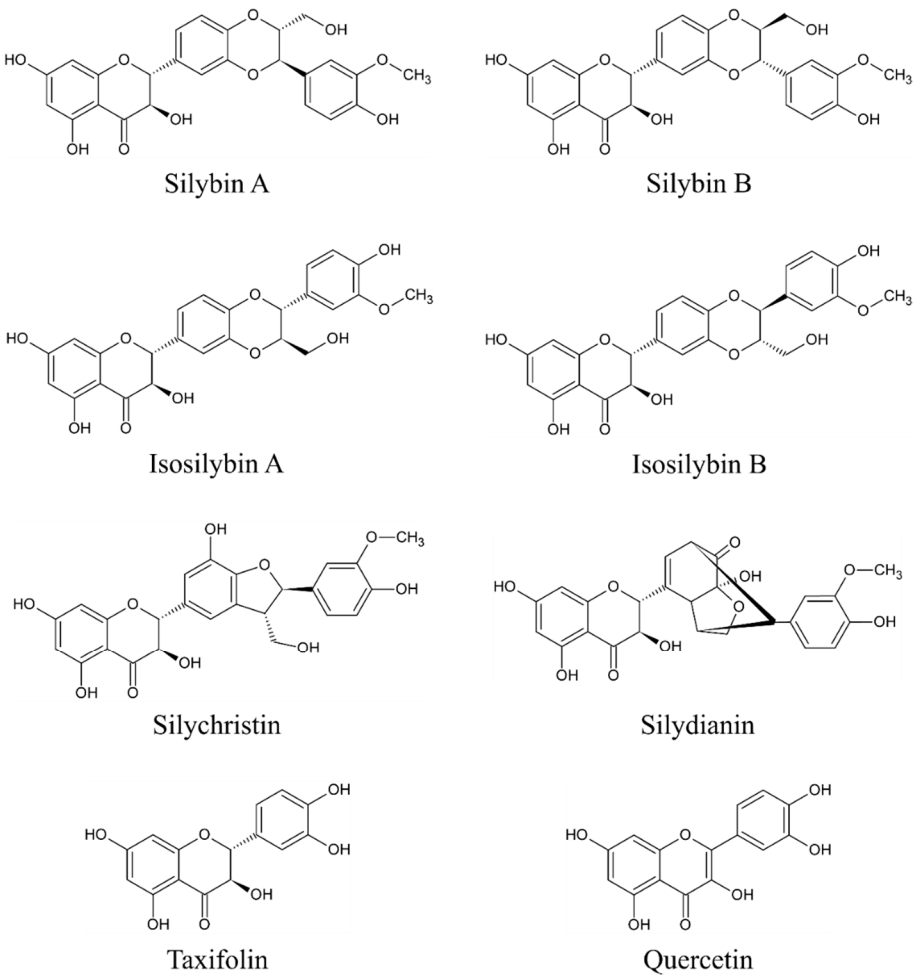

Figure 1. Chemical structures of main flavonolignans and flavonoids contained in silymarin complex.

4. Beneficial Effects of Milk Thistle-Based Supplements

MT has been used as a therapeutic herb for 2000 years [25]. Its main compound silymarin is without a doubt the most popular, most well-researched and potentially most effective herbal product used in the treatment of liver disease in particular, including toxin-induced liver disease, viral hepatitis, liver cirrhosis and hepatocellular carcinoma [5,9,15,25]. In addition, MT is also used in the treatment of kidney, spleen and biliary diseases [25,29]. Besides its well-known hepatoprotective properties, silymarin has also been shown to have antioxidant, antifibrotic, anti-inflammatory, choleretic, and immune-stimulating, regenerative, cytoprotective, cardioprotective, neuroprotective, anti-carcinogenic properties [9,25,29,30]. MT can be used as an antidote or a protective agent against both chemical (metals, fluoride, pesticides, cardiotoxins, neurotoxins, hepatotoxins, and nephrotoxins) and biological (snake and scorpion venoms, bacterial toxins, and mycotoxins) xenobiotics [30]. Due to this wide range of beneficial effects, many recent studies have focused on the effects of silymarin on various health problems. Several studies have demonstrated the neuroprotective effects of silymarin and its potential use in the treatment of Alzheimer's disease [31,32]. Furthermore, positive effects of

silymarin in the treatment of prostatic disorders such as benign prostatic hyperplasia [33], in decreasing frequency and severity of menopausal hot flashes [34], or in alleviating the side effects of the chemotherapeutic drug doxorubicin [35,36] have been demonstrated The possible use of silymarin against solar-induced skin ageing has been demonstrated in a recent study [37]; however, Fidrus et al. warn of increased UVA-induced cytotoxicity after silymarin treatment [38]. Moreover, enhanced proteosynthesis, liver regeneration, increased lactation and immunomodulatory activity have also been associated with the effect of silymarin [9].

The efficacy of silymarin against the adverse effects of some mycotoxins has also been reported. As reviewed by Alhidari et al. (2017), many studies have demonstrated the beneficial effect of silymarin on aflatoxin B_{1}- (AFB_1)-induced reduction of feed intake and weight gain of broilers [39]. Additionally, silymarin has completely prevented the ochratoxin A- (OTA)-induced immunosuppressive effect and has exerted hepatoprotective and nephroprotective effects in broiler chicks [40]. In a recent study, silymarin has been reported to provide cytoprotective activity against OTA, fumonisin B_1 (FB_1) and deoxynivalenol (DON) in porcine kidney-15 (PK-15) cells [41] The alleviating effect of silymarin on zearalenone (ZEA)-induced liver damage and reproductive toxicity in rats has also been reported [42].

MT is marketed as a "dietary supplement" in various forms including seeds, capsules, tablets, granules, extracts or teas. Producers tend to specify the amount of the plant extract contained in the supplement. However, the content of active compounds in the extract itself can vary depending on the conditions (temperature, climate, season, soil, etc.) in which the plant was grown [4]. The recommended daily dose (RDD) of silymarin usually ranges from 420 mg to 600 mg, depending on the application defined by the manufacturer The most common usage is in three doses of 140 mg of silymarin [43]. As demonstrated in a study by Fenclova et al., the content of silymarin compounds can vary considerably (5–393 mg/g), throughout various supplements as well as inter-batch [3] The inconsistency of the number of bioactive compounds may lead to a reduced effect or to an overdose [4], which is manifested with gastrointestinal discomfort (nausea, diarrhea, abdominal pain, etc.) [29].

However, the biomass of MT can also be used in a non-medicinal way, including e.g., human and animal nutrition, bioenergy production, phytoremediation, agriculture, or cosmetic industry [21]. The supplementation of feed with MT/silymarin has proven useful in the livestock diet The improved growth rate and meat quality in pigs [44] and rabbits [45] and increased milk yield and/or quality in cows [46] and sheep [47] have been linked to such supplementation. Moreover, an increase in the egg yield was observed in hens whose feed has been supplemented with MT [48].

5. Methods Used in the Determination of Mycotoxins in Milk Thistle-Based Dietary Supplements

The extraction of mycotoxins from the matrix of MT-based dietary supplements was usually based on the "quick easy cheap effective rugged safe" (QuEChERS) approach [2,3,15] or the dispersive liquid-liquid microextraction (DLLME) approach [2] followed by analysis performed by ultra-high-performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) in studies by Arroyo-Manzanares et al. and Veprikova et al. [2,15], or high-resolution mass spectrometry (UHPLC-HRMS) in a study by Fenclova et al. [3]. A clean-up step based on the immunoaffinity columns followed by separation and quantification using reversed-phase liquid chromatography (RPLC) and determination by post-column photochemical derivatization and fluorescence detection (FLD) was employed in a study by Tournas et al. [17] The enzyme-linked immunosorbent assay (ELISA) method was used after a clean-up step using multifunctional or polyamide columns in a study by Santos et al. [16]. For more details concerning methods used in the determination of mycotoxins in milk thistle-based dietary supplements see Table 1.

Table 1. Overview of the methods used in studies dealing with mycotoxins in milk thistle-based dietary supplements.

Supplement Form	Mycotoxins	Clean-up Method	Analysis	References
Seeds	7 mycotoxins	multifunctional columns (for AFs, ZEA, DON, FBs, T-2); polyamide column (for CIT); no clean-up (for OTA)	ELISA	[16]
Seeds, herbs, tea, alcohol-based liquid seed extract, oil-based liquid seed extract	AFs, AFB_1	immunoaffinity column clean-up	RPLC-FLD	[17]
Seeds, extract	15 mycotoxins	QuEChERS + DLLME (for AFB_1, AFB_2, AFG_1, AFG_2, CIT, HT-2, OTA, STEG, T-2, ZEA)	UHPLC-MS/MS	[2]
Capsules with dried powder/oil-based matrix, seeds, tablets, granules, tea	57 mycotoxins	QuEChERS	UHPLC-MS/MS	[15]
Encapsulated oily paste, capsules with dried powder	55 mycotoxins	QuEChERS	UHPLC-HRMS	[3]

Notes: AFs, aflatoxins; AFB_1, aflatoxin B_1; AFB_2, aflatoxin B_2; AFG_1, aflatoxin G_1; AFG_2, aflatoxin G_2; CIT, citrinin; DON, deoxynivalenol; FBs, fumonisins; HT-2, HT-2 toxin; OTA, ochratoxin A; STEG, sterigmatocystin; T-2, T-2 toxin; ZEA, zearalenone; DLLME, dispersive liquid-liquid microextraction; ELISA, enzyme-linked immunosorbent assay; QuEChERS, quick easy cheap effective rugged safe; RPLC-FLD, reversed-phase liquid chromatography with fluorescence detector; UHPLC-HRMS, ultra-high-performance liquid chromatography-high-resolution mass spectrometry; UHPLC-MS/MS, ultra-high-performance liquid chromatography-mass spectrometry.

6. Micro-fungi in Milk Thistle-Based Dietary Supplements—An Overview

MT has been shown to be infested with numerous saprotrophic and potentially pathogenic molds. *Alternaria* genus, mainly *A. alternata*, is the most prevalent [11–14] The occurrence of *Aspergillus* spp., *Eurotium* spp., *Melanospora* spp., *Mortierella* spp., *Mucor* spp., *Rhizopus* spp., *Ulocladium* spp., *Verticillium* spp., and *Zygorhynchus* spp. is also significant, while the occurrence of *Botrytis* spp., *Phoma* spp., and *Rhizoctonia* spp. is seen rather less often [11,13]. *Cladosporium* spp., *Fusarium* spp., and *Penicillium* spp. have also been found predominant in a study by Rosinska et al. [13], while less often in other studies [11,14]. Other fungi species from the genera of *Acremoniella* spp., *Acremonium* spp., *Arthrinium* spp., *Bipolaris* spp., *Chaetomium* spp., *Epicoccum* spp., *Monascus* spp., *Gliomastix* spp., *Humicola* spp., *Paecilomyces* spp., *Papulaspora* spp., *Phialophora* spp., *Phomopsis* spp., *Sordaria* spp., *Sporotrichum* spp., *Stagnospora* spp., *Stemphylium* spp., *Thamnidium* spp., *Trichoderma* spp., and *Trichothecium* spp. have also been isolated from MT [3,11–14].

The different maximum limits for molds in various herbal materials, based on their intended use, have been set at three levels [49]: the limit of 10^5 colony forming units per gram (CFU/g) for "Raw medicinal plant and herbal materials intended for further processing", 10^4 CFU/g for "Herbal materials that have been pretreated" and "Herbal medicines to which boiling water is added before use", and 10^3 CFU/g for "Other herbal materials for internal use" and "Other herbal medicines" [49]. In a study by Tournas et al. [14], *Aspergillus flavus, A. foetidus, A. penicillioides, A. versicolor, Eurotium amstelodami,* and *E. repens* have exceeded the limit of 10^5 CFU/g. *Alternaria* spp., *Aspergillus candidus*, *A. niger*, *A. tritici*, *Eurotium* spp., *E. rubrum*, *Fusarium* spp., *Fusarium proliferatum*, and *Penicillium chrysogenum* have met or exceeded the limit of 10^4 CFU/g. *Aspergillus* spp., *A. parasiticus*, *A. sydowii*,

A. tamarii, *A. tubingensis*, *Penicillium* spp., *P. diercxii*, *Rhizopus* spp., *Fusarium subglutinans*, and *Eurotium chevalieri* have met or exceeded the limit of 10^3 CFU/g.

7. Mycotoxin Contamination of Dietary Supplements Based on Milk Thistle—An Overview

This review provides a summary of five original papers on mycotoxins in various forms of dietary supplements based on MT The results of the individual original papers have been summarized to create a comprehensive analysis. For the purpose of this review, the various forms have been grouped into six categories as follows: (1) seeds, (2) capsules, (3) tablets, (4) granules, (5) extracts, and (6) herbs.

Throughout all five original studies, a total of 57 mycotoxins have been tested in various MT-based supplements, namely: 3-acetyl deoxynivalenol (3-AcDON), 3/15-acetyl deoxynivalenol (3/15-AcDON), aflatoxins (AFs), AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), agroclavine (AGC), alternariol-methyl-ether (AME), alternariol (AOH), beauvericin (BEA), citrinin (CIT), cyclopiazonic acid (CPA), diacetoxyscirpenol (DAS), DON, deoxynivalenol-3-glucoside (DON-3G), enniatin A (ENNA), enniatin A_1 ($ENNA_1$), enniatin B (ENNB), enniatin B_1 ($ENNB_1$), ergot alcaloids (EA; including ergocornine, ergocorninine, ergocristine, ergocristinine, ergocryptine, ergocryptinine, ergometrine, ergosine, ergosinine, ergotamine, ergotaminine), fumonisins (FBs), FB_1, fumonisin B_2 (FB_2), fumonisin B_3 (FB_3), fusarenon X (FUS-X), gliotoxin (GLI), HT-2 toxin (HT-2), meleagriin (MEL), mycophenolic acid (MPA), neosolaniol (NEO), nivalenol (NIV), OTA, patulin (PAT), paxilline (PAX), penicillic acid (PeA), penitrem A (PenA), phomopsin A (PHO-A), roquefortine C (ROC), sterigmatocystin (STEG), stachybotrylactam (STLAC), T-2 toxin (T-2), tenuazonic acid (TEA), tentoxin (TEN), verrucarol (VER), verruculogen (VERR), ZEA, α-zearalenol (α-ZOL), β-zearalenol (β-ZOL).

A total of 21 mycotoxins (3-AcDON, AFB_1, AME, AOH, BEA, DAS, DON, ENNA, $ENNA_1$, ENNB, $ENNB_1$, FB_3, FUS-X, HT-2, MPA, NEO, STEG, T-2, TEA, TEN, ZEA) have been found positive at least once in one of the forms throughout all five studies. On the contrary, a total of 36 mycotoxins (3/15-AcDON, AFB_2, AFG_1, AFG_2, AGC, CIT, CPA, DON-3G, EA, FB_1, FB_2, GLI, MEL, NIV, OTA, PAT, PAX, PeA, PenA, PHO-A, ROC, STLAC, VER, VERR, α-ZOL, β-ZOL) have been tested in various MT-samples, but have never been confirmed positive. For more details regarding the positivity/negativity and the number of tested samples in the given categories see Figure 2. Among all mycotoxins, AME, AOH, BEA, DON, ENNA, $ENNA_1$, ENNB, $ENNB_1$, HT-2, T-2, TEN, and ZEA seem to be the most significant in MT-based dietary supplements. In this review, special attention will be given to these significant mycotoxins (see Section 8).

As can be seen in Figure 2, AFs (117 samples), AFB_1 (68), OTA (67), DON (67), T-2 (67), ZEA (67), FB_1 (65), FB_2 (65), FUS-X (65), HT-2 (65), and STEG (65) are the most frequently analyzed mycotoxins in MT-based dietary supplements, followed by AME (58), AOH (58), BEA (58), DAS (58), ENNA (58), $ENNA_1$ (58), ENNB (58), $ENNB_1$ (58), FB_3 (58), MPA (58), NEO (58), PAT (58), PenA (58), and TEN (58). Regarding the positivity of samples for a given mycotoxin, the frequency of testing should be taken into consideration as the percentages below are the more conclusive the more samples they are based on. For that reason, the categorization into seven levels: 1) Extremely high (more than 90%), 2) Very high (up to 90%), 3) High (up to 75%), 4) Moderate (up to 50%), 5) Low (up to25%), 6) Rare (up to 5%), and 7) None (0%) are based on data with at least 50 tested samples on a given mycotoxin. Extremely high positivity has been found in case of AME (98.28%, 57/58), $ENNB_1$ (94.83%, 55/58), AOH (93.10%, 54/58), BEA (93.10%, 54/58), and ENNB (93.10%, 54/58), very high positivity in case of $ENNA_1$ (89.66%, 52/58), ENNA (87.93%, 51/58), TEN (86.21%, 50/58), and T-2 (77.61%, 52/67), high positivity in case of HT-2 (73.85%, 48/65), ZEA (73.13%, 49/67), and DON (55.22%, 37/67), low positivity in case of NEO (18.97%, 11/58), AFs (15.38%, 18/117), DAS (6.90%, 4/58), MPA (6.90%, 4/58), and FUS-X (6.15%, 4/65), as rare in case of STEG (4.62%, 3/65), AFB_1 (2.94%, 2/68), and FB_3 (1.72%, 1/58), and none in case of OTA (0%, 0/67), FB_1 (0%, 0/65), FB_2 (0%, 0/65), PAT (0%, 0/58), and PenA (0%, 0/58).

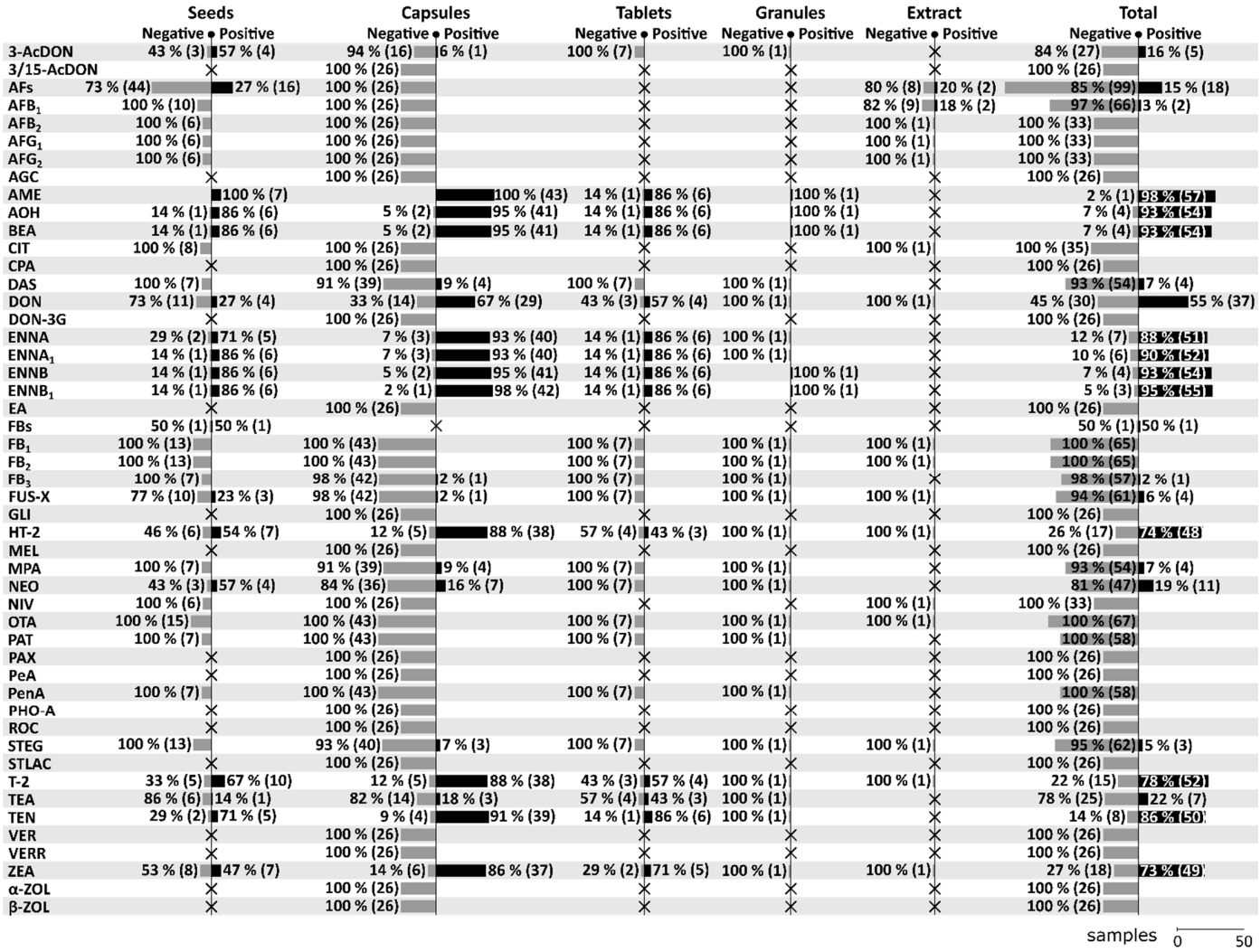

Figure 2. Contamination of milk thistle-based dietary supplement depending on its form. Processed based on the data from original papers [2,3,15–17]. Notes: EA include ergocornine, ergocorninine, ergocristine, ergocristinine, ergocryptine, ergocryptinine, ergometrine, ergosine, ergosinine, ergotamine, and ergotaminine.

7.1. Seeds

Beside seeds [2,15–17], the category *"Seeds"* also includes several samples of seeds intended for the preparation of tea [15,17]. A total of 31 mycotoxins have been analysed in MT seeds of which a total of 16 mycotoxins have been found positive. Compared to other categories, seeds appear to be relatively more contaminated with 3-AcDON, AFs, FUS-X, and NEO. For more details concerning the positivity of seed samples see Figure 2.

In seeds, the highest concentrations have reached up to 1900 µg/kg for AME, 1740 µg/kg for ENNB, 1450 µg/kg for AOH, 975 µg/kg for TEA [15], 943.7 µg/kg for HT-2 [2], 681 µg/kg for $ENNB_1$ [15], 453.9 µg/kg for T-2 [2], 293 µg/kg for DON, 274 µg/kg for $ENNA_1$, 265 µg/kg for 3-AcDON [15], 236.7 µg/kg for FBs [16], 234 µg/kg for BEA, 202 µg/kg for ENNA, 201 µg/kg for TEN, 199 µg/kg for FUS-X, 110 µg/kg for ZEA, 36 µg/kg for NEO [15], 11.5 µg/kg for AFs [16], 1.9 µg/kg for AFB_1 [17].

7.2. Capsules

The category *"Capsules"* consists of capsules with dried powder [3,15], capsules with oil-based matrix [15], and encapsulated oily paste [3]. A total of 57 mycotoxins have been analysed in MT capsules of which a total of 20 mycotoxins have been found positive. Compared to other categories, capsules appear to be relatively more contaminated with DAS, DON, HT-2, MPA, and T-2. For more details concerning the positivity of capsule samples see Figure 2.

The maximum levels have been reaching up to 10,940 µg/kg for $ENNB_1$, 9260 µg/kg for ENNB, 8340 µg/kg for ENNA [15], 6834 µg/kg for AOH, 6477 µg/kg for DON, 5958 µg/kg for T-2, 3891 µg/kg for BEA [3], 3200 for AME [15], 2985 µg/kg for HT-2 [3], 2340 µg/kg for $ENNA_1$, 2140 µg/kg for TEA [15], 2127 µg/kg for TEN [3], 1710 µg/kg for MPA, 751 µg/kg for ZEA, 175 µg/kg for 3-AcDON, 126 µg/kg for NEO, 120 µg/kg for FUS-X [15], 59 µg/kg for DAS [3], 13 µg/kg for FB_3, and 11 µg/kg for STEG [15].

7.3. Tablets

A total of 25 mycotoxins have been analysed in MT-tablets of which a total of 13 mycotoxins have been found positive. Compared to other categories, tablets appear to be relatively more contaminated with TEA. For more details concerning the positivity of tablets samples see Figure 2.

The maximum levels have been reaching up to 2110 µg/kg for ENNB, 2020 µg/kg for AME, 1560 µg/kg for DON, 1370 µg/kg for TEA, 1340 µg/kg for AOH, 988 µg/kg for TEN, 842 µg/kg for BEA, 716 µg/kg for $ENNB_1$, 640 µg/kg for T-2, 582 µg/kg for HT-2, 403 µg/kg for ZEA, 380 µg/kg for $ENNA_1$, and 186 µg/kg for ENNA [15].

7.4. Granules

Only one sample of MT granules have been analysed for a total of 25 mycotoxins of which a total of 5 mycotoxins have been found positive with the following levels: 23 µg/kg for AOH, 16 µg/kg for ENNB, 6 µg/kg for $ENNB_1$, 5 µg/kg for BEA, and 3 µg/kg for AME [15]. For more details concerning the positivity of granule samples see Figure 2.

7.5. Extracts

The category *"Extracts"* covers natural extract in glycerin [2], oil-based liquid seed extract and alcohol-based liquid seed extract [17]. A total of 16 mycotoxins have been analysed in MT extract of which a total of 2 mycotoxins have been found positive The maximum levels have been up to 0.06 µg/kg for AFB_1 (and AFs at the same time) [17]. For more details concerning the positivity of extract samples see Figure 2.

7.6. Herbs

So far, MT herbs (powdered or minced) have been analysed only for AFs and AFB_1, but none of the tested samples has been found positive [17].

8. The Most Significant Mycotoxins in Milk Thistle-Based Dietary Supplements

Based on available studies on the occurrence of mycotoxins in MT-based dietary supplements, the most critical mycotoxins appear to be AME, AOH, and TEN produced by *Alternaria* species and BEA, DON, ENNA, ENNA$_1$, ENNB, ENNB$_1$, HT-2, T-2, and ZEA produced by *Fusarium* species. All of these mycotoxins have shown an overall positivity of more than 50% (at least 55% in case of DON, up to 98% in case of AME) based on at least 58 samples. All of these 12 mycotoxins are considered significant in this study and will be given special attention (see below) The data regarding the positivity and concentrations of *"significant mycotoxins"* are based on original studies reviewed by Arroyo-Manzanares et al. [2], Fenclova et al. [3], Santos et al. [16], and Veprikova et al. [15]. The chemical structure of these significant mycotoxins is shown in Figure 3.

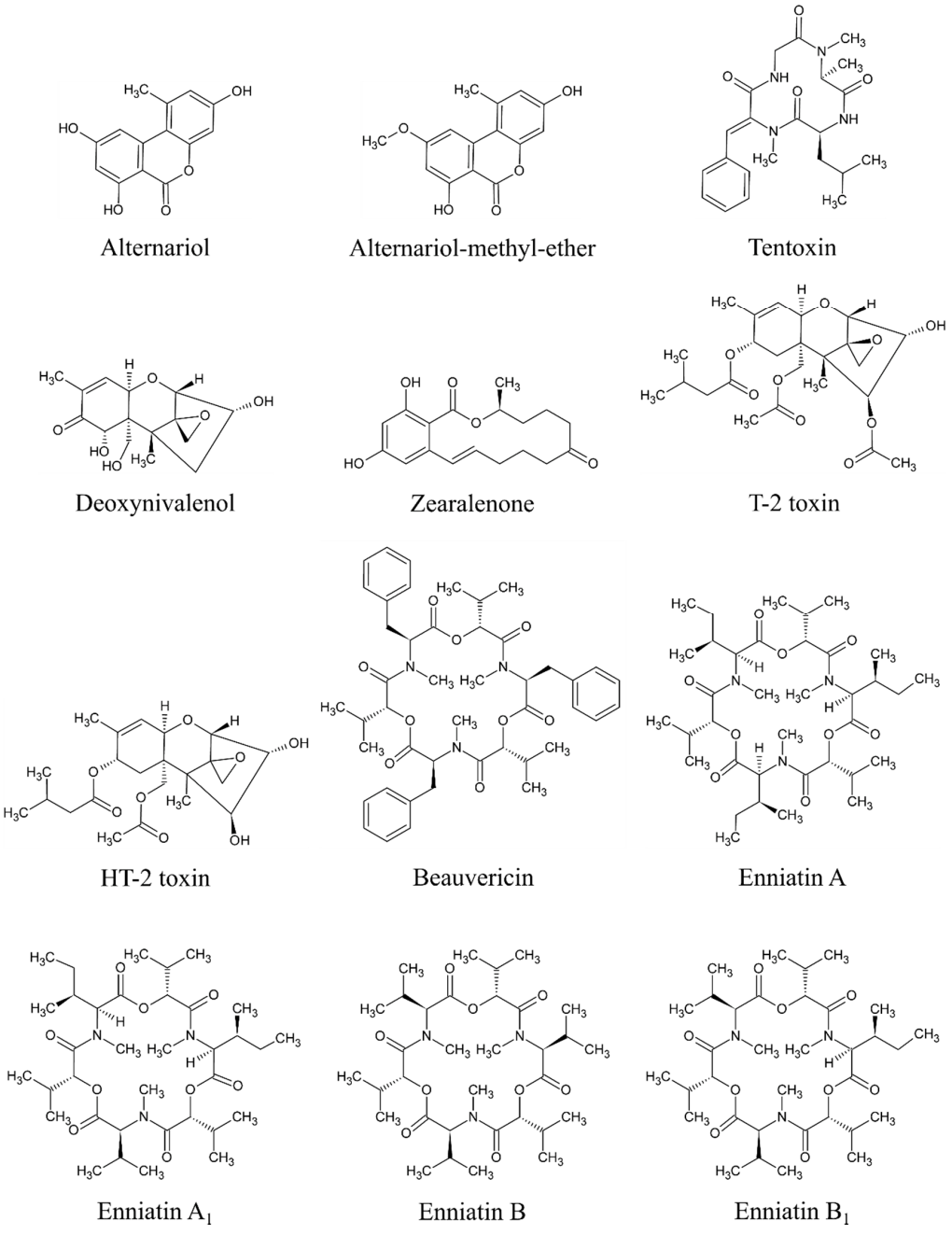

Figure 3. Chemical structures of significant mycotoxins found in milk thistle-based dietary supplements.

8.1. Alternaria Mycotoxins (AME, AOH, TEN)

Alternaria mycotoxins are produced by *Alternaria* genus [50], with *A. alternata* being the most common species [51,52]. However, *A. tenuissima*, *A. arborescens* [53], *A. tangelonis*, and *A. turkisafria* [54] are also significant in food. *Alternaria* fungi produce more than 70 different secondary metabolites [55]. Some of these are significant contaminants in food such as fruits, vegetables, cereals and derived products, and oilseeds [55]. AME (PubChem CID: 5360741), AOH (PubChem CID: 5359485), and TEN (PubChem CID: 5281143) [28] appear to be significant contaminants in MT-based supplements. Generally, AOH and AME are so far the most commonly studied *Alternaria* metabolites [56].

Among the *Alternaria* mycotoxins, hepatotoxic, genotoxic, mutagenic, clastogenic, immunotoxic and dermatoxic effects, reproductive toxicity, as well as an effect on estrogen activity, have been observed. Hepatotoxicity of AOH, AME and TEN have been suggested in vitro on the human hepatoma (HepaRG) cell line [57]. Genotoxicity of *Alternaria* toxin mixtures has been reported in vitro on human endometrial adenocarcinoma (Ishikawa) cells [56] and genotoxicity of AOH and AME has been reported on Chinese hamster (V79) cells, human liver (HepG2) cells and human colon (HT-29) cells [58]. Mutagenic effect of AOH has been observed in vitro on Chinese hamster (V79) cells and mouse lymphoma (L5178Y TK+/−) cells [59]. Clastogenic effect of AOH has been reported in vitro on human endometrial adenocarcinoma (Ishikawa) cells and Chinese hamster (V79) cells [60]. Immunotoxicity of AOH has been demonstrated in vitro on human colon adenocarcinoma (Caco-2) cells [61] or human monocytic (THP-1) cells [62]. Dermal toxicity of AOH has been demonstrated in vivo on mice [63]. Adverse effects on reproductive performance have been suggested in vitro on porcine ovarian cells [64] The effect on estrogen activity has been reported in vitro and in silico on human endometrial adenocarcinoma (Ishikawa) cells and Chinese hamster (V79) cells [56,60,65]. Despite some esophageal carcinogenic effects of *Alternaria* mycotoxins (AOH and AME) having been reported [66], none has been classified by the International Agency for Research on Cancer (IARC) so far.

AME has proved to be the most common mycotoxin in MT-based supplements, occurring in 57 out of 58 total examined samples (7 seeds, 43 capsules, 6 tablets and 1 granule) The only negative sample was a tablet form.

The maximum levels of AME have been found in capsules containing dried powder (3200 μg/kg), followed by oil-based capsules (2110 μg/kg), tablets (2020 μg/kg) and seeds (1900 μg/kg) [15]. In the granule sample, a concentration of 3 μg/kg has been observed [15].

AOH is among the mycotoxins with extremely high positivity in MT-based supplements, with 54 positive samples out of 58 total examined samples (6 out of 7 seed samples, 41 out of 43 capsules, 6 out of 7 tablets and 1 out of 1 granule).

The maximum levels of AOH have been found in capsules containing dried powder (6834 μg/kg), followed by oil-based capsules (1964 μg/kg) [3], seeds (1450 μg/kg) and tablets (1340 μg/kg) [15]. In the granule sample, a concentration of 23 μg/kg has been observed [15].

Although less significant than AME and AOH, the positivity of TEN in MT-based supplements is still very high: 50 positive samples out of 58 total examined samples (5 out of 7 seed samples, 39 out of 43 capsules, 6 out of 7 tablets, and 0 out of 1 granule).

The maximum levels of TEN have been found in capsules containing dried powder (2127 μg/kg) [3], followed by tablets (988 μg/kg), oil-based capsules (772 μg/kg) and seeds (201 μg/kg) [15].

8.2. Fusarium Mycotoxins

Four "common" *Fusarium* mycotoxins occur in MT-based supplements in significant amounts –DON (PubChem CID: 40024), T-2 (PubChem CID: 5759), HT-2 (PubChem CID: 520286), ZEA (PubChem CID: 5281576) [28]. Moreover, some emergent *Fusarium* mycotoxins–BEA (PubChem CID: 3007984), ENNA (PubChem CID: 57339252), $ENNA_1$ (PubChem CID: 57339253), ENNB (PubChem CID: 164754), and $ENNB_1$ (PubChem CID: 11262300) [28] are also significant.

8.3. Trichothecenes (DON, T-2, HT-2)

Trichothecenes (TCT) are a group of chemically related mycotoxins (types A-D). In food, TCT are produced by the *Fusarium* genera. T-2/HT-2 (type A) and DON (type B) are significant contaminants of MT. DON is the most important TCT produced mainly by *F. graminearum* and *F. culmorum*, especially in cereals [67]. T2/HT-2 are produced mainly by *F. sporotrichioides*, *F. landsethiae*, *F. poae*, and *F. sambucinum* [67].

Cytotoxic, hepatotoxic, neurotoxic, and immunotoxic effects, as well as reproductive toxicity and skin toxicity, have been reported for both T-2 and DON. In vivo hepatotoxic effects have been reported on mice in case of DON [68] and on broilers in case of T-2 [69]. Neurotoxic effects in vivo have been reported on chicks in case of DON [70] and on rats in case of T-2 [71] The immunotoxic effect of T-2 has been reported on rainbow trout (*Oncorhynchus mykiss*) in vivo [72] and the cytotoxic effect on monocytes, macrophages, dendritic cells and B and T lymphocytes in vitro [73–75]. DON was reported to be less cytotoxic on dendritic cells in vitro than T-2 [76]. Reproductive toxicity has been reported on male mice in vivo in case of T-2 [77] and on boar semen in vitro in case of DON [78]. Skin toxicity has been demonstrated for T-2 on mice and rabbits in vivo [79,80] and suggested for DON in vitro on human immortalized keratinocytes [81]. Moreover, in vitro, the cytotoxic effect of T-2 and DON on human liver cancer (HepG2) cells has been confirmed [82,83]. T-2(/HT-2)-induced cytotoxicity on human chondrocytes [84] and broiler hepatocytes [85] in vitro has been reported. In terms of carcinogenicity, T-2 and DON are classified by the IARC into group 3 "Not classifiable as to its carcinogenicity to humans" [86], but no data are available on the carcinogenicity of HT-2 [87].

DON is the least occurring among the significant mycotoxins in MT-based dietary supplements, as it has been found only in 37 out of 67 total examined samples (4 out of 15 seed samples, 29 out of 43 capsules, 4 out of 7 tablets, 0 out of 1 granule, and 0 out of 1 extract).

The maximum levels of DON have been found in capsules containing dried powder (6477 µg/kg) [3], followed by oil-based capsules (2890 µg/kg), tablets (1560 µg/kg), and seeds (293 µg/kg) [15].

T-2 has been found in 52 out of 67 total examined samples (10 out of 15 seed samples, 38 out of 43 capsules, 4 out of 7 tablets, 0 out of 1 granule, and 0 out of 1 extract) The maximum levels of T-2 have been found in capsules with dried powder (5958 µg/kg) [3], followed by oil-based capsules (1870 µg/kg), tablets (640 µg/kg) [15], and seeds (453.9 µg/kg) [2].

HT-2 has been found positive in 48 out of 65 total examined samples (7 out of 13 seed samples, 38 out of 43 capsules, 3 out of 7 tablets, 0 out of 1 granule, and 0 out of 1 extract) The maximum levels of HT-2 have been found in capsules with dried powder (2985 µg/kg) [3], followed by oil-based capsules (1530 µg/kg) [15], seeds (943.7 µg/kg) [2], and tablets (582 µg/kg) [15].

8.4. Zearalenone (ZEA)

ZEA is a non-steroidal estrogenic mycotoxin produced mainly by the *Fusarium* genera [88]. *F. graminearum* and *F. culmorum* are the main ZEA producers in food. *F. equiseti* and *F. crookwellense* also produce ZEA [67]. ZEA is a common contaminant in grains, mainly in maize, but also in other cereals such as wheat, barley, oat and sorghum [89,90]. Nevertheless, in the context of this review, ZEA has been shown to be a significant contaminant in MT-supplements.

ZEA is often associated with reproductive disorders in livestock (e.g., pigs, cattle, and sheep) and occasionally exerts hyper-estrogenic syndrome in humans [91]. Recently, ZEA reproductive toxicity has been demonstrated in vitro on boar semen [78,92] and in vivo on rats [42], or model organism *Artemia francisacana* [93] The estrogenic effect has been observed in vitro on human endometrial cancer (Ishikawa) cells [94]. Moreover, developmental toxicity and fetotoxicity have been reported on mice in vivo [95] and embryotoxicity has been observed in vitro on early porcine embryos [96] and human embryonic stem cells (hESC) [97].

Besides reproductive and developmental toxicity, xenoestrogenity, fetotoxicity and embryotoxicity, ZEA was reported to exert cytotoxic, cardiotoxic, nephrotoxic, hepatotoxic, immunotoxic, genotoxic and neurotoxic effects. ZEA-induced cardiotoxicity has been reported in vivo on mice [98]

The nephrotoxicity of ZEA has been reported in vivo on rats [99,100] The hepatotoxic effect was observed in vitro on rats [100] and mice [68] The immunotoxicity of ZEA has been confirmed on mice [101] and rats [102] in vivo and suggested in vitro on swine spleen [103]. ZEA has been found to promote apoptosis, autophagy and DNA damage in porcine blastocysts [96] The cytotoxic effect of ZEA has been demonstrated in vitro on human liver cancer (HepG2) cells [82,104,105], human adrenocortical carcinoma (H295R) cells [106], murine Leukemia virus-induced tumor (RAW 264.7) cells [82] and pig intestinal epithelial (IPEC-J2) cells [107]. ZEA has been reported to affect mouse brain function in vivo [108]. Recent studies confirm a gastro-toxic effect of ZEA on piglets [109] and rats [110] in vivo and reveal in vitro gastro-toxic effects on porcine jejunum explant [111]. From the point of view of the carcinogenicity, ZEA has been classified by IARC into group 3 "Not classifiable as to its carcinogenicity to humans" [86].

ZEA has been found in 49 out of 67 total examined samples (7 out of 15 seed samples, 37 out of 43 capsules, 5 out of 7 tablets, 0 out of 1 granule, and 0 out of 1 extract) The maximum levels of ZEA have been found in capsules with dried powder (751 μg/kg), followed by tablets (403 μg/kg), oil-based capsules (373 μg/kg), and seeds (110 μg/kg) [15].

8.5. Emergent Mycotoxins (BEA, ENNs)

ENNs and BEA are considered emergent in the recent literature [112]. They are non-trichothecene secondary metabolites produced by the *Fusarium* species in particular [113–115]. In food, they are both produced by *Fusarium acuminatum*, *F. avenaceum*, *F. poae*, *F. sambucinum* and *F. sporotrichioides* The other BEA food-born producers are *F. dalminii*, *F. equiseti*, *F. longipes*, *F. nygamai*, *F. oxysporum*, *F.proliferatum*, *F. subglutinans*, *F. verticillioides* The other ENN food-borne producers are *F. langsethiae* and *F. lateritium* [67].

The European Food Safety Authority (EFSA) has concluded that neither BEA nor ENNs indicates a serious problem for human health in acute exposure [114], which may be relevant to their rapid absorption, distribution and elimination [116] The cytotoxic effects in vitro of both BEA and ENNs are widely researched and confirmed by many studies. Their cytotoxic effect has been reported on human colon adenocarcinoma (Caco-2) cells [117–119], human liver cancer (HepG2) cells, human bronchial (BEAS-2B) cells, human gastric (N87) cells, human vascular endothelial cells (HUVEC), and human keratinocytes (HEK) [119]. Moreover, BEA has been reported cytotoxic in human neuroblastoma (SH-SY5Y) cells [120], while ENNs have shown cytotoxic effects on human cervix carcinoma (HeLa) cells (ENNA) [121]. In some cases, both BEA and ENNs (namely ENNA) have been reported to have a mild genotoxic [117,121] or hemolytic [119] effect. In addition, $ENNB_1$ has been reported to induce oxidative stress and immunotoxic effects during mouse embryo development [122]. A recent in vivo study showed an overall toxic effect of BEA on *Caenorhaditis elegans*, reducing its life span and exerting reproductive and developmental toxicity, cyto-toxicity and oxidative stress [123].

Due to the common producers, as well as the similar chemical structure of these mycotoxins, their co-occurrence can be expected. However, they can also occur together with other *Fusarium* mycotoxins [114]. Although they are considered to occur especially in cereal grains and grain-based products [114], they have also been shown as significant in this review concerning MT-based supplements.

BEA has been found in 54 out of 58 total examined samples (6 out of 7 seed samples, 41 out of 43 capsules, 6 out of 7 tablets, and 1 out of 1 granule) The maximum levels of BEA have been found in capsules with dried powder (3891 μg/kg) [3], followed by oil-based capsule (1560 μg/kg), tablets (842 μg/kg), seeds (234 μg/kg), and granules (5 μg/kg) [15].

ENNA has been found in 51 out of 58 total examined samples (5 out of 7 seed samples, 40 out of 43 capsules, 6 out of 7 tablets, 0 out of 1 granule) The maximum levels have been found in oil-based capsules (8340 μg/kg), followed by capsules with dried powder (4240 μg/kg), seeds (202 μg/kg), and tablets (186 μg/kg) [15].

$ENNA_1$ has been found in 52 out of 58 total examined samples (6 out of 7 seed samples, 40 out of 43 capsules, 6 out of 7 tablets, an 0 out of 1 granule) The maximum levels have been found in oil-based capsules (2340 μg/kg), followed by capsules with dried powder (1420 μg/kg), tablets (380 μg/kg), and seeds (274 μg/kg) [15].

ENNB has been found in 54 out of 58 total examined samples (6 out of 7 seed samples, 41 out of 43 capsules, 6 out of 7 tablets, and 1 out of 1 granule) The maximum levels have been found in oil-based capsules (9260 μg/kg), followed by capsules with dried powder (6190 μg/kg), tablets (2110 μg/kg), seeds (1740 μg/kg), and granules (16 μg/kg) [15].

$ENNB_1$ has been found in 55 out of 58 total examined samples (6 out of 7 seed samples, 42 out of 43 capsules, 6 out of 7 tablets) The maximum levels have been found in capsules with dried powder (10,940 μg/kg), followed by oil-based capsules (4750 μg/kg), tablets (716 μg/kg), seeds (681 μg/kg), and granules (6 μg/kg) [15].

9. Mycotoxin Regulations

The presence of mycotoxins in herbal-based food supplements cannot be completely avoided. There is a need to establish maximum levels or action levels of mycotoxins in some kinds of commodities. Risk management is significantly applied here. No regulatory limits for herbal-based food supplements have been incorporated into legislation so far The maximum regulatory limits for certain mycotoxins in foods have been set under EU regulation No. 1881/2006 [124], and later decrees as in force. Nevertheless, in the case of herbs, the legislation covers only AFs and OTA The maximum limits of 5 and 10 μg/kg for AFB_1 and sum of AFs, respectively, have been set for ginger [124]. For OTA, the maximum limit of 20 μg/kg has been set for liquorice root, ingredient for herbal infusion and 80 μg/kg for liquorice extract, for use in food in particular beverages and confectionary [125].

10. Mycotoxin Exposure Assessment and Risk Characterization

The Joint FAO/WHO Expert Committee on Food Additives (JEFCA) established provisional maximum tolerable daily intakes (PMTDI) for DON and its acetylated derivates (3-AcDON and 15-AcDON) of 1 μg/kg body weight (bw) per day [126]. A current group tolerable daily intake (TDI) of 1 μg/kg bw was established for the sum of DON, 3-AcDON, 15-AcDON and DON-3G, based on reduced body weight gain in experimental female and male mice [127].

PMTDI was established for T-2 and HT-2 alone or in combination of 0.06 μg/kg bw per day obtained in a 3-week dietary study in pigs [128]. A new group TDI of 0.02 μg/kg bw was established by EFSA for the sum of T-2 and HT-2 based on an in vivo sub-chronic toxicity study with rats [129].

PMTDI was established for ZEA of 0.5 μg/kg bw based on the no observed effect level (NOEL) of 40 μg/kg bw per day obtained in a 15-day study in pigs [130] The current TDI for ZEA of 0.25 μg/kg bw per day established by EFSA is based on estrogenicity in pigs [131].

The increased incidence of microscopic kidney lesions seen in a 3-month feeding study with pigs [132] was considered as the most appropriate endpoint of non-neoplastic effects of OTA and the resulting benchmark dose limit (BMDL) of 4.73 μg/kg bw per day was used for comparison with chronic exposures.

In the absence of elucidated MoAs for the genotoxicity/carcinogenicity of OTA, the Panel concluded that a margin of exposure (MOE) of 10,000 needs to be applied to the $BMDL_{10}$ of 14.5 μg/kg bw per day for neoplastic effects (kidney tumors) in the rat The Panel points out that this MOE is likely to be particularly conservative in this case, as the evidence for a direct interaction of OTA with the DNA is inconclusive and other threshold mechanisms may play a role in the formation of kidney tumors. As it was not possible to quantify these variables, the default MOE of 10,000 was applied [133].

Based on studies in animals, the CONTAM Panel selected a $BMDL_{10}$ of 0.4 μg/kg bw per day for the incidence of hepatocellular carcinoma (HCC) in male rats following AFB_1 exposure to be used in a MOE approach The calculation of a BMDL from the human data was not appropriate; instead, the cancer potencies estimated by the JECFA in 2016 were used [134].

Studies evaluating the dietary exposure to mycotoxins from MT food supplements are scarce. Several studies have attempted a very rough assessment of dietary exposure based on the RDD of food supplements (e.g., capsules) declared by the manufacturers.

For DON, TDI has been set at 1 μg/kg bw per day [135], which means 70 μg for a 70 kg human. In the worst-case scenario, for a human of this weight, a single RDD of 3 capsules of MT-based supplement has accounted for 23.0% of TDI [3]. On average, 2.1% of TDI is received by the MT-based supplements [3,15].

For ZEA, the TDI has been set at 0.25 μg/kg per day [136], which means 17.5 μg for a 70 kg human. In the worst-case scenario, for a human of this weight, a single RDD of 10 capsules of MT-based supplement has accounted for 5.3% of TDI [15]. On average, 1.0% of TDI is received by the MT-based supplements [3,15].

For the sum of T-2 and HT-2, the TDI has been set at 0.02 μg/kg bw per day [129], which means 1.4 μg for a 70 kg human. In the worst-case scenario, for a human of this weight, a single RDD of 3 capsules of MT-based supplement has accounted for 1590% of TDI [3]. On average, 123% of TDI is received by the MT-based supplements [3,15].

There is insufficient data to establish dietary exposure assessment for any *Alternaria* mycotoxins [137], ENNs or BEA [114].

11. Summary

People use silymarin preparations to prevent or treat various diseases, especially, but not limited to, liver diseases. Although silymarin appears to be effective in this aspect, a number of various mycotoxins with, inter alia, hepatotoxic effects have been found in marketed MT preparations. Studies have shown that silymarin can alleviate the adverse effects of some mycotoxins, notably AFB_1, but also OTA, FB_1, ZEA or DON. However, the latter two have also been shown to occur in MT-based supplements to a considerable extent. In addition, it has been shown that the content of silymarin in the preparations varies considerably The question arises as to whether the consumption of these supplements in order to improve health does not become rather harmful, with a regard to the detected levels of mycotoxins. It is, therefore, necessary to monitor both the content of active compounds in MT-based supplements and the presence of mycotoxins and other contaminants, to assess the intake of the substances into the body, and to evaluate whether the beneficial effects of marketed MT-preparations outweigh the harmful effects of the contaminants. It should also be borne in mind that people may take more than one type of food supplement at the same time, which is worrying if the other supplements are also contaminated with mycotoxins to a similar extent.

This review examined the current state of contamination of MT-based dietary supplements with mycotoxins and, to a lesser extent, micro-fungi The results show that these supplements are mainly infested by micro-fungi of the *Alternaria* genus. Of the 57 mycotoxins monitored across five original studies concerning MT-based supplements in various forms, a total of 21 have been found to be positive in at least one case. A total of 12 (AME, AOH, TEN, DON, HT-2, T-2, ZEA BEA, ENNA, $ENNA_1$, ENNB, $ENNB_1$) of these mycotoxins can be considered significant due to their high occurrence meaning more than 50% of positive samples in the context of this review.

The obtained overview strongly indicates the need for the strict monitoring of mycotoxins in commercially sold MT-based dietary supplements that are used by many people worldwide to treat or prevent liver diseases, and thereby enhance their health.

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References

1. European Parliament and the Council of the European Union. Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the member states relating to food supplements. *Off. J. Eur. Communities* **2002**, *L183*, 51–57.
2. Arroyo-Manzanares, N.; García-Campaña, A.M.; Gámiz-Gracia, L. Multiclass mycotoxin analysis in *Silybum marianum* by ultra high performance liquid chromatography–tandem mass spectrometry using a procedure based on QuEChERS and dispersive liquid–liquid microextraction. *J. Chromatogr. A* **2013**, *1282*, 11–19. [CrossRef]
3. Fenclova, M.; Novakova, A.; Viktorova, J.; Jonatova, P.; Dzuman, Z.; Ruml, T.; Kren, V.; Hajslova, J.; Vitek, L.; Stranska-Zachariasova, M. Poor chemical and microbiological quality of the commercial milk thistle-based dietary supplements may account for their reported unsatisfactory and non-reproducible clinical outcomes. *Sci. Rep.* **2019**, *9*, 11118. [CrossRef]
4. Fibigr, J.; Šatínský, D.; Solich, P. Current trends in the analysis and quality control of food supplements based on plant extracts. *Anal. Chim. Acta* **2018**, *1036*, 1–15. [CrossRef]
5. Seeff, L.B.; Bonkovsky, H.L.; Navarro, V.J.; Wang, G. Herbal products and the liver: A review of adverse effects and mechanisms. *Gastroenterology* **2015**, *148*, 517–532.e3. [CrossRef]
6. Ashiq, S.; Hussain, M.; Ahmad, B. Natural occurrence of mycotoxins in medicinal plants: A review. *Fungal Genet. Biol.* **2014**, *66*, 1–10. [CrossRef] [PubMed]
7. Mavungu, J.D.D.; Monbaliu, S.; Scippo, M.-L.; Maghuin-Rogister, G.; Schneider, Y.-J.; Larondelle, Y.; Callebaut, A.; Robbens, J.; Peteghem, C.V.; Saeger, S.D. LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. *Food Addit. Contam. Part A* **2009**, *26*, 885–895. [CrossRef]
8. Smith, T.; Gillespie, M.; Eckl, V.; Knepper, J.; Reynolds, C.M. Herbal supplement sales in US increase by 9.4% in 2018. *HerbalGram* **2019**, *123*, 62–73.
9. Abenavoli, L.; Capasso, R.; Milic, N.; Capasso, F. Milk thistle in liver diseases: Past, present, future. *Phytother. Res.* **2010**, *24*, 1423–1432. [CrossRef] [PubMed]
10. Asrani, S.K.; Devarbhavi, H.; Eaton, J.; Kamath, P.S. Burden of liver diseases in the world. *J. Hepatol.* **2019**, *70*, 151–171. [CrossRef] [PubMed]
11. Cwalina-Ambroziak, B.; Wierzbowska, J.; Damszel, M.; Bowszys, T The effect of mineral fertilization on achenes yield and fungal communities isolated from the stems of milk thistle *Silybum marianum* (L.) Gaertner. *Acta Sci. Pol. Hortorum Cultus* **2012**, *11*, 157–168.
12. Rosińska, A.; Dorna, H.; Szopińska, D.; Seidler-Łożykowska, K. Experimental paper The effect of colour grading of milk thistle (*Silybum marianum* (L.) Gaertn.) seeds on their quality for sowing. *Herba Pol.* **2017**, *63*, 7–19. [CrossRef]
13. Rosińska, A.; Dorna, H.; Szopińska, D.; Irzykowska, L.; Seidler-Łożykowska, K. Evaluation of milk thistle (*Silybum marianum* (L.) Gaertn.) seed germination in relation to seed health and seedling emergence. *Herba Pol.* **2018**, *64*, 1–10. [CrossRef]
14. Tournas, V.H.; Calo, J.R.; Sapp, C. Fungal profiles in various milk thistle botanicals from US retail. *Int. J. Food Microbiol.* **2013**, *164*, 87–91. [CrossRef] [PubMed]
15. Veprikova, Z.; Zachariasova, M.; Dzuman, Z.; Zachariasova, A.; Fenclova, M.; Slavikova, P.; Vaclavikova, M.; Mastovska, K.; Hengst, D.; Hajslova, J. Mycotoxins in plant-based dietary supplements: Hidden health risk for consumers. *J. Agric. Food Chem.* **2015**, *63*, 6633–6643. [CrossRef]
16. Santos, L.; Marín, S.; Sanchis, V.; Ramos, A.J. Screening of mycotoxin multicontamination in medicinal and aromatic herbs sampled in Spain. *J. Sci. Food Agric.* **2009**, *89*, 1802–1807. [CrossRef]
17. Tournas, V.H.; Sapp, C.; Trucksess, M.W. Occurrence of aflatoxins in milk thistle herbal supplements. *Food Addit. Contam. Part A* **2012**, *29*, 994–999. [CrossRef]
18. Capriotti, A.L.; Caruso, G.; Cavaliere, C.; Foglia, P.; Samperi, R.; Laganà, A. Multiclass mycotoxin analysis in food, environmental and biological matrices with chromatography/mass spectrometry. *Mass Spectrom. Rev.* **2012**, *31*, 466–503. [CrossRef]
19. Steyn, P.S. Mycotoxins, general view, chemistry and structure. *Toxicol. Lett.* **1995**, *82–83*, 843–851. [CrossRef]

20. Wianowska, D.; Wiśniewski, M. Simplified procedure of silymarin extraction from *Silybum marianum* L. Gaertner. *J. Chromatogr. Sci.* **2015**, *53*, 366–372. [CrossRef]
21. Andrzejewska, J.; Martinelli, T.; Sadowska, K. *Silybum marianum*: Non-medical exploitation of the species. *Ann. Appl. Biol.* **2015**, *167*, 285–297. [CrossRef]
22. Karkanis, A.; Bilalis, D.; Efthimiadou, A. Cultivation of milk thistle (*Silybum marianum* L. Gaertn.), a medicinal weed. *Ind. Crops Prod.* **2011**, *34*, 825–830. [CrossRef]
23. Bijak, M. Silybin, a major bioactive component of milk thistle (*Silybum marianum* L. Gaernt.)—Chemistry, bioavailability, and metabolism. *Molecules* **2017**, *22*, 1942. [CrossRef] [PubMed]
24. Gresta, F.; Avola, G.; Guarnaccia, P. Agronomic characterization of some spontaneous genotypes of milk thistle (*Silybum marianum* L. Gaertn.) in Mediterranean environment. *J. Herbs Spices Med. Plants* **2006**, *12*, 51–60. [CrossRef]
25. Abenavoli, L.; Izzo, A.A.; Milić, N.; Cicala, C.; Santini, A.; Capasso, R. Milk thistle (*Silybum marianum*): A concise overview on its chemistry, pharmacological, and nutraceutical uses in liver diseases. *Phytother. Res.* **2018**, *32*, 2202–2213. [CrossRef] [PubMed]
26. Fibigr, J.; Šatínský, D.; Solich, P. A new approach to the rapid separation of isomeric compounds in a *Silybum marianum* extract using UHPLC core-shell column with F5 stationary phase. *J. Pharm. Biomed. Anal.* **2017**, *134*, 203–213. [CrossRef]
27. Javed, S.; Kohli, K.; Ali, M. Reassessing bioavailability of silymarin. *Altern. Med. Rev. J. Clin. Ther.* **2011**, *16*, 239–249.
28. PubChem. Available online: https://pubchem.ncbi.nlm.nih.gov/ (accessed on 23 July 2020).
29. Mitchell, S.T. Chapter 231. Silymarin or Milk Thistle (*Silybum Marianum*). In *Poisoning & Drug Overdose*; Olson, K.R., Ed.; The McGraw-Hill Companies: New York, NY, USA, 2012; pp. 551–552, ISBN 0-07-166833-0.
30. Fanoudi, S.; Alavi, M.S.; Karimi, G.; Hosseinzadeh, H. Milk thistle (*Silybum Marianum*) as an antidote or a protective agent against natural or chemical toxicities: A review. *Drug Chem. Toxicol.* **2020**, *43*, 240–254. [CrossRef]
31. Aboelwafa, H.R.; El-kott, A.F.; Abd-Ella, E.M.; Yousef, H.N The possible neuroprotective effect of silymarin against aluminum chloride-prompted Alzheimer's-like disease in Rats. *Brain Sci.* **2020**, *10*, 628. [CrossRef]
32. Guo, H.; Cao, H.; Cui, X.; Zheng, W.; Wang, S.; Yu, J.; Chen, Z. Silymarin's inhibition and treatment effects for Alzheimer's disease. *Molecules* **2019**, *24*, 1748. [CrossRef]
33. El-Ashmawy, N.E.; Khedr, E.G.; El-Bahrawy, H.A.; Helmy, N.N. Modulatory effect of silymarin on apoptosis in testosterone -induced benign prostatic hyperplasia in rats. *Pathol. Oncol. Res.* **2020**, *26*, 1947–1956. [CrossRef]
34. Saberi, Z.; Gorji, N.; Memariani, Z.; Moeini, R.; Shirafkan, H.; Amiri, M. Evaluation of the effect of *Silybum Marianum* extract on menopausal symptoms: A randomized, double-blind placebo-controlled trial. *Phytother. Res.* **2020**, 1–8. [CrossRef]
35. Othman, S.; Ali, S.M.; Deeb, N.M.E. Protective effect of *Silybum marianum* extract against doxorubicin induced toxicity in male rats. *PSM Biol. Res.* **2020**, *5*, 14–21.
36. Rašković, A.; Stilinović, N.; Kolarović, J.; Vasović, V.; Vukmirović, S.; Mikov, M The protective effects of silymarin against doxorubicin-induced cardiotoxicity and hepatotoxicity in rats. *Molecules* **2011**, *16*, 8601–8613. [CrossRef] [PubMed]
37. Vostálová, J.; Tinková, E.; Biedermann, D.; Kosina, P.; Ulrichová, J.; Rajnochová Svobodová, A. Skin protective activity of silymarin and its flavonolignans. *Molecules* **2019**, *24*, 1022. [CrossRef]
38. Fidrus, E.; Ujhelyi, Z.; Fehér, P.; Hegedűs, C.; Janka, E.A.; Paragh, G.; Vasas, G.; Bácskay, I.; Remenyik, É. Silymarin: Friend or foe of UV exposed keratinocytes? *Molecules* **2019**, *24*, 1652. [CrossRef]
39. Alhidary, I.A.; Rehman, Z.; Khan, R.U.; Tahir, M. Anti-aflatoxin activities of milk thistle (*Silybum marianum*) in broiler. *Worlds Poult. Sci. J.* **2017**, *73*, 559–566. [CrossRef]
40. Stoev, S.D.; Njobeh, P.; Zarkov, I.; Mircheva, T.; Zapryanova, D.; Denev, S.; Dimitrova, B. Selected herbal feed additives showing protective effects against ochratoxin A toxicosis in broiler chicks. *World Mycotoxin J.* **2019**, *12*, 257–268. [CrossRef]
41. Ledur, P.C.; Santurio, J.M. Cytoprotective effects of curcumin and silymarin on PK-15 cells exposed to ochratoxin A, fumonisin B1 and deoxynivalenol. *Toxicon* **2020**, *185*, 97–103. [CrossRef]

42. Gao, X.; Xiao, Z.-H.; Liu, M.; Zhang, N.-Y.; Khalil, M.M.; Gu, C.-Q.; Qi, D.-S.; Sun, L.-H. Dietary silymarin supplementation alleviates zearalenone-induced hepatotoxicity and reproductive toxicity in rats. *J. Nutr.* **2018**, *148*, 1209–1216. [CrossRef]
43. Gillessen, A.; Schmidt, H.H.-J. Silymarin as supportive treatment in liver diseases: A narrative review. *Adv. Ther.* **2020**, *37*, 1279–1301. [CrossRef] [PubMed]
44. Grela, E.R.; Świątkiewicz, M.; Florek, M.; Wojtaszewska, I. Impact of milk thistle (*Silybum marianum* L.) seeds in fattener diets on pig performance and carcass traits and fatty acid profile and cholesterol of meat, backfat and liver. *Livest. Sci.* **2020**, *239*, 104180. [CrossRef]
45. Kosina, P.; Dokoupilová, A.; Janda, K.; Sládková, K.; Silberová, P.; Pivodová, V.; Ulrichová, J. Effect of *Silybum marianum* fruit constituents on the health status of rabbits in repeated 42-day fattening experiment. *Anim. Feed Sci. Technol.* **2017**, *223*, 128–140. [CrossRef]
46. Tedesco, D.; Tava, A.; Galletti, S.; Tameni, M.; Varisco, G.; Costa, A.; Steidler, S. Effects of silymarin, a natural hepatoprotector, in periparturient Dairy Cows. *J. Dairy Sci.* **2004**, *87*, 2239–2247. [CrossRef]
47. Khamisabadi, H. Effects of Silymarin on milk production, liver enzymes, oxidative status and HSP70 gene expression in postparturient Sanjabi ewes. *Cell. Mol. Biol.* **2020**, *66*, 76–81. [CrossRef] [PubMed]
48. Šťastník, O.; Mrkvicová, E.; Pavlata, L.; Roztočilová, A.; Umlášková, B.; Anzenbacherová, E. Performance, biochemical profile and antioxidant activity of hens supplemented with addition of milk thistle (*Silybum marianum*) seed cakes in diet. *Acta Univ. Agric. Silvic. Mendel. Brun.* **2019**, *67*, 993–1003. [CrossRef]
49. World Health Organization. *WHO Guidelines for Assessing Quality of Herbal Medicines with Reference to Contaminants and Residues*; World Health Organization: Geneva, Switzerland, 2007.
50. Ostry, V. *Alternaria* mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J.* **2008**, *1*, 175–188. [CrossRef]
51. Logrieco, A.; Bottalico, A.; Mulé, G.; Moretti, A.; Perrone, G. Epidemiology of toxigenic fungi and their associated mycotoxins for some mediterranean Crops. *Eur. J. Plant Pathol.* **2003**, *109*, 645–667. [CrossRef]
52. Romero, S.M.; Comerio, R.M.; Larumbe, G.; Ritieni, A.; Vaamonde, G.; Fernández Pinto, V. Toxigenic fungi isolated from dried vine fruits in Argentina. *Int. J. Food Microbiol.* **2005**, *104*, 43–49. [CrossRef]
53. Andersen, B.; Krøger, E.; Roberts, R.G. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycol. Res.* **2002**, *106*, 170–182. [CrossRef]
54. Andersen, B.; Hansen, M.E.; Smedsgaard, J. Automated and unbiased image analyses as tools in phenotypic classification of small-spored *Alternaria* spp. *Phytopathology* **2005**, *95*, 1021–1029. [CrossRef] [PubMed]
55. European Food Safety Authority. Dietary exposure assessment to *Alternaria* toxins in the European population. *EFSA J.* **2016**, *14*, e04654. [CrossRef]
56. Aichinger, G.; Krüger, F.; Puntscher, H.; Preindl, K.; Warth, B.; Marko, D. Naturally occurring mixtures of *Alternaria* toxins: Anti-estrogenic and genotoxic effects in vitro. *Arch. Toxicol.* **2019**, *93*, 3021–3031. [CrossRef] [PubMed]
57. Hessel-Pras, S.; Kieshauer, J.; Roenn, G.; Luckert, C.; Braeuning, A.; Lampen, A. In vitro characterization of hepatic toxicity of *Alternaria* toxins. *Mycotoxin Res.* **2019**, *35*, 157–168. [CrossRef]
58. Pfeiffer, E.; Eschbach, S.; Metzler, M. *Alternaria* toxins: DNA strand-breaking activity in mammalian cells in vitro. *Mycotoxin Res.* **2007**, *23*, 152. [CrossRef]
59. Brugger, E.-M.; Wagner, J.; Schumacher, D.M.; Koch, K.; Podlech, J.; Metzler, M.; Lehmann, L. Mutagenicity of the mycotoxin alternariol in cultured mammalian cells. *Toxicol. Lett.* **2006**, *164*, 221–230. [CrossRef]
60. Lehmann, L.; Wagner, J.; Metzler, M. Estrogenic and clastogenic potential of the mycotoxin alternariol in cultured mammalian cells. *Food Chem. Toxicol.* **2006**, *44*, 398–408. [CrossRef]
61. Schmutz, C.; Cenk, E.; Marko, D The *Alternaria* mycotoxin alternariol triggers the immune response of IL-1β-stimulated, differentiated Caco-2 cells. *Mol. Nutr. Food Res.* **2019**, *63*, 1900341. [CrossRef]
62. Kollarova, J.; Cenk, E.; Schmutz, C.; Marko, D The mycotoxin alternariol suppresses lipopolysaccharide-induced inflammation in THP-1 derived macrophages targeting the NF-κB signalling pathway. *Arch. Toxicol.* **2018**, *92*, 3347–3358. [CrossRef]
63. Bansal, M.; Singh, N.; Alam, S.; Pal, S.; Satyanarayana, G.N.V.; Singh, D.; Ansari, K.M. Alternariol induced proliferation in primary mouse keratinocytes and inflammation in mouse skin is regulated via PGE2/EP2/cAMP/p-CREB signaling pathway. *Toxicology* **2019**, *412*, 79–88. [CrossRef]

64. Tiemann, U.; Tomek, W.; Schneider, F.; Müller, M.; Pöhland, R.; Vanselow, J The mycotoxins alternariol and alternariol methyl ether negatively affect progesterone synthesis in porcine granulosa cells in vitro. *Toxicol. Lett.* **2009**, *186*, 139–145. [CrossRef]
65. Dellafiora, L.; Warth, B.; Schmidt, V.; Del Favero, G.; Mikula, H.; Fröhlich, J.; Marko, D. An integrated in silico/in vitro approach to assess the xenoestrogenic potential of *Alternaria* mycotoxins and metabolites. *Food Chem.* **2018**, *248*, 253–261. [CrossRef] [PubMed]
66. Liu, G.T.; Qian, Y.Z.; Zhang, P.E.; Dong, W.H.; Qi, Y.M.; Guo, H. Etiological role of *Alternaria alternata* in human esophageal cancer. *Chin. Med. J.* **1992**, *105*, 394–400. [PubMed]
67. Frisvad, J.C.; Thrane, U.; Samson, R.A. Mycotoxin producers. In *Food Mycology: A Multifaceted Approach to Fungi and Food*; Dijksterhuis, J., Samson, R.A., Eds.; CRC Press: Boca Raton, FL, USA, 2007; pp. 135–159.
68. Sun, L.-H.; Lei, M.; Zhang, N.-Y.; Zhao, L.; Krumm, C.S.; Qi, D.-S. Hepatotoxic effects of mycotoxin combinations in mice. *Food Chem. Toxicol.* **2014**, *74*, 289–293. [CrossRef] [PubMed]
69. Yin, H.; Han, S.; Chen, Y.; Wang, Y.; Li, D.; Zhu, Q. T-2 Toxin induces oxidative stress, apoptosis and cytoprotective autophagy in chicken hepatocytes. *Toxins* **2020**, *12*, 90. [CrossRef] [PubMed]
70. Wang, X.; Tang, J.; Geng, F.; Zhu, L.; Chu, X.; Zhang, Y.; Rahman, S.U.; Chen, X.; Jiang, Y.; Zhu, D.; et al. Effects of deoxynivalenol exposure on cerebral lipid peroxidation, neurotransmitter and calcium homeostasis of chicks in vivo. *Toxicon* **2018**, *150*, 60–65. [CrossRef]
71. Guo, P.; Liu, A.; Huang, D.; Wu, Q.; Fatima, Z.; Tao, Y.; Cheng, G.; Wang, X.; Yuan, Z. Brain damage and neurological symptoms induced by T-2 toxin in rat brain. *Toxicol. Lett.* **2018**, *286*, 96–107. [CrossRef]
72. Modra, H.; Palikova, M.; Hyrsl, P.; Bartonkova, J.; Papezikova, I.; Svobodova, Z.; Blahova, J.; Mares, J. Effects of trichothecene mycotoxin T-2 toxin on haematological and immunological parameters of rainbow trout (*Oncorhynchus mykiss*). *Mycotoxin Res.* **2020**, *36*, 319–326. [CrossRef]
73. Hymery, N.; Léon, K.; Carpentier, F.-G.; Jung, J.-L.; Parent-Massin, D. T-2 toxin inhibits the differentiation of human monocytes into dendritic cells and macrophages. *Toxicol. In Vitro* **2009**, *23*, 509–519. [CrossRef]
74. Vlata, Z.; Porichis, F.; Tzanakakis, G.; Tsatsakis, A.; Krambovitis, E. In vitro cytopathic effects of mycotoxin T-2 on human peripheral blood T lymphocytes. *Toxicol. Lett.* **2005**, *160*, 60–68. [CrossRef]
75. Minervini, F.; Fornelli, F.; Lucivero, G.; Romano, C.; Visconti, A. T-2 toxin immunotoxicity on human B and T lymphoid cell lines. *Toxicology* **2005**, *210*, 81–91. [CrossRef]
76. Hymery, N.; Sibiril, Y.; Parent-Massin, D. In vitro effects of trichothecenes on human dendritic cells. *Toxicol. In Vitro* **2006**, *20*, 899–909. [CrossRef] [PubMed]
77. Yang, X.; Zhang, X.; Yao, Q.; Song, M.; Han, Y.; Shao, B.; Li, Y. T-2 toxin impairs male fertility by disrupting hypothalamic-pituitary-testis axis and declining testicular function in mice. *Chemosphere* **2019**, *234*, 909–916. [CrossRef] [PubMed]
78. Tassis, P.D.; Tsakmakidis, I.A.; Nagl, V.; Reisinger, N.; Tzika, E.; Gruber-Dorninger, C.; Michos, I.; Mittas, N.; Basioura, A.; Schatzmayr, D. Individual and combined in vitro effects of deoxynivalenol and zearalenone on boar semen. *Toxins* **2020**, *12*, 495. [CrossRef]
79. Agrawal, M.; Yadav, P.; Lomash, V.; Bhaskar, A.S.B.; Lakshmana Rao, P.V. T-2 toxin induced skin inflammation and cutaneous injury in mice. *Toxicology* **2012**, *302*, 255–265. [CrossRef] [PubMed]
80. Hemmati, A.A.; Kalantari, H.; Jalali, A.; Rezai, S.; Zadeh, H.H. Healing effect of quince seed mucilage on T-2 toxin-induced dermal toxicity in rabbit. *Exp. Toxicol. Pathol.* **2012**, *64*, 181–186. [CrossRef] [PubMed]
81. Cho, U.M.; Choi, J.H.; Hwang, H.S. Deoxynivalenol impair skin barrier function through the down regulation of filaggrin and claudin 1/8 in HaCaT keratinocyte. *Biotechnol. Bioprocess Eng.* **2017**, *22*, 693–699. [CrossRef]
82. Zhou, H.; George, S.; Hay, C.; Lee, J.; Qian, H.; Sun, X. Individual and combined effects of aflatoxin B1, deoxynivalenol and zearalenone on HepG2 and RAW 264.7 cell lines. *Food Chem. Toxicol.* **2017**, *103*, 18–27. [CrossRef]
83. Fernández-Blanco, C.; Elmo, L.; Waldner, T.; Ruiz, M.-J. Cytotoxic effects induced by patulin, deoxynivalenol and toxin T2 individually and in combination in hepatic cells (HepG2). *Food Chem. Toxicol.* **2018**, *120*, 12–23. [CrossRef]
84. Yu, F.-F.; Lin, X.-L.; Wang, X.; Ping, Z.-G.; Guo, X. Comparison of apoptosis and autophagy in human chondrocytes Induced by the T-2 and HT-2 Toxins. *Toxins* **2019**, *11*, 260. [CrossRef]
85. Yang, L.; Tu, D.; Zhao, Z.; Cui, J. Cytotoxicity and apoptosis induced by mixed mycotoxins (T-2 and HT-2 toxin) on primary hepatocytes of broilers in vitro. *Toxicon* **2017**, *129*, 1–10. [CrossRef] [PubMed]

86. International Agency for Research on Cancer. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occuring Substances: Food Items and Costituents, Heterocyclic Aromatic Amines and Mycotoxins*; IARC Press: Lyon, France, 1993; Volume 56, ISBN 92-832-1256-8.
87. European Food Safety Authority. Scientific opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. *EFSA J.* **2011**, *9*, 2481. [CrossRef]
88. Poór, M.; Kunsági-Máté, S.; Sali, N.; Kőszegi, T.; Szente, L.; Peles-Lemli, B. Interactions of zearalenone with native and chemically modified cyclodextrins and their potential utilization. *J. Photochem. Photobiol. B* **2015**, *151*, 63–68. [CrossRef] [PubMed]
89. Kotowicz, N.K.; Frac, M.; Lipiec, J The importance of *Fusarium* fungi in wheat cultivation-pathogenicity and mycotoxins production: A review. *J. Anim. Plant Sci.* **2014**, *21*, 3326–3343.
90. Rai, A.; Das, M.; Tripathi, A. Occurrence and toxicity of a *fusarium* mycotoxin, zearalenone. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2710–2729. [CrossRef]
91. Zinedine, A.; Soriano, J.M.; Moltó, J.C.; Mañes, J. Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. *Food Chem. Toxicol.* **2007**, *45*, 1–18. [CrossRef]
92. Krejcárková, A.; Šimoník, O.; Šašková, M.; Krejčířová, R.; Drábek, O.; Rajmon, R. Effects of zearalenone, α-zearalenol, and genistein on boar sperm motility in vitro. *Czech J. Anim. Sci.* **2017**, *62*, 435–445. [CrossRef]
93. Harčárová, M.; Čonková, E.; Proškovcová, M.; Falis, M. In vivo assessment of zearalenone toxicity. *Folia Vet.* **2020**, *64*, 60–65. [CrossRef]
94. Aichinger, G.; Pantazi, F.; Marko, D. Combinatory estrogenic effects of bisphenol A in mixtures with alternariol and zearalenone in human endometrial cells. *Toxicol. Lett.* **2020**, *319*, 242–249. [CrossRef]
95. Althali, N.J.; Hassan, A.M.; Abdel-Wahhab, M.A. Effect of grape seed extract on maternal toxicity and in utero development in mice treated with zearalenone. *Environ. Sci. Pollut. Res.* **2019**, *26*, 5990–5999. [CrossRef]
96. Yao, X.; Jiang, H.; Gao, Q.; Li, Y.-H.; Xu, Y.N.; Kim, N.-H. Melatonin alleviates defects induced by zearalenone during porcine embryo development. *Theriogenology* **2020**, *151*, 66–73. [CrossRef] [PubMed]
97. Cao, H.; Zhi, Y.; Xu, H.; Fang, H.; Jia, X. Zearalenone causes embryotoxicity and induces oxidative stress and apoptosis in differentiated human embryonic stem cells. *Toxicol. In Vitro* **2019**, *54*, 243–250. [CrossRef] [PubMed]
98. Salem, I.B.; Boussabbeh, M.; Neffati, F.; Najjar, M.; Abid-Essefi, S.; Bacha, H. Zearalenone-induced changes in biochemical parameters, oxidative stress and apoptosis in cardiac tissue: Protective role of crocin. *Hum. Exp. Toxicol.* **2016**, *35*, 623–634. [CrossRef]
99. Jia, Z.; Liu, M.; Qu, Z.; Zhang, Y.; Yin, S.; Shan, A. Toxic effects of zearalenone on oxidative stress, inflammatory cytokines, biochemical and pathological changes induced by this toxin in the kidney of pregnant rats. *Environ. Toxicol. Pharmacol.* **2014**, *37*, 580–591. [CrossRef] [PubMed]
100. Szabó, A.; Szabó-Fodor, J.; Fébel, H.; Mézes, M.; Balogh, K.; Bázár, G.; Kocsó, D.; Ali, O.; Kovács, M. Individual and combined effects of fumonisin B1, deoxynivalenol and zearalenone on the hepatic and renal membrane lipid integrity of rats. *Toxins* **2018**, *10*, 4. [CrossRef] [PubMed]
101. Islam, M.R.; Kim, J.W.; Roh, Y.-S.; Kim, J.-H.; Han, K.M.; Kwon, H.-J.; Lim, C.W.; Kim, B. Evaluation of immunomodulatory effects of zearalenone in mice. *J. Immunotoxicol.* **2017**, *14*, 125–136. [CrossRef] [PubMed]
102. Hueza, I.M.; Raspantini, P.C.F.; Raspantini, L.E.R.; Latorre, A.O.; Górniak, S.L. Zearalenone, an estrogenic mycotoxin, is an immunotoxic compound. *Toxins* **2014**, *6*, 1080–1095. [CrossRef]
103. Pistol, G.C.; Braicu, C.; Motiu, M.; Gras, M.A.; Marin, D.E.; Stancu, M.; Calin, L.; Israel-Roming, F.; Berindan-Neagoe, I.; Taranu, I. Zearalenone mycotoxin affects immune mediators, MAPK signalling molecules, nuclear receptors and genome-wide gene expression in pig spleen. *PLoS ONE* **2015**, *10*, e0127503. [CrossRef]
104. Bouaziz, C.; Sharaf el dein, O.; El Golli, E.; Abid-Essefi, S.; Brenner, C.; Lemaire, C.; Bacha, H. Different apoptotic pathways induced by zearalenone, T-2 toxin and ochratoxin A in human hepatoma cells. *Toxicology* **2008**, *254*, 19–28. [CrossRef]
105. Marin, D.E.; Pistol, G.C.; Bulgaru, C.V.; Taranu, I. Cytotoxic and inflammatory effects of individual and combined exposure of HepG2 cells to zearalenone and its metabolites. *Naunyn. Schmiedebergs Arch. Pharmacol.* **2019**, *392*, 937–947. [CrossRef]

106. Frizzell, C.; Ndossi, D.; Verhaegen, S.; Dahl, E.; Eriksen, G.; Sørlie, M.; Ropstad, E.; Muller, M.; Elliott, C.T.; Connolly, L. Endocrine disrupting effects of zearalenone, alpha- and beta-zearalenol at the level of nuclear receptor binding and steroidogenesis. *Toxicol. Lett.* **2011**, *206*, 210–217. [CrossRef] [PubMed]
107. Wang, X.; Yu, H.; Fang, H.; Zhao, Y.; Jin, Y.; Shen, J.; Zhou, C.; Zhou, Y.; Fu, Y.; Wang, J.; et al. Transcriptional profiling of zearalenone-induced inhibition of IPEC-J2 cell proliferation. *Toxicon* **2019**, *172*, 8–14. [CrossRef] [PubMed]
108. Ren, Z.H.; Deng, H.D.; Deng, Y.T.; Deng, J.L.; Zuo, Z.C.; Yu, S.M.; Shen, L.H.; Cui, H.M.; Xu, Z.W.; Hu, Y.C. Effect of the *Fusarium* toxins, zearalenone and deoxynivalenol, on the mouse brain. *Environ. Toxicol. Pharmacol.* **2016**, *46*, 62–70. [CrossRef] [PubMed]
109. Jia, R.; Liu, W.; Zhao, L.; Cao, L.; Shen, Z. Low doses of individual and combined deoxynivalenol and zearalenone in naturally moldy diets impair intestinal functions via inducing inflammation and disrupting epithelial barrier in the intestine of piglets. *Toxicol. Lett.* **2020**, *333*, 159–169. [CrossRef]
110. Zhang, W.; Zhang, S.; Wang, J.; Shan, A.; Xu, L. Changes in intestinal barrier functions and gut microbiota in rats exposed to zearalenone. *Ecotoxicol. Environ. Saf.* **2020**, *204*, 111072. [CrossRef]
111. Lahjouji, T.; Bertaccini, A.; Neves, M.; Puel, S.; Oswald, I.P.; Soler, L. Acute exposure to zearalenone disturbs intestinal homeostasis by modulating the Wnt/β-Catenin signaling pathway. *Toxins* **2020**, *12*, 113. [CrossRef]
112. Jajić, I.; Dudaš, T.; Krstović, S.; Krska, R.; Sulyok, M.; Bagi, F.; Savić, Z.; Guljaš, D.; Stankov, A. Emerging *Fusarium* mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin in Serbian maize. *Toxins* **2019**, *11*, 357. [CrossRef]
113. Tonshin, A.A.; Teplova, V.V.; Andersson, M.A.; Salkinoja-Salonen, M.S The *Fusarium* mycotoxins enniatins and beauvericin cause mitochondrial dysfunction by affecting the mitochondrial volume regulation, oxidative phosphorylation and ion homeostasis. *Toxicology* **2010**, *276*, 49–57. [CrossRef]
114. European Food Safety Authority. Scientific opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA J.* **2014**, *12*, 3802. [CrossRef]
115. Jestoi, M. Emerging *Fusarium* -mycotoxins fusaproliferin, beauvericin, enniatins, and moniliformin—A review. *Crit. Rev. Food Sci. Nutr.* **2008**, *48*, 21–49. [CrossRef]
116. Devreese, M.; Broekaert, N.; De Mil, T.; Fraeyman, S.; De Backer, P.; Croubels, S. Pilot toxicokinetic study and absolute oral bioavailability of the *Fusarium* mycotoxin enniatin B1 in pigs. *Food Chem. Toxicol.* **2014**, *63*, 161–165. [CrossRef] [PubMed]
117. Prosperini, A.; Juan-García, A.; Font, G.; Ruiz, M.J. Beauvericin-induced cytotoxicity via ROS production and mitochondrial damage in Caco-2 cells. *Toxicol. Lett.* **2013**, *222*, 204–211. [CrossRef] [PubMed]
118. Prosperini, A.; Font, G.; Ruiz, M.J. Interaction effects of *Fusarium* enniatins (A, A1, B and B1) combinations on in vitro cytotoxicity of Caco-2 cells. *Toxicol. In Vitro* **2014**, *28*, 88–94. [CrossRef] [PubMed]
119. Olleik, H.; Nicoletti, C.; Lafond, M.; Courvoisier-Dezord, E.; Xue, P.; Hijazi, A.; Baydoun, E.; Perrier, J.; Maresca, M. Comparative structure–activity analysis of the antimicrobial activity, cytotoxicity, and mechanism of action of the fungal cyclohexadepsipeptides enniatins and eeauvericin. *Toxins* **2019**, *11*, 514. [CrossRef]
120. Agahi, F.; Font, G.; Juan, C.; Juan-García, A. Individual and combined effect of zearalenone derivates and beauvericin mycotoxins on SH-SY5Y Cells. *Toxins* **2020**, *12*, 212. [CrossRef]
121. Mamur, S.; Yuzbasioglu, D.; Yılmaz, S.; Erikel, E.; Unal, F. Assessment of cytotoxic and genotoxic effects of enniatin—A in vitro. *Food Addit. Contam. Part A* **2018**, *35*, 1633–1644. [CrossRef]
122. Huang, C.-H.; Wang, F.-T.; Chan, W.-H. Enniatin B1 exerts embryotoxic effects on mouse blastocysts and induces oxidative stress and immunotoxicity during embryo development. *Environ. Toxicol.* **2019**, *34*, 48–59. [CrossRef]
123. Büchter, C.; Koch, K.; Freyer, M.; Baier, S.; Saier, C.; Honnen, S.; Wätjen, W The mycotoxin beauvericin impairs development, fertility and life span in the nematode *Caenorhabditis elegans* accompanied by increased germ cell apoptosis and lipofuscin accumulation. *Toxicol. Lett.* **2020**, *334*, 102–109. [CrossRef]
124. European Commission. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union* **2006**, *L364*, 5–24.
125. European Commission. European Union Commission Regulation (EU) No. 105/2010 of 5 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. *Off. J. Eur. Union* **2010**, *L35*, 7–8.

126. JECFA FAO/WHO. *Evaluation of Certain Contaminants in Food Seventy-Second Report of the Joint FAO/WHO Expert Committee on Food Additives*; WHO Technical Report Series 959; World Health Organization: Geneva, Switzerland, 2011; ISBN 978-92-4-120959-5.
127. European Food Safety Authority. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. *EFSA J.* **2017**, *15*, e04718. [CrossRef]
128. JECFA FAO/WHO. *Evaluation of Certain Mycotoxins in Food. Fifty-Sixth Report of the Joint FAO/WHO Expert Committee on Food Additives*; WHO Technical Report Series 906; World Health Organization: Geneva, Switzerland, 2002; ISBN 92-4-120906-2.
129. European Food Safety Authority. Human and animal dietary exposure to T-2 and HT-2 toxin. *EFSA J.* **2017**, *15*, e04972. [CrossRef]
130. JECFA FAO/WHO. *Evaluation of Certain food Additives and Contaminants. Fifty-Third Report of the Joint FAO/WHO Expert Committee on Food Additives*; WHO Technical Report Series 896; World Health Organization: Geneva, Switzerland, 2000; ISBN 92-4-120896-1.
131. European Food Safety Authority. Appropriateness to set a group health-based guidance value for zearalenone and its modified forms. *EFSA J.* **2016**, *14*, 1–46. [CrossRef]
132. Krogh, P.; Axelsen, N.H.; Elling, F.; Gyrd-Hansen, N.; Hald, B.; Hyldgaard-Jensen, J.; Larsen, A.E.; Madsen, A.; Mortensen, H.P.; Moller, T.; et al. Experimental porcine nephropathy. Changes of renal function and structure induced by ochratoxin A- contaminated feed. *Acta Pathol. Microbiol. Scand. Suppl.* **1974**, *84*, 1–21.
133. European Food Safety Authority. Risk assessment of ochratoxin A in food. *EFSA J.* **2020**, *18*, 1–150. [CrossRef]
134. European Food Safety Authority. Risk assessment of aflatoxins in food. *EFSA J.* **2020**, *18*, 1–112. [CrossRef]
135. EU. *SCF Opinion of the Scientific Committee on Food on Fusarium-Toxins Part 1: Deoxynivalenol (DON)*; (Expressed on 2 December 1999); EU: Brussel, Belgium, 1999.
136. European Food Safety Authority. Scientific opinion on the risks for public health related to the presence of zearalenone in food. *EFSA J.* **2011**, *9*, 2197. [CrossRef]
137. Ostry, V.; Skarkova, J.; Ruprich, J. *Alternaria Mycotoxins in Foodstuffs–Current Information for Health Risk Assessment*; Mycotoxin: Bratislava, Slovakia, 2009; pp. 1–9.

Příloha 2

A review on mycotoxins and microfungi in spices in the light of the last five years

toxins

MDPI

Review

A Review on Mycotoxins and Microfungi in Spices in the Light of the Last Five Years

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Abstract: Spices are imported worldwide mainly from developing countries with tropical and/or subtropical climate. Local conditions, such as high temperature, heavy rainfall, and humidity, promote fungal growth leading to increased occurrence of mycotoxins in spices. Moreover, the lack of good agricultural practice (GAP), good manufacturing practice (GMP), and good hygienic practice (GHP) in developing countries are of great concern. This review summarizes recent data from a total of 56 original papers dealing with mycotoxins and microfungi in various spices in the last five years. A total of 38 kinds of spices, 17 mycotoxins, and 14 microfungi are discussed in the review. Worldwide, spices are rather overlooked in terms of mycotoxin regulations, which usually only cover aflatoxins (AFs) and ochratoxin A (OTA). In this paper, an extensive attention is devoted to the limits on mycotoxins in spices in the context of the European Union (EU) as well as other countries. As proven in this review, the incidence of AFs and OTA, as well as other mycotoxins, is relatively high in many spices; thus, the preparation of new regulation limits is advisable.

Keywords: spices; contamination; microfungi; mycotoxin

Key Contribution: This review provides a summary of the results of recent original papers focusing on the occurrence of mycotoxins and microfungi in spices published since 2015 and presents the results as a summarized comprehensive output, which gives a clear insight on the current state of mycotoxin contamination. Moreover, this review provides an extensive overview of national legal limits on mycotoxins, which, to our knowledge, has not been recently published at all.

1. Introduction

The attention of the professional public has been focused on systematic control of the presence of xenogenous substances in foodstuffs which might endanger the health state of the population. This is also the case of mycotoxins, toxic secondary metabolites of microfungi. Specific problems and risks arise from the global climate change and globalization of the food market—these processes may result in an increased occurrence of mycotoxins due to many reasons including the extension of the scale of foodstuffs from regional sources, changes of food storage, transportation or dietary patterns [1,2]. Spices have been widely used since ancient times and that, primarily, for their unique flavoring, coloring, and aromatizing properties and, secondarily, for preservative, antimicrobial, and antioxidant effects. Moreover, their beneficial effect on human health is valued both in traditional and modern

medicine [3,4]. One of the definitions describes spices as non-leafy parts of a plant such as bud, fruit, seed, bark, rhizome or bulb; parts derived from leaf or flower of a plant are considered to form a distinct group—herbs [5]. However, all parts of a plant should be considered to be spices if they possess the aforementioned properties for meal enhancement, such as its color, flavor, or even texture [4]. In this review, spices have been selected in line with the definition by Uhl [4] and at the discretion of the authors.

Unfortunately, certain spices are very susceptible to toxigenic microfungi growth and thus potential mycotoxin development [3,6–8]. It is known that "spices" are generally more susceptible to contamination than "herbs" [9,10]. Moreover, spices purchased in open markets are confirmed to be significantly more contaminated than spices purchased in supermarkets [11].

Agricultural land with infected plant residues serves as the main reservoir of microfungi. Agricultural products can be infected with spores in situ or ex situ via dust or insects [12]. The mycotoxin contamination of agricultural commodities is a common phenomenon and despite of various prevention technologies and recommendations cannot be completely avoided [7]. However, some preventing physical, chemical, and biological strategies have been developed [7,13,14]. Nevertheless, in the EU, chemical treatments are not allowed for the decontamination of foodstuffs [15]. Appropriate and well-designed strategies could result in the reduction of mycotoxins in spices [7]. Beside innovative technologies, following GAP, GMP, and GHP is also necessary to prevent mold growth and mycotoxin production [7]. Inappropriate conditions during pre-harvest, harvest, and post-harvest can affect the quality of the spices. Good hygienic and physical separation are the best approaches for mycotoxin management in spices [7]. Maintaining good practices can, however, be problematic as spices are mainly grown in the developing countries from where they are exported and distributed worldwide. Moreover, their contamination is further supported by local subtropical and tropical climate characterized by high temperatures, heavy rainfalls, and relative humidity providing suitable conditions for fungal growth and thus mycotoxin production [1,2,16,17]. Fungal growth is also affected by the landform, soil types, and its properties, as well as interactions between the microfungus and micro- and macro-organisms in the soil [18,19]. Mycotoxins in the soil can be absorbed by plant roots and transported via the xylem to plant tissues [20].

This review summarizes only recent relevant original papers published in the last five years (since 2015). We consider this time span to be appropriate in terms of reflecting the current situation. A fair deal of studies concerning mycotoxins and/or microfungi in spices has been published. In the last five years, a total of 147 and 127 papers dealing with "mycotoxins" and "fungi" in "spices" have been found in the Web of Science database and a total of 52 and 45 publications in PubMed database, respectively. In total, 56 relevant papers were selected as the basis for this review. The quality criteria for the comparative analysis of individual studies were validation of analytical methods and quality of analytical results of mycotoxin determination.

2. Spices as a Part of the Worldwide Diet

Spices, as an essential part of the human diet, are normally used in small amounts for food flavoring [21]. Spice consumption varies worldwide, depending on the country and local eating habits [22]; however, there is a limited number of scientific publications concerning spice consumption providing comprehensive data on its intake into the human body.

As for European and American countries, oregano is considered the most consumed herbal spice, followed by basil, bay leaf, parsley, thyme, and chives [22]. In the recent study, pepper, paprika, parsley, and basil were labeled the most commonly used spices in the European Union (EU) [23].

As for Asia, commonly used spices include black pepper, cardamom, cinnamon, cassia, chili pepper, cloves, coriander, cumin, garlic, ginger, nutmeg, mace, turmeric, and vanilla [5]. Chili pepper is the most commonly used spice in India, consumed in much higher amounts per portion than other spices. Based on the total amount of consumed spice (amount per portion and frequency of consumption), chili pepper (on average 3.0 g per portion), cumin (1.64 g), turmeric (0.71 g), coriander (1.37 g) and

mustard (1.07 g) can be considered the top five most important spices in India. Caraway, cinnamon, cardamom, cloves, black pepper, garlic, and ginger are also commonly used in India [21,24]. Less used are asafetida, carom, mace, and nutmeg [21]. Fenugreek is also among the less important spices in India [21]; however, apart from its use as a spice, people also consume its seeds as food [25]. In China, commonly used spices and herbs include garlic, onions, chili pepper, coriander, basil, cinnamon, star anise, and ginger [26,27]. In addition, some herbs and spices are used in traditional Chinese medicine, e.g., galangal or nutmeg [28]. In Thailand, chili pepper, onion (shallot), and garlic are the most used spices. Other common spices include lemongrass, galangal, basil, mint, and fennel [29].

As for African countries, many commonly used spices are world-known such as garlic, ginger, chili pepper (*Capsicum frutescens*), onion, nutmeg or pepper (Ashanti pepper, *Piper guineese*) [30–33], while some spices are typical for Africa, such as, e.g., dawadawa, ogiri, okpehe, hwentia, soro wisa or fem wisa [31,34]. Based on a study by Nguegwouo et al. [35], cloves, white pepper, and black pepper are also common in Africa. The daily intake of white pepper (mean 1.924 g) is approximately two times higher than the daily intake of black pepper (mean 0.939 g) in Cameroon [35].

As evident, chili pepper (*Capsicum* spp.) and peppers (*Piper* spp.) are ubiquitous spices, normally consumed in quantities of a few grams per day in many places around the world. Moreover, garlic and onion (*Allium* spp.) can be considered to be one of the most used spices worldwide [36]. This makes *Capsicum* spp., *Piper* spp. and *Allium* spp. one of the most important spices from the perspective of xenogenous substance and thus also mycotoxin studies. However, many other world-known spices as well as local and traditional spices are also consumed in relatively high amounts and should be taken into consideration.

3. The Worldwide Spice Production

According to the data available over the last 5 years (the latest available data from the years 2014–2018), the average worldwide production of spices was c. 12.3 million tonnes per year (13.0 million tonnes in 2018) and consisted especially of the following spices: "Anise, badian, fennel, coriander", "Chilies and peppers, dry" "Cinnamon", "Cloves", "Ginger", "Nutmeg, mace, cardamoms", "Mustard seed", "Pepper, *Piper* spp.","Peppermint", "Vanilla" and "Spice not elsewhere specified". The items such as "Garlic" and "Onions dry" were not included, as their production of 27.8 million tonnes and 84.3 million tonnes, respectively (2018), would increase the total spice production approximately ten times. Asia, with its production share of 78.2% (10.2 million tonnes in 2018), is undoubtedly the largest producer of spices in the present world—see Figure 1. India contributes most to this share (5.4 million tonnes in 2018), by far followed by China (1.2 million tonnes in 2018) [37]. Top 20 world producers are shown in Table 1.

Table 1. Top 20 spice producers in the world in the last available year 2018.

Country	Category of Spice	Production (Tonnes)	Country	Category of Spice	Production (Tonnes)
India	1, 2, 5, 7, 8, 11	5,393,231	Pakistan	2, 5, 11	225,682
China	1, 2, 3, 4, 5, 6, 8, 9, 10, 11	1,163,542	Mexico	1, 2, 5, 6, 8, 9, 10, 11	206,232
Indonesia	3, 4, 5, 7, 8, 10, 11	651,075	Myanmar	2, 6, 11	186,190
Nepal	2, 5, 6, 7, 11	550,070	Canada	1, 6	186,052
Nigeria	2, 5, 11	446,793	Morocco	1, 2, 9, 11	157,365
Thailand	2, 5, 8, 11	419,348	Russian Fed.	1, 6	133,653
Vietnam	1, 2, 3, 8	397,770	Côte d'Ivoire	2, 5, 8, 11	125,097
Bangladesh	2, 5, 11	393,694	Ghana	2, 5, 8	119,388
Ethiopia	1, 2, 5, 6, 7, 8, 11	356,239	Brazil	8	101,274
Turkey	1, 2, 10, 11	299,487	Sri Lanka	3, 4, 5, 6, 7, 8, 11	100,745

Notes: Number of spice category: (1) Anise, badian, fennel, coriander; (2) Chilies and peppers, dry; (3) Cinnamon; (4) Cloves; (5) Ginger; (6) Mustard seed; (7) Nutmeg, mace, cardamoms; (8) Pepper, *Piper* spp.; (9) Peppermint, (10) Vanilla; (11) Spice not elsewhere specified. Processed according to FAOSTAT [37].

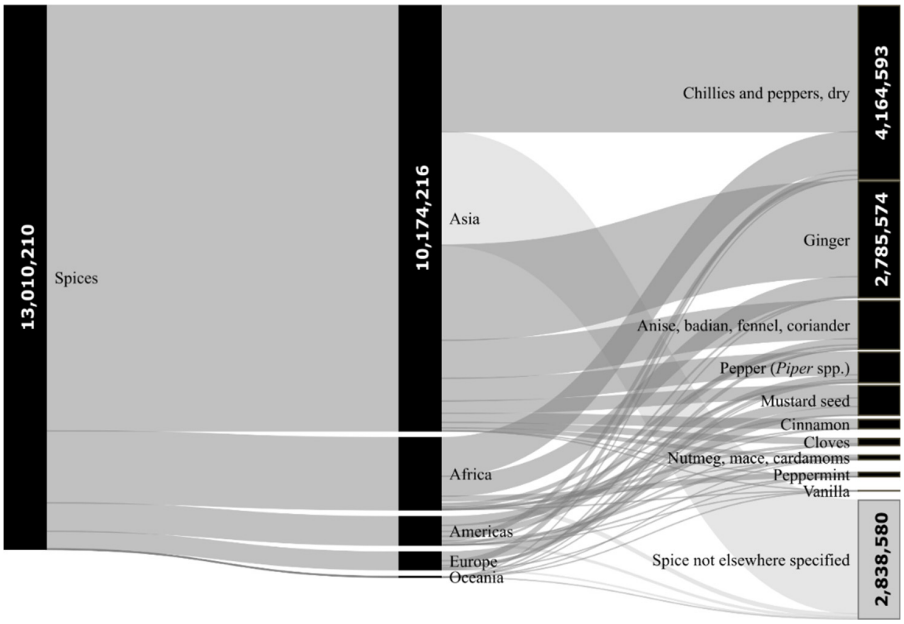


Figure 1. The share of spice production in tonnes in the last available year 2018. Note: Number of tonnes produced in brackets: Africa (1,722,909); Americas (679,830); Europe (420,188); Oceania (13,067); Anise, badian, fennel, coriander (1,165,683); Pepper, *Piper* spp. (732,523); Mustard seed (710,350); Cinnamon (221,815); Cloves (167,506); Nutmeg, mace, cardamoms (109,284); Peppermint (106,728); Vanilla (7574). Processed according to FAOSTAT [37].

4. Characterization of Mycotoxins and Their Producers Included in This Review

Mycotoxins, one of the most serious contaminants of natural origin [38], are produced by toxigenic microfungi, mostly by *Aspergillus*, *Penicillium*, and *Fusarium* [8,39] and to a certain extent *Alternaria* genera [40] as their secondary metabolites [38,41]. Moreover, some highly mycotoxigenic microfungi have not been described yet [42].

Currently, more than 500 mycotoxins have been identified, but only a few of them normally occur in the human diet in significant amounts and, consequently, pose a potential threat to human and/or animal health [43]. AFs, OTA, fumonisins (FMNs), zearalenone (ZEA), citrinin (CIT), and trichothecenes (TCT)—deoxynivalenol (DON) and nivalenol (NIV) are considered to be some of the most important in terms of toxic effect and high prevalence in the agro-food commodities [44,45], including spices [44]. In addition, *Alternaria* mycotoxins are also common contaminants in spices and other agricultural products [46]. Mycotoxins dealt with in this review are listed below; their chemical structures are shown in Table 2.

Table 2. Chemical characterization of mycotoxins.

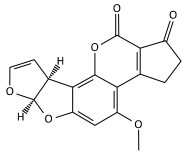
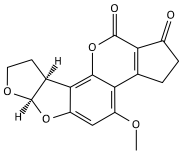
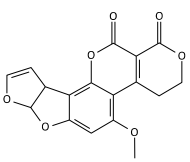
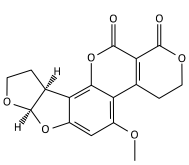
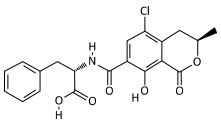
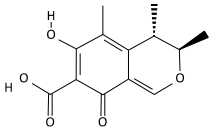
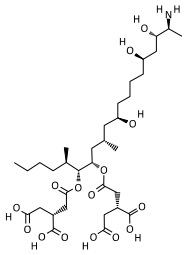
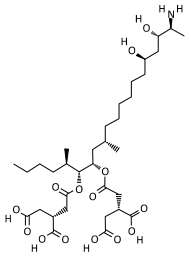
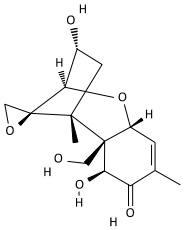
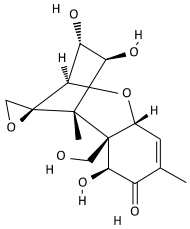
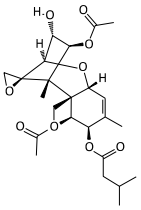
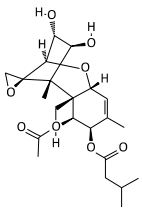
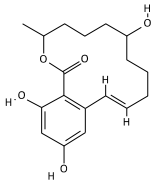
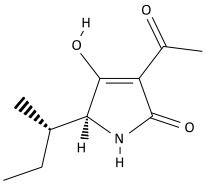
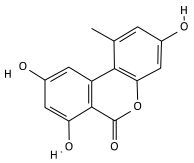
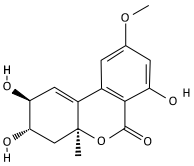
Mycotoxin	Chemical Structure	Mycotoxin	Chemical Structure
AFB_1		AFB_2	
AFG_1		AFG_2	

Table 2. *Cont.*

Mycotoxin	Chemical Structure	Mycotoxin	Chemical Structure
OTA		CIT	
FB_1		FB_2	
DON		NIV	
T-2		HT-2	
ZEA		TEA	
AOH		ALT	

Note: AF—Aflatoxin B_1, B_2, G_1, G_2; OTA—Ochratoxin A; CIT—Citrinin; F—Fumonisin B_1, B_2; DON—Deoxynivalenol; NIV—Nivalenol; T-2—T-2 toxin; HT-2—HT-2 toxin; ZEA—Zearalenone; TEA—Tenuazonic acid; AOH—Alternariol; ALT—Altenuene. Processed according to PubChem [47].

4.1. Aflatoxins

AFs are the world´s most studied mycotoxins [48] as they have been shown to have hepatotoxic, genotoxic, mutagenic, teratogenic, immunosuppressive, nephrotoxic, cytotoxic, and mainly carcinogenic effects [49–51]. According to the International Agency for Research on Cancer (IARC), all mentioned AFs are classified into group 1 "*Carcinogenic to humans*" [52].

The most common AFs include aflatoxin B_1 (AFB_1) (PubChem CID: 186907), aflatoxin B_2 (AFB_2) (PubChem CID: 2724360), aflatoxin G_1 (AFG_1) (PubChem CID: 14421) and aflatoxin G_2 (AFG_2) (PubChem CID: 2724362) [47]. AFB_1 is thought to be the most significant [50].

AFs are produced by *Aspergillus* species, mainly by *A. flavus*, and *A. parasiticus* [53–55]. *A. nomius* [55] and *A. pseudotamarii* have also been reported to be aflatoxigenic in food [42,56].

4.2. Ochratoxin A

OTA (PubChem CID: 442530) [47] is the second most important mycotoxin from the public health perspective [39]. It is mainly nephrotoxic [57] and hepatotoxic [58]. Furthermore, it exhibits genotoxic, teratogenic, immunosuppressive, and neurotoxic effects [57,59] and they have been confirmed by Arenas-Huertero et al. [49] and by EFSA [60]. According to the IARC, OTA is classified in group 2B "*Possibly carcinogenic to humans*" [52].

OTA producers of *Aspergillus* species, especially *A. carbonarius*, *A. ochraceus*, *A. westerdijkiae*, *A steynii*, *A. lacticoffeatus*, *A. niger*, *A. sclerotioniger*, and *A. tubingensis*, are typical for areas with subtropical and tropical climate while producers of *Penicillium* species, such as mainly *P. verrucosum* and *P. nordicum*, are typical for areas with temperate or colder climate [59,61]. *Aspergillus melleus* and *A. alliaceus* are less typical OTA producers [62].

4.3. Citrinin

CIT (PubChem CID: 54680783) [47] is reported to have nephrotoxic, hepatotoxic, genotoxic, mutagenic, teratogenic, and cytotoxic effects [63,64]; they have been confirmed by EFSA [65]. According to IARC, CIT is classified in group 3 "*Not classifiable as to its carcinogenicity to humans*" [52].

CIT is produced primarily by *Penicillium citrinum* [61,66]. Other fungi from *Penicillium* species such as *P. expansum and P. verrucosum* are also able to produce CIT [61]. In addition, *Monascus purpureus* and *M. ruber* have also been confirmed to produce CIT [61,67].

4.4. Fumonisins

FMNs, of which fumonisin B_1 (FB_1) (PubChem CID: 2733487) and fumonisin B_2 (FB_2) (PubChem CID: 2733489) [47] are discussed in this review, are reported to have nephrotoxic, hepatotoxic, cardiotoxic, immunosuppressive, neurotoxic, teratogenic, embryotoxic, pulmotoxic, and cytotoxic effects [49,68,69]. According to IARC, FMNs are classified in group 2B "*Possibly carcinogenic to humans*" [52].

FMNs are primarily produced by *Fusarium* species, mainly represented by *F. verticillioides* [70,71] and *F. proliferatum* [54]. Furthermore, *Aspergillus niger* has been reported to produce FB_2 [72,73].

4.5. Trichothecenes

TCT involved in this review (DON (PubChem CID: 40024), NIV (PubChem CID: 5284433), T-2 toxin (T-2) (PubChem CID: 5284461) and HT-2 toxin (HT-2) (PubChem CID: 10093830) [47]) are reported to have genotoxic, mutagenic, teratogenic, immunosuppressive, hepatotoxic, neurotoxic, and hematoxic effects [68,74–76]. According to IARC, TCT (DON, NIV, T-2) are classified in group 3 "*Not classifiable as to its carcinogenicity to humans*" [52].

In food, TCT are produced primarily by *Fusarium* species [70,77,78], such as *F. graminearum* [54], *F. culmorum*, *F. cerealis* [70,78], and *F. crookwellense* in case of DON or NIV, and *F. poae, F. equiseti* and *F. acuminatum* in case of T-2 and its metabolite HT-2 [70,77].

4.6. Zearalenone

ZEA (PubChem CID: 5933650) [47] has been reported to have estrogenic, genotoxic, mutagenic, teratogenic, immuno-suppressive, and hematoxic effects [68,79]. According to IARC, ZEA is classified in group 3 "*Not classifiable as to its carcinogenicity to humans*" [52]. In food, ZEA is produced by *Fusarium* species represented by *F. graminearum*, *F. culmorum*, and *F. crookwellense* [70,77,80].

4.7. Alternaria Mycotoxins

Alternaria mycotoxins, such as alternariol (AOH) (PubChem CID: 5359485), altenuene (ALT) (PubChem CID: 5359485), or tenuazonic acid (TEA) (PubChem CID: 54683011) [47], have been reported to be genotoxic, mutagenic, teratogenic, and cytotoxic [49,81]. As for IARC classification, none of the *Alternaria* mycotoxins is listed, although supposed esophageal carcinogenic effects were reported [82].

Alternaria mycotoxins are produced by the *Alternaria* species [81]. Those that contaminate foods include *A. alternata* [83,84], *A. tenuissima, A. arborescent* [85], *A. tangelonis*, and *A. turkisafria* [86].

4.8. Sterigmatocystin

Sterigmatocystin (STEG) (PubChem CID: 5280389) [47] has been reported to possess hepatotoxic, nephrotoxic, genotoxic, mutagenic, and teratogenic effects [87]. According to IARC, STEG is classified in group 2B "*Possibly carcinogenic to humans*" [52] because it can induce tumors including hepatocellular carcinomas, liver haemangiosarcomas, angiosarcomas in brown fat, and lung adenomas in several species [87]. However, in comparison with AFB_1, STEG toxicity has been assessed to be 10 or even up to 100 times lower [88,89]. Due to the minor significance of STEG in this review, its chemical structure is not shown in Table 2.

STEG is produced by more than 50 fungal species [87]. *Aspergillus versicolor* and *Emericella nidulans* (*anamorph: A. nidulans*) [62] are the main producers in food commodities as they can produce STEG in high amounts, compared to *A. flavus* and *A. parasiticus* which convert a part of STEG into O-methylsterigmatocystin a direct precursor of AFB_1, resulting in lower STEG production [90,91].

5. International Regulation of Aflatoxins and Ochratoxin A in Spices

On the global level, the debate on fixing the limits on mycotoxins in spices seems to be relatively recent. In 2015, the Codex Alimentarius Commission or, more precisely, its Committee on Contaminants in Foods (CCCF) agreed to start working on a Code of practice for the prevention and reduction of mycotoxin contamination in spices and combinations of spices [92]. In the same year, the feasibility of establishing the maximum levels for selected spices was also discussed as a separate topic in the CCCF. It was India which, in 2014, initiated the discussion (the 8th Session; March 2014) and which simultaneously proposed to establish the maximum levels with respect to (i) total AFs, (ii) AFB_1, and (iii) OTA in five different spices occupying a prominent place in the global trade with spices, namely, in dried or dehydrated forms of nutmeg, chili/paprika, ginger, pepper, and turmeric [92]. Upon the Indian proposal, the electronic working group was set up to deal with the issue. Reaching a consensus on establishing the maximum limits for total AFs and OTA in the proposed spices, however, turned out to be a complex process. While some states argued that more conclusive data on the occurrence of mycotoxins in spices were needed, others opined that the general level of the consumption of spices was too low to justify establishing the maximum limits for mycotoxins contained in spices. Due to the diverging views of different states, in 2018, the CCCF decided to temporarily discontinue works on establishing the maximum limits and give time to member states to implement the Code of Practice for the prevention and reduction of mycotoxins in spices adopted in 2017 [93]. Upon the implementation of the Code of Practice for the prevention and reduction of mycotoxins in spices, in a three-year horizon, the new data on the occurrence of mycotoxins in spices should be obtained, and based on them, the issue of establishing their maximum limits in spices should be re-examined by the CCCF. Nevertheless, the levels of 20/30 µg/kg for total AFs and the level of 20 µg/kg for OTA have been retained as the points of departure for future discussion [93].

Thus, for the time being, the most extensive regulation of the presence of mycotoxins in spices on an international level can be found in EU law. On the grounds of powers conferred by Article 2(3) of Council Regulation (EEC) No 315/93 [94], the European Commission adopted Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs [15].

Section 2 of the Annex to the Regulation No 1881/2006 fixes the maximum levels of selected mycotoxins in different foodstuffs including spices and licorice. Section 2 primarily establishes the maximum levels of AFs in *Capsicum* spp. (including chilies, chili powder, cayenne, and paprika), peppers, nutmeg, ginger, turmeric, and in the mixtures of these spices; see Table 3. In addition, Section 2 lays down the maximum limits for OTA in the same spices, their mixtures, and also in licorice; see Table 4. The maximum limits for OTA in spices evolved in a rather complex way. They were first laid down in Regulation No 105/2010 [95], later, they were amended by Regulation No 594/2012 [96]. The present maximum levels have been established by Regulation No 2015/1137.

In other regions, the regulation of the presence of mycotoxins in spices mostly depends on the sole appreciation of individual states. In this respect, data gathered by the CCCF in a direct link with the discussion on establishing the maximum limits for mycotoxins in spices provide an interesting insight on the existing limits in the Codex member states [93].

As it is evident from these data, at present, a fair number of the Codex member states have fixed the maximum limits on AFs in spices. These limits range from 1 μg/kg (Honduras) to 30 μg/kg (India).

In the case of OTA, the same data suggest the situation is different in that the number of states which have laid down the maximum limits for the presence of OTA in spices seems to be markedly lower. The lowest limit of 10 μg/kg has been reported from Armenia while the highest one of 30 μg/kg has been reported from Brazil. However, even a lower limit has applied in South Korea where, at least in some spices (such as red pepper), the maximum limit for the presence of OTA has been related to be 7 μg/kg [97].

When it comes to identifying spices in which the presence of AFs and OTA is regulated, the approaches differ. While many states have limits fixed for all the foodstuffs, in other states, there are specific limits for spices in general or only for specific spices (such as chili or nutmeg).

These observations can be exemplified by the regulatory practice of several states which play a prominent role in the global trade in spices.

In India, which has initiated the discussion on the regulation of mycotoxins in spices on the global level, the maximum limits are prescribed for AFs in spices by the Food Safety and Standards Authority [98]. Currently, the maximum limit is 30 μg/kg. However, no limits on OTA in spices have been reported.

In China, the presence of mycotoxins in foodstuffs is currently regulated by the National Food Safety Standard of Maximum Levels of Mycotoxin in Foods (GB 2761-2012) which is based on comparative analysis of international and national standards and came into force in October 2011 [99].

In 2017, the National Standard has been updated by the National Food Safety Standard for Maximum Levels of Mycotoxins in Foods (GB 2761-2017), and in January 2020, the public consultation on its revision was launched [100]. While under the National Standard, the maximum level is set at 5.0 μg/kg for AFB_1 in spices, it does not seem that specific maximum limits would apply with regard to other mycotoxins in spices.

In Brazil, before 2011, only the presence of AFs in some selected commodities such as peanuts was subject to legal regulation. Under the impact of the introduction of regulatory limits on international and the EU level, in 2011, however, the Brazilian Surveillance Agency (ANVISA) established the limits for six mycotoxins, which were amended in 2017 [101]. The existing regulation now applies to more than 20 categories of foodstuffs including spices. As for AFs (B_1, B_2, G_1, G_2), the maximum limits are fixed at 20 μg/kg; as for OTA, the maximum limit equals 30 μg/kg.

In the USA, the world's largest spice consumer, in case of AFs (B_1, B_2, G_1, G_2), the action levels for their presence in foodstuffs have been laid down by the Food and Drug Administration (FDA) since 1965. Since 1969, the action level has been set at 20 μg/kg for all foodstuffs intended for human consumption, except milk [102]. The action levels are understood as levels above which the foodstuffs will be considered to be adulterated, which means the FDA is allowed to bring regulatory and enforcement action under the Federal Food, Drug, and Cosmetic Act (FFDC Act). As far as OTA is concerned, the FDA has not been reported to have established any action or guidance levels.

Table 3. Maximum levels of aflatoxins in spices under EU legislation (Regulation No 1881/2006, as in force).

Foodstuff	AFB_1 (μg/kg)	Total AFs [a] (μg/kg)	Reference
Capsicum spp. (dried fruits thereof, whole or ground, including chilies, chili powder, cayenne, and paprika) *Piper* spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	5	10	[15]
Mixtures of spices containing one or more of the abovementioned spices	5	10	[103]

Note: [a] AFs = Sum of aflatoxins B_1, B_2, G_1 and G_2.

Table 4. Maximum levels of ochratoxin A in spices and licorice under EU legislation (Regulation No 1881/2006, as in force).

Foodstuff	OTA (μg/kg)	Reference
Piper spp. (fruits thereof, including white and black pepper) *Myristica fragrans* (nutmeg) *Zingiber officinale* (ginger) *Curcuma longa* (turmeric)	15	[96]
Capsicum spp. (dried fruits thereof, whole or ground, including chilies, chili powder, cayenne, and paprika)	20	[104]
Mixtures of spices containing one of the abovementioned spices	15	[96]
Licorice (*Glycyrrhiza glabra, Glycyrrhiza inflate* and other species)		[95]
Licorice root, ingredient for herbal infusion	20	
Licorice extract, for use in food in particular beverages and confectionary	80	

6. Mycotoxins and Microfungi in Spices from the Perspective of Research in the Last Five Years (Since 2015)

This review summarizes the studies concerning mycotoxins and their producers in spices over the last five years—since 2015. For the evaluation of the positivity on microfungi or mycotoxins, the following six-level scale was established: (i) none (0%), (ii) rare (up to 5%), (iii) low (up to 25%), (iv) moderate (up to 50%), (v) high (up to 75%), and (vi) very high (more than 75%) occurrence of positive results. This scale was used for the evaluation of the percentage of studies with a positive incidence of microfungi/mycotoxins in a given spice (a study with at least one positive sample, hereinafter referred to as "positive study") in the total number of publications dealing with related microfungi/mycotoxins in spice. The same scale was used in case of the percentage of samples with a positive finding on mycotoxins in the total number of samples throughout all publications involved. However, it is important to consider the number of baseline studies, because the listed percentages are the more conclusive, the more studies they are based on.

6.1. Mycotoxins in Spices Overview

A total of 48 studies altogether covering 17 mycotoxins in 38 spices were included. Namely, these publications cover (the numbers in brackets indicate the number of publications related to the kind of spice or type of mycotoxin) allspice (*Pigmenta officinalis*) (2), anise (*Pimpinella anisum*) (5), basil (*Ocimum basilicum*) (5), bay leaf (*Laurus nobilis*) (6), caraway (*Carum carvi*) (7), cardamom (*Elateria cardamomum*) (7), carom (*Trachyspermum ammi*) (1), chili (*Capsicum* spp.) (30), cinnamon (*Cinnamomum burmannii*) (11), cloves (*Eugenia caryophyllata*) (8), coriander (*Coriandrum sativum*) (10), cumin (*Cuminum cyminum*) (9), cumin black (*Nigella sativa*) (3), curry (3), dawdawa (*Parkia biglobosa*) (2), fennel (*Foeniculum vulgare*)

(12), fenugreek (*Trigonella foenum-graecum*) (4), garlic (*Allium sativum*) (5), ginger (*Zingiber officinale*) (14), licorice (*Glycyrrhiza glabra*) (3), mace (*Myristica fragrans*) (1), marjoram (*Majorana hortensis*) (3), mint (*Mentha piperita*) (5), mustard (*Sinapis* spp.) (3), nutmeg (*Myristica fragrans*) (12), onion (*Allium* spp.) (3), oregano (*Origanum vulgare*) (5), paprika (*Capsicum* spp.) (6), parsley (*Allium schoenoprasum*) (3), pepper black (*Piper nigrum*) (23), pepper white (*Piper nigrum*) (6), rosemary (*Salvia rosmarinus*) (6), saffron (*Crocus* spp.) (2), sage (*Salvia* spp.) (4), star anise (*Illicium verum*) (1), sumac (*Rhus coriaria*) (3), thyme (*Thymus vulgaris*) (10), and turmeric (*Curcuma longa*) (11) in which the following mycotoxins were analyzed: AFB_1 (33), AFB_2 (19), AFG_1 (19), AFG_2 (18), OTA (20), CIT (4), ZEA (5), FB1 (9), FB2 (7), DON (4), NIV (3), T-2 (5), HT-2 (4), ALT (2), AOH (3), TEA (2), and STEG (4).

The percentage of positive studies of the total number of studies dealing with related mycotoxin and spice are shown in Table S1 of the Supplementary Materials (for mycotoxins produced by *Aspergillus* and *Penicillium* genera), Table S2 of the Supplementary Materials (for *Fusarium* mycotoxins) and Table S3 of the Supplementary Materials (for *Alternaria* mycotoxins). Similarly, the percentage of the total sum of positive samples to the total sum of tested samples for each unique spice and mycotoxin combination throughout all included publications are shown in Tables 5–7.

6.1.1. Aflatoxins

As can be seen above, AFs (mainly AFB_1) are undoubtedly the most frequently analyzed mycotoxins in spices. In terms of AFs, studies are most often concerned with chili, black pepper, ginger, fennel, turmeric, coriander, cinnamon, nutmeg, and thyme, in descending order. The occurrence of total AFs in the above-mentioned spices is usually high to very high. In the following summaries of positive findings, only aflatoxin occurrence supported by at least 5 studies or at least 30 samples will be described in more detail.

Aflatoxin B_1. Number of AFB_1-positive studies has been proven as very high in ginger, chili, and turmeric; as high in black pepper, cumin, coriander, and cinnamon; and as moderate in fennel, caraway, thyme, and nutmeg—see Table S1 of the Supplementary Materials.

The AFB_1 occurrence has been proven as high in ginger, chili, fenugreek, turmeric, and coriander; as moderate in paprika, cumin, black pepper, nutmeg, and fennel; as low in caraway, cinnamon, and white pepper; as rare in licorice and thyme, and none in oregano and basil—see Table 5.

The highest AFB_1 concentrations in different spices have been reported in nutmeg (1632.2 μg/kg) in Indonesia [105], chili (156.0 μg/kg) in Nigeria [106], paprika (155.7 μg/kg) in Italy [107], black pepper (75.8 μg/kg) in Pakistan [108], licorice (57.0 μg/kg) in Egypt, black cumin (56.8 μg/kg) in Egypt [109], ginger (39.8 μg/kg) in Iran [110], parsley (27.4 μg/kg) in Egypt [109], saffron (26.5 μg/kg) in Algeria [111], fennel (21.7 μg/kg) in Malaysia [112], mustard (18.2 μg/kg) and thyme (16.8 μg/kg) in Egypt [109], and coriander (11.0 μg/kg) in Malaysia [112].

Aflatoxin B_2. Several AFB_2-positive studies have been proven as high in chili, turmeric, ginger, and black pepper; as moderate in coriander and fennel; and as low in cinnamon—see Table S1 of the Supplementary Materials.

The AFB_2 occurrence has been proven as moderate in ginger; as low in turmeric, chili, caraway, paprika, coriander, fenugreek, black pepper, nutmeg, fennel, and cumin; as rare in white pepper; and as none in cinnamon and licorice—see Table 5.

The highest AFB_2 concentrations in different spices have been reported in chili (33.3 μg/kg) in Indonesia [113], paprika (9.9 μg/kg) in Italy [107], parsley (2.5 μg/kg) in Egypt [109], and fennel (2.3 μg/kg), turmeric (1.7 μg/kg) and coriander (1.6 μg/kg) in Malaysia [112].

Aflatoxin G_1. Number of AFG_1-positive studies has been proven as high in turmeric and cumin and as moderate in chili, black pepper, fennel, cinnamon, and ginger—see Table S1 of the Supplementary Materials.

The AFG_1 occurrence has been proven as moderate in fennel and white pepper; as low in cumin, turmeric, paprika, fenugreek, cinnamon, ginger, chili, coriander, and black pepper; and as rare in nutmeg, caraway, and licorice—see Table 5.

The highest AFG_1 concentrations in different spices have been reported in paprika (318.1 μg/kg) in Italy [107], anise (157.5 μg/kg), thyme (41.2 μg/kg), black pepper (31.5 μg/kg), rosemary (12.9 μg/kg), mustard (10.5 μg/kg) and parsley (8.1 μg/kg) in Egypt [109], and chili (7.0 μg/kg) in Malaysia [114].

Aflatoxin G_2. Number of AFG_2-positive studies has been proven as moderate in chili, cumin, ginger, coriander, black pepper, and fennel and as rare in cinnamon and turmeric—see Table S1 of the Supplementary Materials.

The AFG_2 occurrence has been proven as moderate in white pepper; as low in fenugreek, turmeric, coriander, paprika, black pepper, and chili; as rare in fennel, cumin, ginger, and caraway; and none in nutmeg, cinnamon, and licorice—see Table 5.

The highest AFG_2 concentrations in different spices have been reported in paprika (45.4 μg/kg) in Italy [107], black pepper (16.0 μg/kg) in Egypt, mustard (7.6 μg/kg) in Egypt [109], chili (1.5 μg/kg) in Turkey [115], and cinnamon (0.4 μg/kg) in Iran [116].

6.1.2. Ochratoxin A

OTA is the second most frequently analyzed mycotoxin in spices, after AFs. In terms of OTA, studies are most often concerned with black pepper, chili, ginger, fennel, and turmeric, in descending order, where its occurrence is high to very high. In the following summaries of positive findings, only OTA occurrence supported by at least 5 studies or at least 30 samples will be described in more detail.

The number of OTA-positive studies has been proven as very high in turmeric, chili, and ginger and as high in black pepper and fennel—see Table S1 of the Supplementary Materials.

The OTA occurrence has been proven as high in paprika and mace; as moderate in turmeric, ginger, fenugreek, cardamom, chili, black pepper, caraway, licorice, coriander, and fennel; as low in white pepper, cinnamon, and cumin; and none in oregano, clove, thyme, and basil—see Table 5.

The highest OTA concentrations in different spices have been reported in chili (907.5 μg/kg) in Ivory Coast [117], paprika (177.4 μg/kg) in Italy [107], black pepper (79.0 μg/kg) in Sri Lanka [118], cardamom (78.0 μg/kg) in Saudi Arabia [119], nutmeg (60.7 μg/kg) and licorice (36.7 μg/kg) in the Czech Republic [120], cumin (20.4 μg/kg) in Malaysia [112], cinnamon (16.1 μg/kg) in Iran [121], ginger (12.7 μg/kg) in the Czech Republic [120], curry (9.6 μg/kg) in Malaysia [112], turmeric (8.5 μg/kg) in Iran [121], garlic (5.1 μg/kg) in Lebanon [9], and white pepper (4.9 μg/kg) in Cameroon [35].

6.1.3. Citrinin

Very few studies deal with CIT in spices—only 1 to 3 studies pertain to a single spice at a time. Publications mentioning CIT-positive findings deal with black pepper, chili, coriander, cumin, fenugreek, ginger, and licorice. On the contrary, CIT has not been found in basil, caraway, fennel, nutmeg, oregano, thyme, and turmeric, although they have been tested—see Table S1 of the Supplementary Materials.

The CIT occurrence has been proven as moderate in chili, ginger, coriander, and fenugreek; as low in black pepper and licorice; and none in basil, nutmeg, oregano, thyme, and turmeric—see Table 5.

Table 5. Samples positivity: Natural occurrence of mycotoxins produced by *Aspergillus* and *Penicillium* species in spices in the last 5 years (since 2015).

Mycotoxin [a]/Spice	AFB_1			AFB_2			AFG_1			AFG_2			AFs			OTA			CIT			Reference
	Positive [b] (%)		n [c]	Positive (%)		n	Positive (%)		n	Positive (%)		n	Positive (%)		n	Positive (%)		n	Positive (%)		n	
Allspice	-	-	0	-	-	0	-	-	0	-	-	0	●	66.7	3	×	0.0	3	-	-	0	[9]
Anise	●	80.0	5	×	0.0	1	●	100	1	×	0.0	1	○	25.0	8	◌	33.3	3	-	-	0	[9,109,111,122]
Basil	×	0.0	56	-	-	0	-	-	0	-	-	0	×	0.0	2	×	0.0	52	×	0.0	50	[9,110,123]
Bay leaf	×	0.0	25	×	0.0	18	○	11.1	18	○	22.2	18	×	0.0	6	×	0.0	2	-	-	0	[9,110,122,124]
Caraway	○	25.0	56	○	24.1	54	☆	3.7	54	☆	1.9	54	◌	39.3	56	◌	35.9	39	×	0.0	25	[8,9,111,120,124,125]
Cardamom	×	0.0	2	×	0.0	1	×	0.0	1	×	0.0	1	●	63.9	122	◌	42.2	116	-	-	0	[9,109,119,122,126,127]
Carom	◌	50.0	20	×	0.0	20	×	0.0	20	×	0.0	20	◌	50.0	20	-	-	0	-	-	0	[125]
Chili	●	61.2	957	○	24.3	267	○	10.9	311	○	5.1	293	●	58.9	638	◌	41.6	586	◌	47.3	55	[8,9,106,108,110–118,120–122,128–138]
Cinnamon	○	17.6	51	×	0.0	39	○	15.4	39	×	0.0	39	◌	32.3	62	○	20.5	39	-	-	0	[9,110–112,116,121,122,125,127,131]
Cloves	×	0.0	13	×	0.0	12	×	0.0	12	×	0.0	12	○	11.1	18	×	0.0	54	-	-	0	[9,122,127,131]
Coriander	●	56.5	46	○	19.0	42	○	8.1	62	○	6.5	62	●	53.1	64	◌	31.1	45	◌	40.0	30	[8,9,109,111,112,120,124,125]
Cumin	◌	33.3	69	○	8.8	57	○	24.6	57	☆	3.5	57	●	56.5	62	○	5.7	35	○	21.4	28	[8,9,109–112,122,125]
Cumin, black	○	14.3	7	☆	4.8	21	●	100	1	☆	4.8	21	●	81.0	21	-	-	0	-	-	0	[109,110,125]
Curry	●	84.6	13	●	61.5	13	○	23.1	13	○	7.7	13	●	84.6	13	●	100	8	-	-	0	[112]
Dawadawa	●	100	12	-	-	0	-	-	0	-	-	0	-	-	0	○	16.7	12	-	-	0	[130]
Fennel	◌	25.3	91	○	9.2	76	◌	30.3	76	☆	3.9	76	●	54.0	113	◌	29.1	79	×	0.0	25	[8,9,109–112,124–127,131]
Fenugreek	●	58.3	36	○	16.7	36	○	16.7	36	○	13.9	36	●	62.5	40	◌	46.2	39	◌	37.1	35	[8,9,109]
Garlic	-	-	0	-	-	0	-	-	0	-	-	0	×	0.0	2	◌	50.0	2	-	-	0	[9]
Ginger	●	63.1	217	◌	29.7	192	○	13.0	192	☆	2.6	192	●	59.4	165	◌	47.9	213	◌	44.4	36	[8,9,109–111,117,120,122,130,139,140]
Licorice	☆	3.1	32	×	0.0	32	☆	3.1	32	×	0.0	32	☆	3.1	32	◌	32.6	43	○	6.5	31	[109,120,141]
Mace	-	-	0	-	-	0	-	-	0	-	-	0	●	63.3	30	●	60.0	30	-	-	0	[126]
Marjoram	●	100	1	×	0.0	1	×	0.0	1	×	0.0	1	◌	33.3	3	◌	50.0	2	-	-	0	[9,109]
Mint	×	0.0	25	×	0.0	16	×	0.0	16	×	0.0	16	×	0.0	19	×	0.0	3	-	-	0	[9,110,124]
Mustard	◌	50.0	2	×	0.0	1	●	100	1	●	100	1	●	100	1	○	25.0	12	-	-	0	[109,120,127]
Nutmeg	◌	27.9	104	○	13.2	53	☆	3.8	53	×	0.0	53	●	55.7	131	●	92.9	14	×	0.0	50	[9,105,109,120,123,127,135]
Onion	×	0.0	8	-	-	0	×	0.0	8	-	-	0	×	0.0	12	×	0.0	12	-	-	0	[9,133]
Oregano	×	0.0	79	×	0.0	29	×	0.0	29	×	0.0	29	☆	3.1	32	×	0.0	65	×	0.0	50	[9,123,124,131]
Paprika	◌	47.6	42	○	22.6	31	○	18.4	38	○	6.5	31	◌	43.9	41	●	60.4	53	-	-	0	[9,107,111,120,133]
Parsley	●	100	1	●	100	1	●	100	1	×	0.0	1	◌	50.0	2	×	0.0	1	-	-	0	[9,109]
Pepper, black	◌	31.0	226	○	13.8	80	○	7.5	120	○	5.7	140	◌	44.8	203	◌	36.0	264	○	20.7	92	[8,9,35,108–112,116,117,120–123,125–127,129–131,134]
Pepper, white	○	5.3	38	☆	2.6	38	◌	26.3	38	◌	26.3	38	●	55.0	40	○	21.1	38	-	-	0	[9,35,112,125,131]
Rosemary	○	14.8	27	◌	29.6	27	☆	3.7	27	◌	33.3	27	◌	27.8	18	○	5.9	17	-	-	0	[9,109,124,131]
Saffron	◌	50.0	4	-	-	0	-	-	0	-	-	0	×	0.0	1	×	0.0	1	-	-	0	[9,111]
Sage	◌	33.3	3	×	0.0	1	●	100	1	×	0.0	1	◌	50.0	4	◌	33.3	3	-	-	0	[9,109,110]
Star anise	×	0.0	1	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[127]
Sumac	×	0.0	9	-	-	0	-	-	0	-	-	0	×	0.0	2	×	0.0	2	-	-	0	[9,110]
Thyme	☆	2.7	73	×	0.0	13	○	7.7	13	×	0.0	13	○	6.3	16	×	0.0	53	×	0.0	50	[9,109,110,123,124]
Turmeric	●	57.1	70	○	24.6	65	○	24.6	65	○	8.2	85	●	51.9	129	◌	49.0	104	×	0.0	35	[8,9,109,110,112,116,121,122,125,126]

Notes: [a] AFB_1 = Aflatoxin B_1, AFB_2 = Aflatoxin B_2, AFG_1 = Aflatoxin G_1, AFG_2 = Aflatoxin G_2, AFs = Aflatoxins, OTA = Ochratoxin A, CIT = Citrinin; [b] Positive = the percentage of positive samples; [c] n = the total number of samples related to mycotoxin and spice from all publications involved; × = none occurrence (0%), ☆ = rare occurrence (up to 5%), ○ = low occurrence (up to 25%), ◌ = moderate occurrence (up to 50%), ● = high occurrence (up to 75%), ● = very high occurrence (more than 75%).

6.1.4. Fumonisins

As in the case of CIT, there are not many studies for FMNs in spices—only 1-4 and 1-2 studies dealing with FB_1 and FB_2, respectively, pertain to a single spice at a time. Studies with positive findings of FMNs in spices are rather rare; however, some publications in connection with positive findings in black pepper, licorice, nutmeg, mint, and thyme for FB_1; chili for FB_2; and paprika, onion spice and dawadawa for both of them have been published. On the contrary, neither of FMNs have been found in many kinds of spices—see Table S2 of the Supplementary Materials.

The FB_1 occurrence has been proven as moderate in paprika and licorice; as low in mint and garlic; as rare in thyme; and as none in black pepper, oregano, and basil—see Table 6.

The FB_2 occurrence has been proven as high in paprika and as none in garlic and licorice.

The highest FB_1 concentrations in different spices have been reported in onion (591.0 µg/kg) in South Africa [133], garlic (540.0 µg/kg) of unknown origin [142], mint (256.0 µg/kg) in Turkey [143], paprika (243.9 µg/kg) in Italy [107], dawadawa (165.0 µg/kg) in Nigeria [34], black pepper (135.0 µg/kg) from Sri Lanka [118], thyme (125.0 µg/kg) in Turkey [143], licorice (39.3 µg/kg) in China [141], and nutmeg (25.0 µg/kg) originated in Indonesia [123].

The highest FB_2 concentrations in different spices have been reported in onion (4537.0 µg/kg) in South Africa, chili (425.0 µg/kg) in South Africa [133], paprika (176.9 µg/kg) in Italy [107], and dawadawa (170.0 µg/kg) in Nigeria [34].

6.1.5. Trichothecenes (DON, NIV, T-2, HT-2)

As with CIT and FMNs, there are not many studies for TCT in spices, including DON, NIV, T-2, and HT-2—see Table S2 of the Supplementary Materials. None of the TCT has been detected in basil, nutmeg, black pepper, and oregano, while all of the above-mentioned toxins have been detected in paprika at low to moderate levels. For thyme, DON has been detected at a low level, while none of the other TCT has been detected—see Table 6.

The highest concentrations in different spices have been reported in paprika (59.8 µg/kg) in Italy [107] and licorice (11.0 µg/kg) in China [141] for DON, in paprika (243.9 µg/kg) in Italy [107] for NIV, in dawadawa (32.0 µg/kg) in Nigeria [34] and paprika (27.1 µg/kg) in Italy [107] for T-2, and in paprika (75.9 µg/kg) in Italy [107] and dawadawa (58.0 µg/kg) in Nigeria [34] for HT-2.

6.1.6. Zearalenone

ZEA is one of the least analyzed mycotoxins in this review. No more than one study pertains to a single spice—see Table S2 of the Supplementary Materials. The ZEA occurrence has been proven as very high in paprika (up to 53.6 µg/kg) in Italy [107]; as moderate in dawadawa (up to 86.0 µg/kg) in Nigeria [34]; as low in thyme (up to 209.0 µg/kg) originated in Poland [123] and licorice (up to 8.8 µg/kg) in China [141]; and as none in chili originated in Korea [137], basil originated in India, nutmeg originated in Indonesia, oregano originated in Turkey, and black pepper originated in Brazil and Vietnam [123]—see Table 6.

Table 6. Samples positivity: Natural occurrence of *Fusarium* mycotoxins in spices in the last 5 years (since 2015).

Mycotoxin [a]/Spice	FB_1			FB_2			DON			NIV			T-2			HT-2			ZEA			Reference
	Positive [b]	(%)	n [c]	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	
Basil	×	0.0	55	×	0.0	5	×	0.0	50	×	0.0	50	×	0.0	50	×	0.0	50	×	0.0	50	[123,143]
Bay leaf	×	0.0	19	×	0.0	1	-	-	0	-	-	0	×	0.0	18	×	0.0	18	-	-	0	[124,143]
Caraway	×	0.0	9	-	-	0	-	-	0	-	-	0	×	0.0	9	×	0.0	9	-	-	0	[124]
Chili	×	0.0	18	○	5.6	18	-	-	0	-	-	0	-	-	0	-	-	0	×	0.0	56	[133,137]
Coriander	×	0.0	17	×	0.0	8	-	-	0	-	-	0	◔	33.3	9	×	0.0	9	-	-	0	[124,143]
Dawadawa	◔	47.1	17	◕	58.8	17	×	0.0	17	×	0.0	17	◔	35.3	17	○	5.9	17	◔	35.3	17	[34]
Fennel	×	0.0	11	-	-	0	-	-	0	-	-	0	×	0.0	11	×	0.0	11	-	-	0	[124]
Garlic	○	5.4	56	×	0.0	56	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[142]
Licorice	◔	38.7	31	×	0.0	31	☆	3.2	31	-	-	0	×	0.0	31	-	-	0	○	12.9	31	[141]
Mint	○	6.5	31	×	0.0	15	-	-	0	-	-	0	○	18.8	16	×	0.0	16	-	-	0	[124,143]
Nutmeg	-	-	0	-	-	0	×	0.0	50	×	0.0	50	×	0.0	50	×	0.0	50	×	0.0	50	[123]
Onion	◔	37.5	8	●	87.5	8	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[133]
Oregano	×	0.0	67	-	-	0	×	0.0	50	×	0.0	50	×	0.0	67	×	0.0	67	×	0.0	50	[123,124]
Paprika	◔	50.0	38	◕	73.7	38	◔	38.7	31	◔	48.4	31	○	19.4	31	○	19.4	31	●	87.1	31	[107,133]
Pepper, black	×	0.0	50	-	-	0	×	0.0	50	×	0.0	50	×	0.0	50	×	0.0	50	×	0.0	50	[123]
Rosemary	×	0.0	11	-	-	0	-	-	0	-	-	0	×	0.0	11	×	0.0	11	-	-	0	[124]
Thyme	☆	1.3	76	×	0.0	14	○	14.0	50	×	0.0	50	×	0.0	62	×	0.0	62	○	18.0	50	[123,124,143]

Notes: [a] FB_1 = Fumonisin B_1, FB_2 = Fumonisin B_2, DON = Deoxynivalenol, NIV = Nivalenol, T-2 = T-2 toxin, HT-2 = HT-2 toxin, ZEA = Zearalenone; [b] Positive = the percentage of positive samples; [c] n = the total number of samples related to mycotoxin and spice from all publications involved; × = none occurrence (0%), ☆ = rare occurrence (up to 5%), ○ = low occurrence (up to 25%), ◔ = moderate occurrence (up to 50%), ◕ = high occurrence (up to 75%), ● = very high occurrence (more than 75%).

6.1.7. *Alternaria* Mycotoxins

Alternaria mycotoxins (ALT, AOH, TEA) are rarely studied in spices, as most data originated in just one publication [10]—see Table S3 of the Supplementary Materials. Moreover, very few samples per single spice have been tested.

All above mentioned *Alternaria* mycotoxins have been confirmed in cinnamon, ginger, chili and paprika, but no other findings have been found in anise, basil and parsley. In addition, among *Alternaria* mycotoxins, TEA has been found in most of spice samples: bay leaf, caraway, cardamom, cinnamon, cloves, coriander, cumin, fennel, fenugreek, garlic, ginger, chili, marjoram, mint, nutmeg, onion, oregano, paprika, black pepper, white pepper, rosemary, sage, sumac, thyme, and turmeric. More details about single *Alternaria* toxins and other spices are shown in Table 7.

Table 7. Samples positivity: Natural occurrence of *Alternaria* mycotoxins in spices in the last 5 years (since 2015).

Mycotoxin [a]/Spice	ALT			AOH			TEA			Reference
	Positive [b]	(%)	n [c]	Positive	(%)	n	Positive	(%)	n	
Allspice	×	0.0	3	◌	33.3	3	×	0.0	3	[10]
Anise	×	0.0	3	×	0.0	3	×	0.0	3	[10]
Basil	×	0.0	2	×	0.0	2	×	0.0	2	[10]
Bay leaf	×	0.0	2	×	0.0	2	◌	50.0	2	[10]
Caraway	×	0.0	2	×	0.0	2	●	100	2	[10]
Cardamom	×	0.0	4	×	0.0	4	◍	75.0	4	[10]
Chili	○	14.3	7	◌	42.9	7	●	100	7	[10]
Cinnamon	◍	66.7	3	◍	66.7	3	◍	66.7	3	[10]
Cloves	◌	50.0	2	×	0.0	2	◌	50.0	2	[10]
Coriander	×	0.0	2	×	0.0	2	●	100	2	[10]
Cumin	×	0.0	5	×	0.0	5	●	100	5	[10]
Fennel	×	0.0	2	×	0.0	2	●	100	2	[10]
Fenugreek	×	0.0	4	×	0.0	4	◌	50.0	4	[10]
Garlic	×	0.0	2	◌	50.0	2	●	100	2	[10]
Ginger	◌	33.3	3	◌	33.3	3	◍	66.7	3	[10]
Licorice	-	-	0	◌	45.2	31	-	-	0	[141]
Marjoram	×	0.0	2	×	0.0	2	●	100	2	[10]
Mint	×	0.0	3	◌	33.3	3	◍	66.7	3	[10]
Nutmeg	×	0.0	2	◌	50.0	2	◌	50.0	2	[10]
Onion	×	0.0	4	◌	50.0	4	◌	50.0	4	[10]
Oregano	×	0.0	3	◌	33.3	3	●	100	3	[10]
Paprika	○	5.9	34	◍	61.8	34	●	100	34	[10,107]
Parsley	×	0.0	1	×	0.0	1	×	0.0	1	[10]
Pepper, black	×	0.0	4	○	25.0	4	◍	75.0	4	[10]
Pepper, white	×	0.0	2	◌	50.0	2	◌	50.0	2	[10]
Rosemary	×	0.0	2	×	0.0	2	◌	50.0	2	[10]
Sage	×	0.0	3	◍	66.7	3	◍	66.7	3	[10]
Sumac	×	0.0	2	◌	50.0	2	●	100	2	[10]
Thyme	×	0.0	3	×	0.0	3	●	100	3	[10]
Turmeric	◌	50.0	2	×	0.0	2	●	100	2	[10]

Notes: [a] ALT = Altenuene, AOH = Alternariol, TEA = Tenuazonic acid; [b] Positive = the percentage of positive samples; [c] n = the total number of samples related to mycotoxin and spice from all publications involved; × = none occurrence (0%), ☆ = rare occurrence (up to 5%), ○ = low occurrence (up to 25%), ◌ = moderate occurrence (up to 50%); ◍ = high occurrence (up to 75%), ● = very high occurrence (more than 75%).

The highest ALT concentrations in different spices have been reported in clove (11.7 µg/kg) in Lebanon [10]; paprika (40.3 µg/kg) in Italy [107]; and ginger (5.2 µg/kg), chili (3.6 µg/kg), and turmeric (2.8 µg/kg) in Lebanon [10]. The highest AOH concentrations in different spices have been reported in licorice (520.6 µg/kg) in China [141]; paprika (428.4 µg/kg) in Italy [107]; and white pepper (319.7 µg/kg), black pepper (89.0 µg/kg), garlic (57.4 µg/kg), oregano (13.5 µg/kg), nutmeg (12.7 µg/kg),

mint (11.8 µg/kg), allspice (8.0 µg/kg), sumac (6.6 µg/kg), and ginger (5.4 µg/kg) in Lebanon [10]. The highest TEA concentrations in different spices have been reported in paprika (8248.5 µg/kg) in Italy [107] and rosemary (50.4 µg/kg), bay leaf (48.2 µg/kg), nutmeg (22.0 µg/kg), white pepper (20.3 µg/kg), and clove (14.9 µg/kg) in Lebanon [10].

6.1.8. Sterigmatocystin

STEG has been found in oregano (unknown positivity, up to 28.0 µg/kg) originated in Turkey [123], at low level in paprika (14.3%, 1/7, 18.0 µg/kg) in South Africa [133], and at rare level in thyme (4%, 2/50, up to 14 µg/kg) originated in Poland [123]. Black pepper and chili have been found positive in Sri Lanka at moderate levels (43.9%, 36/82, 49.0 µg/kg and 38.4%, 33/86, up to 32 µg/kg, respectively) [118], while no STEG has been detected in 50 samples of black pepper originated in Brazil and Vietnam [123] and in 18 samples of chili in South Africa [133]. STEG has been detected in none of the following spices: 50 basil samples originated in India [123], 31 licorice samples from China [141], 50 nutmeg samples originated in Indonesia [123], or 8 onion samples from South Africa [133]. For very little data, STEG is further discussed neither in the text nor in the table.

6.2. Microfungi in Spices Overview

A total of 25 studies altogether covering 14 microfungi in 33 spices were included. These publications cover (the numbers in brackets indicate the number of publications related to the kind of spice or microfungi) anise (3), basil (1), bay leaf (2), caraway (6), cardamom (6), chili (14), cinnamon (8), cloves (8), coriander (6), cumin (5), cumin black (2), curry (4), fennel (8), fenugreek (3), garlic (3), ginger (7), licorice (1), mace (1), marjoram (1), mint (1), mustard (3), nutmeg (10), oregano (2), paprika (2), parsley (1), pepper black (12), pepper white (6), rosemary (3), saffron (3), star anise (1), sumac(2), thyme (3), and turmeric (5) in which the following microfungi were analyzed: *Aspergillus flavus* (20), *A. parasiticus* (13), *A. niger* (20), *A. carbonarius* (4), *A. tamarii* (8), *A. terreus* (6), *A. versicolor* (7), *A. ochraceus* (8), *Penicillium citrinum* (13), *P. verrucosum* (3), *Fusarium verticillioides* (3), *Alternaria alternata* (5), *Rhizopus nigricans* (3), and *R. oryzae* (4).

The percentage of positive studies to the total number of studies concerning each unique spice and microfungi combination are shown in Table 8 (for *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp.), Table 9 (for *Aspergillus* species), and Table 10 (for *Penicillium, Fusarium, Alternaria*, and *Rhizopus* species).

Aspergillus, Penicillium, and *Fusarium* genera are the most important mycotoxin producers in various commodities [39], which also applies to spices in which they are commonly present, as can be seen in Table 8. Out of the mentioned microfungi genera, spices are predominantly contaminated by *Aspergillus* followed by *Penicillium* and then by *Fusarium* strains. In the following summary, only microfungi occurrences supported by at least 5 individual studies are described in more detail.

6.2.1. *Aspergillus* Species

Aspergillus species are in the vast majority of spices. The occurrence is very high in chili, fennel, ginger, caraway, coriander, white pepper, turmeric, black pepper, nutmeg, cardamom, and cumin and moderate in cinnamon and cloves. Based on all included studies, some *Aspergillus* strains were isolated from all spices involved in this review except for star anise, which was only analyzed once and with negative results—see Table 8.

Of the *Aspergillus* species, *A. niger* is most common in spices, followed by *A. flavus* and *A. ochraceus*. The occurrence of *A. niger* is very high in black pepper, cardamom, chili, and fennel, high in cinnamon, ginger, and nutmeg, and low in cloves. The occurrence of *A. flavus* is very high in chili, black pepper, cardamom, and white pepper; high in nutmeg and fennel; and moderate in cloves and cinnamon. The occurrence of *A. ochraceus* is very high in black pepper and moderate in chili and fennel. As for the less significant species, the occurrence of *A. tamarii* is very high in chili and high in nutmeg, and the occurrence of *A. parasiticus* is high in black pepper, ginger, and chili; moderate in cloves; and low in

fennel. The other *Aspergillus* species and data supported by less than five studies are shown in more detail in Table 9.

Table 8. Fungi: Natural occurrence of *Aspergillus*, *Penicillium* and *Fusarium* genera in spices in the last 5 years (since 2015).

Microfungi/Spice	*Aspergillus* spp.			*Penicillium* spp.			*Fusarium* spp.			Reference
	Positive [a] (%)		n [b]	Positive (%)		n	Positive (%)		n	
Anise	●	100	3	●	100	2	◌	50.0	2	[109,111,144]
Basil	●	100	1	●	100	1	×	0.0	1	[109]
Bay leaf	●	100	2	●	100	2	◌	50.0	2	[109,145]
Caraway	●	100	6	●	80.0	5	○	20.0	5	[8,109,111,144–146]
Cardamom	●	83.3	6	◌	50.0	6	◌	33.3	6	[109,119,126,127,145,146]
Chili	●	100	15	◍	66.7	9	●	100	6	[8,106,109,111,113,131,132,144,146–151]
Cinnamon	◌	50.0	8	◌	50.0	6	×	0.0	5	[109,111,127,131,144–146,149]
Cloves	◌	37.5	8	○	14.3	7	×	0.0	4	[109,127,131,145–149]
Coriander	●	100	6	◍	60.0	5	×	0.0	5	[8,109,111,144–146]
Cumin	●	80.0	5	◍	75.0	4	◌	50.0	4	[8,109,111,144,146]
Cumin, black	●	100	2	◌	50.0	2	◌	50.0	2	[109,145]
Curry	◍	75.0	4	×	0.0	4	×	0.0	2	[144,146–148]
Fennel	●	100	8	◌	50.0	6	◍	60.0	5	[8,109,111,126,127,131,145,149]
Fenugreek	●	100	3	◍	66.7	3	◌	33.3	3	[8,109,146]
Garlic	●	100	3	×	0.0	3	×	0.0	1	[109,147,148]
Ginger	●	100	7	◌	33.3	6	◌	50.0	4	[8,109,111,144,146–148]
Licorice	●	100	1	●	100	1	×	0.0	1	[109]
Mace	●	100	1	●	100	1	●	100	1	[126]
Marjoram	●	100	1	●	100	1	×	0.0	1	[109]
Mint	●	100	1	●	100	1	×	0.0	1	[109]
Mustard	◍	66.7	3	◍	66.7	3	×	0.0	3	[109,127,146]
Nutmeg	●	90.0	10	◍	60.0	10	×	0.0	4	[105,109,127,144,146–148,152–154]
Oregano	●	100	2	●	100	1	-	-	0	[131,149]
Paprika	●	100	2	●	100	1	●	100	1	[107,111]
Parsley	●	100	1	●	100	1	×	0.0	1	[109]
Pepper, black	●	91.7	12	◍	75.0	8	◌	33.3	6	[8,109,111,118,126,127,131,144,146,149,151,155]
Pepper, white	●	100	6	◌	50.0	4	×	0.0	2	[118,131,144,146,149,151]
Rosemary	●	100	3	◌	50.0	2	×	0.0	1	[109,131,149]
Saffron	◍	66.7	3	◌	50.0	2	×	0.0	2	[109,111,146]
Star anise	×	0.0	1	×	0.0	1	×	0.0	1	[127]
Sumac	◌	50.0	2	×	0.0	2	◌	50.0	2	[109,145]
Thyme	●	100	3	◌	33.3	3	●	100	1	[109,147,148]
Turmeric	●	100	5	●	80.0	5	◍	60.0	5	[8,109,126,144,146]

Notes: [a] Positive = the percentage of studies with at least one related spice sample positive on related mold; [b] n = number of studies concerning related spice and mold; × = none occurrence (0%); ☆ = rare occurrence (up to 5%); ○ = low occurrence (up to 25%); ◌ = moderate occurrence (up to 50%); ◍ = high occurrence (up to 75%); ● = very high occurrence (more than 75%).

Table 9. Fungi: Natural occurrence of *Aspergillus* species in spices in the last 5 years (since 2015).

Microfungi/Spice	*A. flavus*			*A. parasiticus*			*A. niger*			*A. tamari*			*A. terreus*			*A. versicolor*			*A. ochraceus*			*A. carbonarius*			Reference
	Positive [a]	(%)	n [b]	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	
Anise	×	0.0	1	×	0.0	1	●	100	1	◌	50.0	2	×	0.0	1	×	0.0	1	●	100	1	-	-	0	[109,144]
Basil	×	0.0	1	×	0.0	1	●	100	1	×	0.0	1	×	0.0	1	●	100	1	●	100	1	-	-	0	[109]
Bay leaf	◌	50.0	2	×	0.0	1	●	100	2	×	0.0	1	●	100	1	×	0.0	1	●	100	1	-	-	0	[109,145]
Caraway	◌	33.3	3	×	0.0	3	●	100	4	×	0.0	3	◌	50.0	2	×	0.0	2	◌	50.0	2	-	-	0	[8,109,144–146]
Cardamom	●	83.3	6	◌	50.0	4	●	100	6	×	0.0	1	◌	33.3	3	◌	50.0	2	◍	66.7	3	-	-	0	[109,119,126,127,145,146]
Chili	●	90.0	10	◍	55.6	9	●	90.9	11	●	80.0	5	◌	50.0	4	◍	75.0	4	◌	40.0	5	◌	50.0	4	[8,109,113,131,132,144,146–151]
Cinnamon	◌	33.3	6	○	25.0	4	◍	66.7	6	◌	33.3	3	×	0.0	2	×	0.0	2	◍	66.7	3	×	0.0	2	[109,127,131,144–146,149]
Cloves	◌	37.5	8	◌	33.3	6	○	25.0	8	×	0.0	2	×	0.0	2	×	0.0	3	×	0.0	3	×	0.0	2	[109,127,131,145–149]
Coriander	◌	33.3	3	◌	33.3	3	◍	75.0	4	×	0.0	3	×	0.0	2	×	0.0	2	×	0.0	2	-	-	0	[8,109,144–146]
Cumin	◌	50.0	2	×	0.0	3	◍	66.7	3	◌	33.3	3	◌	50.0	2	◌	50.0	2	●	100	2	-	-	0	[8,109,144,146]
Cumin, black	◌	50.0	2	×	0.0	1	●	100	2	×	0.0	1	×	0.0	1	●	100	1	×	0.0	1	-	-	0	[109,145]
Curry	◌	33.3	3	◌	33.3	3	◌	33.3	3	●	100	1	-	-	0	×	0.0	1	-	-	0	-	-	0	[144,146–148]
Fennel	◍	66.7	6	○	20.0	5	●	85.7	7	×	0.0	3	◍	75.0	4	○	25.0	4	◌	40.0	5	×	0.0	2	[8,109,126,127,131,145,149]
Fenugreek	◌	50.0	2	×	0.0	3	●	100	3	×	0.0	2	×	0.0	2	×	0.0	2	◌	50.0	2	-	-	0	[8,109,146]
Garlic	◌	33.3	3	◍	66.7	3	◍	66.7	3	×	0.0	1	×	0.0	1	◌	50.0	2	×	0.0	1	-	-	0	[109,147,148]
Ginger	◍	75.0	4	◍	60.0	5	◍	60.0	5	×	0.0	3	◌	50.0	2	◌	33.3	3	◌	50.0	2	-	-	0	[8,109,144,146–148]
Licorice	●	100	1	×	0.0	1	●	100	1	●	100	1	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Mace	●	100	1	●	100	1	●	100	1	-	-	0	×	0.0	1	●	100	1	×	0.0	1	-	-	0	[126]
Marjoram	×	0.0	1	×	0.0	1	●	100	1	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Mint	×	0.0	1	×	0.0	1	●	100	1	×	0.0	1	×	0.0	1	●	100	1	×	0.0	1	-	-	0	[109]
Mustard	×	0.0	3	◌	50.0	2	◌	33.3	3	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109,127,146]
Nutmeg	◍	66.7	9	◍	75.0	4	◍	55.6	9	◍	60.0	5	×	0.0	1	◌	50.0	4	◍	66.7	3	-	-	0	[105,109,127,144,146–148,152–154]
Oregano	●	100	2	×	0.0	2	●	100	2	●	100	1	●	100	1	●	100	1	●	100	2	×	0.0	2	[131,149]
Parsley	×	0.0	1	×	0.0	1	●	100	1	●	100	1	×	0.0	1	×	0.0	1	●	100	1	-	-	0	[109]
Pepper, black	●	88.9	9	◍	75.0	8	●	88.9	9	◌	50.0	4	◌	50.0	4	●	100	4	●	100	5	●	100	2	[8,109,118,126,127,131,144,146,149,151,155]
Pepper, white	●	80.0	5	◍	75.0	4	●	100	4	●	100	2	●	100	1	●	100	1	×	0.0	2	×	0.0	2	[118,131,144,146,149,151]
Rosemary	◍	66.7	3	◍	66.7	3	●	100	3	×	0.0	2	×	0.0	2	◌	50.0	2	×	0.0	3	×	0.0	2	[109,131,149]
Saffron	×	0.0	2	×	0.0	2	◌	50.0	2	×	0.0	1	×	0.0	1	●	100	1	×	0.0	1	-	-	0	[109,146]
Star anise	×	0.0	1	-	-	0	×	0.0	1	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[127]
Sumac	◌	50.0	2	×	0.0	1	×	0.0	2	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109,145]
Thyme	●	100	3	◍	66.7	3	◌	33.3	3	×	0.0	1	×	0.0	1	◌	50.0	2	●	100	1	-	-	0	[109,147,148]
Turmeric	◌	33.3	3	◌	50.0	4	◌	50.0	4	×	0.0	3	×	0.0	3	×	0.0	3	●	100	3	-	-	0	[8,109,126,144,146]

Notes: [a] Positive = the percentage of studies with at least one related spice sample positive on related mold; [b] n = number of studies concerning related spice and mold; × = none occurrence (0%); ☆ = rare occurrence (up to 5%); ○ = low occurrence (up to 25%); ◌ = moderate occurrence (up to 50%); ◍ = high occurrence (up to 75%); ● = very high occurrence (more than 75%)

Table 10. Fungi: Natural occurrence of *Penicillium*, *Fusarium*, *Alternaria*, and *Rhizopus* species in spices in the last 5 years (since 2015).

Microfungi/Spice	*Penicillium citrinum*			*Penicillium verrucosum*			*Fusarium verticillioides*			*Alternaria alternata*			*Rhizopus nigricans*			*Rhizopus oryzae*			Reference
	Positive [a]	(%)	n [b]	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	Positive	(%)	n	
Anise	×	0.0	1	-	-	0	×	0.0	1	●	100	1	×	0.0	1	-	-	0	[109]
Basil	×	0.0	1	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Bay leaf	×	0.0	1	-	-	0	×	0.0	1	×	0.0	2	×	0.0	1	●	100	1	[109,145]
Caraway	◌	50.0	2	×	0.0	1	×	0.0	2	×	0.0	3	×	0.0	2	×	0.0	2	[8,109,145]
Cardamom	◌	50.0	4	●	100	2	◌	50.0	2	◍	66.7	3	×	0.0	2	●	100	3	[109,119,126,127,145]
Chili	◌	33.3	6	●	100	1	●	100	2	◍	66.7	3	×	0.0	2	●	100	1	[8,109,132,147–149]
Cinnamon	×	0.0	3	-	-	0	×	0.0	1	×	0.0	2	×	0.0	1	×	0.0	1	[109,127,145,149]
Cloves	×	0.0	5	-	-	0	×	0.0	1	×	0.0	2	×	0.0	1	×	0.0	1	[109,127,145,147–149]
Coriander	◌	50.0	2	●	100	1	×	0.0	2	◌	33.3	3	◌	50.0	2	◌	50.0	2	[8,109,145]
Cumin	◌	50.0	2	×	0.0	1	×	0.0	2	◌	50.0	2	●	100	2	●	100	1	[8,109]
Cumin, black	×	0.0	1	-	-	0	×	0.0	1	×	0.0	2	×	0.0	1	×	0.0	1	[109,145]
Curry	×	0.0	2	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[147,148]
Fennel	○	20.0	5	×	0.0	2	◍	66.7	3	×	0.0	3	×	0.0	3	◍	66.7	3	[8,109,126,127,145,149]
Fenugreek	◌	50.0	2	●	100	1	◌	50.0	2	◌	50.0	2	×	0.0	2	●	100	1	[8,109]
Garlic	×	0.0	3	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109,147,148]
Ginger	○	25.0	4	●	100	1	◌	50.0	2	×	0.0	2	×	0.0	2	●	100	1	[8,109,147,148]
Licorice	×	0.0	1	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Mace	●	100	1	●	100	1	●	100	1	-	-	0	●	100	1	●	100	1	[126]
Marjoram	●	100	1	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Mint	×	0.0	1	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Mustard	◌	50.0	2	-	-	0	×	0.0	1	●	100	1	●	100	1	-	-	0	[109,127]
Nutmeg	◌	50.0	8	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[105,109,127,147,148,152–154]
Oregano	×	0.0	1	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[131,149]
Parsley	×	0.0	1	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Pepper, black	◌	40.0	5	●	100	2	◍	66.7	3	×	0.0	2	◍	66.7	3	●	100	2	[8,109,126,127,149]
Pepper, white	●	100	1	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[149]
Rosemary	◌	50.0	2	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109,149]
Saffron	×	0.0	1	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	-	-	0	[109]
Star anise	×	0.0	1	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[127]
Sumac	×	0.0	1	-	-	0	×	0.0	1	×	0.0	2	×	0.0	1	●	100	1	[109,145]
Thyme	×	0.0	3	-	-	0	●	100	1	×	0.0	1	×	0.0	1	-	-	0	[109,147,148]
Turmeric	●	100	3	●	100	2	◍	66.7	3	×	0.0	2	×	0.0	3	◌	50.0	2	[8,109,126]

Notes: [a] Positive = the percentage of studies with at least one related spice sample positive on related mold; [b] n = number of studies concerning related spice and mold; × = none occurrence (0%); ☆ = rare occurrence (up to 5%); ○ = low occurrence (up to 25%); ◌ = moderate occurrence (up to 50%); ◍ = high occurrence (up to 75%); ● = very high occurrence (more than 75%).

6.2.2. *Penicillium* Species

Compared to *Aspergillus* spp., the occurrence of *Penicillium* spp. is slightly lower but overall, still significant, as it has been very high in caraway and turmeric; high in black pepper, chili, nutmeg, and coriander; moderate in cardamom, cinnamon, fennel, and ginger; and low in cloves.

Based on all included studies, *Penicillium* spp. was isolated from all spices included in the present review except for four cases: curry, garlic, star anise, and sumac—see Table 8.

The two *Penicillium* species considered in this review were *P. citrinum* and *P. verrucosum*, among which the second mentioned is studied rather rarely but in most cases came out positive. The occurrence of *P. citrinum* is moderate in nutmeg, black pepper, and chili; low in fennel; and none in cloves—see Table 10.

6.2.3. *Fusarium* Species

Fusarium spp. occurs in spice substantially less than *Aspergillus* spp. or *Penicillium* spp., although its occurrence is still very high in chili, then high in fennel and turmeric, moderate in cardamom and black pepper, and low in caraway. Apart from oregano which has not been tested for *Fusarium* spp. and again considering all included studies, this genus has been confirmed in 16 out of 32 involved spices—see Table 8. *F. verticillioides* (= *F. moniliforme)* is very little studied with only 1-3 relevant studies per spice. It was confirmed to occur in at least one case in cardamom, fennel, fenugreek, ginger, chili, mace, black pepper, thyme, and turmeric—see Table 10.

6.2.4. Other Microfungi (*Alternaria alternata*, *Rhizopus nigricans* and *Rhizopus oryzae*)

Only a few publications deal with *Alternaria alternata* and *Rhizopus nigricans* and even fewer with *R. oryzae* in spices; therefore, it is not possible to summarize them based on the previously established threshold of five studies. All three have been confirmed to appear in cumin and coriander. In addition, *A. alternata* has been found in anise, cardamom, fenugreek, chili, and mustard; *R. nigricans* in mace, mustard, and black pepper; and *R. oryzae* in bay leaf, cardamom, fennel, fenugreek, ginger, chili, mace, black pepper, sumac, and turmeric—see Table 10.

7. Mycotoxin Levels in Spices in Relation to European Legislation

The concentrations of AFs and/or OTA in spices often exceeded the maximum permissible limit (MPL) set by EU legislation in involved studies where MPL for AFs and AFB_1 were exceeded more often than in case of OTA. Chili and paprika (*Capsicum* spp.) seem to be the most problematic spices. Aflatoxin concentrations exceeded MPL in 10 of 12 studies (83.3%) and 3 of 3 studies (100%) for total AFs and 13 of 18 studies (72.2%) and 2 of 3 studies (66.7%) for AFB_1, respectively. In the case of OTA, MPL was exceeded by 50.0% for both chili (6/12) and paprika (2/4). Nutmeg seems to be also problematic, as its concentration exceeded MPL in 3 of 4 studies (75.0%) for total AFs and 2 of 3 studies (66.7%) for OTA. However, the concentration of AFB_1 exceeded MPL only in one of 6 studies (16.7%). On the contrary, in the case of white pepper, MPL was exceeded in a single study dealing with total AFs (1/4, 25%) and was not exceeded in any of 3 studies concerning AFB_1 and 4 studies concerning OTA—see Table 11.

Table 11. Summary of studies in which above-the-limit values of mycotoxins have been recorded in relation to the European Union legislation.

Mycotoxin/Spice	AFB_1					Total AFs					OTA					Reference
	Positive		Over MPL [a]		n_T [c]	Positive		Over MPL		n_T	Positive		Over MPL		n_T	
	%	n [b]	%	n [b]		%	n	%	n		%	n	%	n		
Pepper, black	58.3	7	◔ 41.7	5	12	60.0	6	◔ 30.0	3	10	66.7	8	○ 25.0	3	12	[8,9,35,108–112,116–118,120–123,125–127,129–131]
Pepper, white	33.3	1	× 0.0	0	3	50.0	2	○ 25.0	1	4	25.0	1	× 0.0	0	4	[9,35,112,125,131]
Nutmeg	33.3	2	○ 16.7	1	6	75.0	3	◕ 75.0	3	4	100	3	◕ 66.7	2	3	[9,105,109,120,123,127,135,152,153]
Ginger	100	7	◔ 42.9	3	7	66.7	4	◔ 33.3	2	6	83.3	5	○ 16.7	1	6	[8,9,109–111,117,120,122,130,139,140,148]
Turmeric	83.3	5	◔ 50.0	3	6	75.0	6	◔ 37.5	3	8	100	5	◔ 40.0	2	5	[8,9,109,110,112,116,121,122,125,126]
Chili	94.4	17	◕ 72.2	13	18	91.7	11	● 83.3	10	12	83.3	10	◔ 50.0	6	12	[8,9,106,108,110–118,120–122,128–133,135,136]
Paprika	100	3	◕ 66.7	2	3	100	3	● 100	3	3	100	4	◔ 50.0	2	4	[9,107,111,120,133]
Licorice	50.0	1	no MPL		2	50.0	1	no MPL		2	100	2	◔ 50.0	1	2	[109,120,141]

Notes: [a] MPL = maximum permissible limit; [b] n = number of studies; [c] n_T = total number of publications related to mycotoxins in spice, with mean or maximum value available or with no mycotoxin occurrence; × = none over-MPL occurrence (0%); ☆ = rare over-MPL occurrence (up to 5%); ○ = low over-MPL occurrence (up to 25%); ◔ = moderate over-MPL occurrence (up to 50%); ◕ = high over-MPL occurrence (up to 75%); ● = very high over-MPL occurrence (more than 75%).

8. Mycotoxins in Spices Based on RASFF

Based on Rapid Alert System for Food and Feed (RASFF) database from the last five years (2015-2019), in terms of several of mycotoxin notifications, the category "Herbs and spices" ranks third after categories "Nuts, nut products and seeds" and "Fruits and vegetables". A total of 219 (80.2%) and 54 (19.8%) mycotoxin notifications relate to AFs and OTA in spices respectively, with 18 of the notifications concerning both. More than a half (51.3%) of the notifications include chilies (powdered, whole, and crushed), followed by nutmeg (20.5%). Each of the other spices, such as berbere spice, sweet powder, ginger, pepper, curry, and turmeric, represents less than 5% and cumin and mace even less than 1%. The most notifications originated in India (38.5%), far followed by Indonesia (13.6%), Ethiopia (11.7%), Sri Lanka (9.9%), Pakistan (5.9%), China (4.0%), and Nigeria (1.8%) and other countries—see Figure 2 [156]. Some of the highest values of aflatoxin contamination are shown in Table 12 [156].

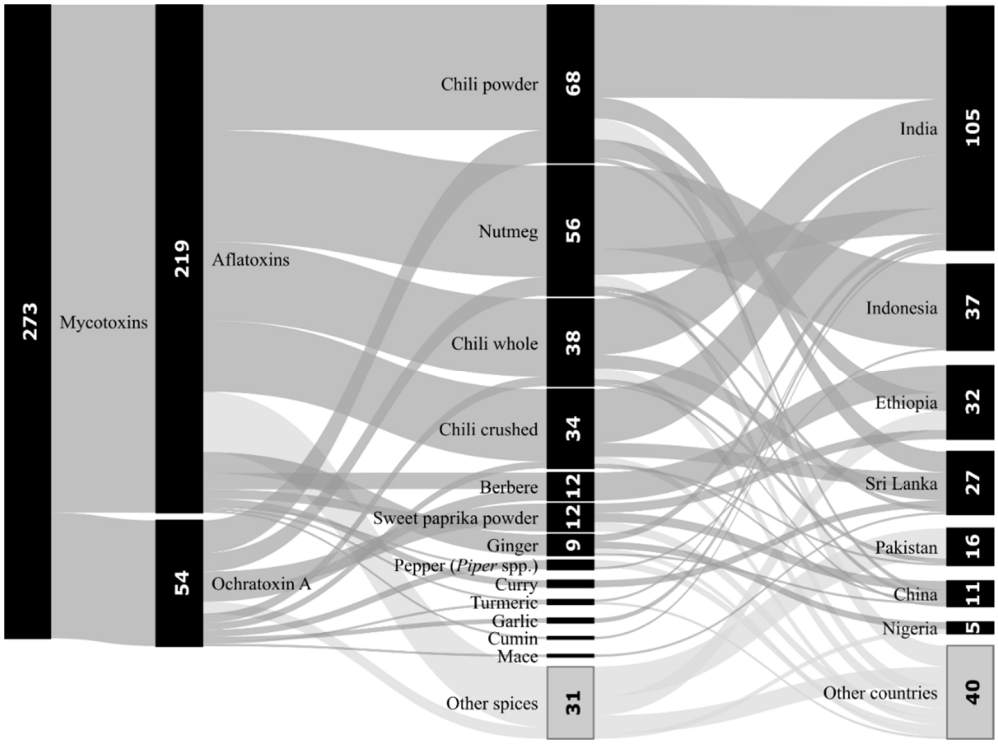


Figure 2. Notifications of aflatoxins and ochratoxin A in spices by the Rapid Alert System for Food and Feed (RASFF) in 2015–2019. Notes: Number of notifications in brackets: Pepper, *Piper* spp. (4), Curry (3), Turmeric (2), Garlic (2), Cumin (1), and Mace (1). The category "Other spices" includes fasika spice, kebab spice, suya pepper, and other various spice mixtures. The category "Other countries" includes all countries with less than 5 mycotoxin notifications for spices: Bangladesh, Croatia, France, Germany, Ghana, Grenada, Hong Kong, Italy, Kosovo, Kuwait, Lebanon, Malawi, Netherlands, Peru, Spain, Thailand, Turkey, United Kingdom, and Vietnam. Processed according to RASFF [156].

Table 12. Some of the highest values of aflatoxin B_1 and total aflatoxins contamination in spices, based on the RASFF database in 2015–2019.

No.	Origin	Spice	Maximum Level of AFB_1 (μg/kg)	Maximum Level of Total AFs [a] (μg/kg)	Classification [b]	Date of Case
1	Nigeria	Suya pepper	300.00	360.00	I	15/02/2017
2	Indonesia, Sri Lanka [c]	Nutmeg	180.00	210.60	BR, BR [c]	21/09/2015, 27/01/2016 [c]
3	Malawi	Chilies	96.20	116.00	A	29/08/2017
4	Ghana, Ghana [c]	Kebab spice	93.40	112.30	A, BR [c]	14/07/2015, 12/10/2016 [c]
5	Ethiopia	Paprika powder	73.44	239.57	BR	19/01/2016
6	Ethiopia	Berbere spice	35.00	91.00	BR	13/05/2016
7	Sri Lanka	Curry powder	34.30	36.50	A	25/01/2018
8	Netherlands	White pepper	23.90	54.70	A	09/12/2015
9	Nigeria	Ginger	22.70	48.70	A	06/04/2017
10	India	Turmeric powder	14.80	16.30	A	05/01/2017
11	India	Cayenne pepper	11.10	11.60	BR	19/07/2019
12	India	Ground cumin	8.82	12.19	A	02/08/2019

Notes: [a] Total AFs = sum of aflatoxins B_1, B_2, G_1, and G_2; [b] I = Information, BR = Border rejection, A = alert; [c] comma-separated data correspond to value of AFB_1 and total AFs, respectively, in case of data originated from separate notifications. Processed according to RASFF [156].

9. Discussion

Mycotoxins in spices are quite often notified by the RASFF. Unfortunately, the RASFF data can be difficult to grasp due to occasional data inconsistency—e.g., inconsistent data format, missing unit, and inconsistent use of decimal point and comma (possibly leading to misunderstanding decimal for thousands separator). Data containing one of these ambiguities could not be included in overall data analysis due to possible distortion of the results—however, the amount of omitted data was not significant. Obviously, the frequency of notifications alone is not conclusive, as it is directly affected by the volume of production of the spice. Unfortunately, the worldwide production could not be carried out for each individual spice, since the FAOSTAT data of certain kinds of spices are grouped—e.g., in the group of four single spices "*Anise, badian, fennel, and coriander*" or the group of three single spices "*Nutmeg, mace, and cardamoms*".

As evident from the studies included in this review, AFs and OTA are the most-commonly researched mycotoxins in spices, especially in chili and black pepper. However, for a better summary of all data, complete and accurate data (mainly concentration ranges, percentages of positive samples, and total numbers of analyzed samples) are needed, some of which are often lacking in many publications. Due to missing data, several publications in this summary had to be omitted.

In general, most spices appear to be prone to fungal infection and thus potentially mycotoxin contamination. Paprika, chili pepper, black pepper, white pepper, ginger, and turmeric seem to be one of the most critical in terms of mycotoxin contamination—often contaminated with all AFs and OTA. In addition, licorice is usually not contaminated with AFs, but quite often with OTA. All above-mentioned mycotoxins in spices are handled in EU legislation; however, many other spices are often contaminated not only with both AFs and OTA but even with other mycotoxins. None of those other spices and mycotoxins are handled in the EU legislation. Among others, e.g., AFs and OTA mainly in cardamom, mace, fenugreek, and other spices and CIT in chili, ginger, coriander, or fenugreek and *Fusarium* mycotoxins in paprika, onion, or chili pepper are all quite common; however, they are not addressed in the EU legislation.

Most original papers deal with spices that are already infamous for their mycotoxin contamination, namely, chilies and black pepper. Although these spices indeed appear to be the most crucial in terms of spice-related human mycotoxin exposure, and their analysis can obviously be expected to produce highly positive results, there are many other important spices. Of course, regional spices such as dawadawa can also be of a big concern in a given region and deserve no less attention than the major ones.

On the contrary, certain spices appear to be either resistant to fungal infection or possess the ability to inhibit mycotoxin production. In this review, these spices mainly include basil, cloves, mint, oregano, and thyme, which are only very rarely contaminated with any mycotoxins. Cases of uncontaminated spices remain in the background and are not discussed in the literature to a greater extent. Usually, these spices are only mentioned in relation to their essential oils, which can supposedly inhibit fungal activity and could possibly be the cause of those certain spices being contaminated rarely.

The essential oils of oregano, basil, and sage with their major compounds, thymol, methyl-cavicol, and thujone, respectively, supposedly inhibit *A. ochraceus* growth and its OTA production [157], so do essential oils of cinnamon, thyme, cloves, caraway, and anise [158,159]. On the contrary, oils of mint and oregano with major compounds menthol and linalool were reported to have no important inhibitory effect on the growth [157]. In this review, an inhibitory effect on the fungal growth can be partially confirmed in the case of coriander, cloves, and mint in which *A. ochraceus* was not detected in any of involved studies, while its presence was confirmed in case of oregano, basil, cinnamon, thyme, caraway, and anise. No data were available for confirming this effect for sage.

Similarly, essential oils of cinnamon and cloves (major compounds cinnamic aldehyde and eugenol, respectively [157]) and also thyme, mint, basil, caraway, and anise (thymol, menthon, methyl-chavicol, anithol) supposedly inhibit the growth of *A. parasiticus* [157,159]. In this review, the inhibitory effect can be supported in the case of caraway, mint, basil, and anise, where *A. parasiticus* was not detected.

Its occurrence was relatively low in the case of cinnamon, which may be due to the inhibitory effect of the essential oil. However, in the case of both thyme and cloves, this inhibitory effect cannot be confirmed.

The inhibitory effect on the growth of *A. flavus* and *F. verticillioides* was reported in the case of essential oils of thyme, cinnamon, mint, basil, caraway, and anise [159]. In this review, the inhibitory effect can be supported only in the case of mint, basil, and anise for *A. flavus* and in the case of cinnamon, mint, basil, anise, and caraway for *F. verticillioides*, as the respective mold was not detected in the mentioned spices.

In addition, in case of cloves, thyme, and oregano, the occurrence of OTA and all AFs was mostly none or rare, although the occurrence of various fungi of *Aspergillus* and *Penicillium* genera has been confirmed, which may indicate the inhibitory effect on the mycotoxin production rather than the growth of the fungi.

10. Conclusions

Mycotoxins are considered potent pathogens. Some of them are highly carcinogenic. Food is the main source of mycotoxins in human body. Spices are a small but integral part of the diet of all people in the world. Therefore, spices are certainly not the main source of the supply of mycotoxins to the human body, but they can contribute to a considerable extent through continuous consumption. The control of mycotoxins in spices is a constantly evolving process, and the obtained data are very important not only for the realization of the dietary exposure to mycotoxins and health risk assessment but also for setting relevant legislation. AFs (mainly AFB_1) and OTA are the most common mycotoxins in spices. However, compared to AFs and OTA, other mycotoxins have been insufficiently studied in spices, and thus, their share in the supply of mycotoxins is difficult to evaluate under the existing data. Among *Alternaria* mycotoxins, an honorable mention belongs to TEA due to its high incidence. Unfortunately, this fact is supported by very few studies. Even less data have been available in the case of CIT and *Fusarium* mycotoxins. As for microfungi, the most common species isolated from spices belong to *Aspergillus* and *Penicillium* and less to *Fusarium* genera. *A. niger* and *A. flavus* are considered to be dominant species isolated from the spice, followed by *A. ochraceus* and *P. citrinum*.

Based on the EU RASFF data, chili, nutmeg, and paprika powder have been the most problematic spices in terms of the frequency of exceeding maximum EU limits. Data from original papers, on which this review has been based, only confirm this conclusion. However, AFs, OTA, and also other mycotoxins have been proven to be present in relatively high amounts in many other spices as well.

This review documents and emphasizes the importance of further monitoring of mycotoxins in common as well as less common spices. As proven, many spices are neglected in terms of mycotoxin monitoring but also have the potential for high contamination. Similarly, many mycotoxins are insufficiently monitored in spices, although their presence has been proven in this review. Given the findings from the included studies, it seems that the current legislation is rather incomplete, and inclusion of both less-common spices and less-common mycotoxins should be considered. It is therefore justified to advise authors to provide complete statistical data or full datasets in their studies, which may potentially be useful for setting new limits. Moreover, there is a need for regulation to be harmonized from country to country, depending on local dietary habits and needs. Nevertheless, more studies are needed to fix these maximum limits. Last but not least, it is also urgent to increase consumer awareness of the risk posed by mycotoxins in spices and their potential health impact.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/12/12/789/s1. Table S1: Studies positivity: Natural occurrence of mycotoxins produced by *Aspergillus* and *Penicillium* species in spices in the last 5 years (since 2015). Table S2: Studies positivity: Natural occurrence of *Fusarium* mycotoxins in spices in the last 5 years (since 2015). Table S3: Studies positivity: Natural occurrence of *Alternaria* mycotoxins in spices in the last 5 years (since 2015).

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References

1. Marroquín-Cardona, A.G.; Johnson, N.M.; Phillips, T.D.; Hayes, A.W. Mycotoxins in a changing global environment—A review. *Food Chem. Toxicol.* **2014**, *69*, 220–230. [CrossRef]
2. Botana, L.M.; Sainz, M.J. (Eds.) *Climate Change and Mycotoxins*; Walter de Gruyter GmbH: Berlin, Germany, 2015; ISBN 978-3-11-033305-3.
3. Kabak, B.; Dobson, A.D. Mycotoxins in spices and herbs–An update. *Crit. Rev. Food Sci. Nutr.* **2017**, *57*, 18–34. [CrossRef] [PubMed]
4. Uhl, S.R. *Handbook of Spices, Seasonings, and Flavorings*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2006; ISBN 978-1-4200-0436-6.
5. Chomchalow, N. Spice Production in Asia—An Overview. *AU J. Technol.* **2001**, *5*, 1–14.
6. Abd El-Tawab, A.A.; El-Diasty, E.M.; Khater, D.F.; Al-baaly, Y.M. Mycological identification of some fungi isolated from meat products and spices with molecular identification of some *Penicillium* isolates. *Adv. Anim. Vet. Sci.* **2020**, *8*, 124–129. [CrossRef]
7. Iha, M.H.; Trucksess, M.W. Management of mycotoxins in spices. *J. AOAC Int.* **2019**, *102*, 1732–1739. [CrossRef]
8. Jeswal, P.; Kumar, D. Mycobiota and natural incidence of aflatoxins, ochratoxin A, and citrinin in Indian spices confirmed by LC-MS/MS. *Int. J. Microbiol.* **2015**, *2015*, 242486. [CrossRef]
9. El Darra, N.; Gambacorta, L.; Solfrizzo, M. Multimycotoxins occurrence in spices and herbs commercialized in Lebanon. *Food Control* **2019**, *95*, 63–70. [CrossRef]
10. Gambacorta, L.; El Darra, N.; Fakhoury, R.; Logrieco, A.F.; Solfrizzo, M. Incidence and levels of *Alternaria* mycotoxins in spices and herbs produced worldwide and commercialized in Lebanon. *Food Control* **2019**, *106*, 106724. [CrossRef]
11. Jalili, M.; Jinap, S. Natural occurrence of aflatoxins and ochratoxin A in commercial dried chili. *Food Control* **2012**, *24*, 160–164. [CrossRef]
12. Winter, G.; Pereg, L. A review on the relation between soil and mycotoxins: Effect of aflatoxin on field, food and finance. *Eur. J. Soil Sci.* **2019**, *70*, 882–897. [CrossRef]
13. Udomkun, P.; Wiredu, A.N.; Nagle, M.; Müller, J.; Vanlauwe, B.; Bandyopadhyay, R. Innovative technologies to manage aflatoxins in foods and feeds and the profitability of application—A review. *Food Control* **2017**, *76*, 127–138. [CrossRef] [PubMed]
14. Sanatombi, K.; Rajkumari, S. Effect of processing on quality of pepper: A review. *Food Rev. Int.* **2019**, *36*, 626–643. [CrossRef]
15. European Commission. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Off. J. Eur. Union* **2006**, *L364*, 5–24.
16. Yogendrarajah, P.; Van Poucke, C.; De Meulenaer, B.; De Saeger, S. Development and validation of a QuEChERS based liquid chromatography tandem mass spectrometry method for the determination of multiple mycotoxins in spices. *J. Chromatogr. A* **2013**, *1297*, 1–11. [CrossRef]
17. Oguntoyinbo, F.A. Safety challenges associated with traditional foods of West Africa. *Food Rev. Int.* **2014**, *30*, 338–358. [CrossRef]
18. Pfliegler, W.P.; Pócsi, I.; Győri, Z.; Pusztahelyi, T. The *Aspergilli* and their mycotoxins: Metabolic interactions with plants and the soil biota. *Front. Microbiol.* **2020**, *10*, 2921. [CrossRef]
19. Zhang, C.; Selvaraj, J.N.; Yang, Q.; Liu, Y. A survey of aflatoxin-producing *Aspergillus* sp. from peanut field soils in four agroecological zones of China. *Toxins* **2017**, *9*, 40. [CrossRef]
20. Snigdha, M.; Hariprasad, P.; Venkateswaran, G. Transport via xylem and accumulation of aflatoxin in seeds of groundnut plant. *Chemosphere* **2015**, *119*, 524–529. [CrossRef]

21. Siruguri, V.; Bhat, R.V. Assessing intake of spices by pattern of spice use, frequency of consumption and portion size of spices consumed from routinely prepared dishes in southern India. *Nutr. J.* **2015**, *14*, 7. [CrossRef]
22. Shylaja, M.R.; Peter, K.V. The functional role of herbal spices. In *Handbook of Herbs and Spices: Volume 2*; Peter, K.V., Ed.; Woodhead Publishing Limited: Cambridge, UK, 2004; Volume 2, pp. 26–45. ISBN 978-1-85573-721-1.
23. Szűcs, V.; Szabó, E.; Lakner, Z.; Székács, A. National seasoning practices and factors affecting the herb and spice consumption habits in Europe. *Food Control* **2018**, *83*, 147–156. [CrossRef]
24. Pradeep, K.U.; Geervani, P.; Eggum, B.O. Common Indian spices: Nutrient composition, consumption and contribution to dietary value. *Plant Foods Hum. Nutr.* **1993**, *44*, 137–148. [CrossRef] [PubMed]
25. Mathur, P.; Choudhry, M. Consumption pattern of fenugreek seeds in Rajasthani families. *J. Hum. Ecol.* **2009**, *25*, 9–12. [CrossRef]
26. Lu, M.; Yuan, B.; Zeng, M.; Chen, J. Antioxidant capacity and major phenolic compounds of spices commonly consumed in China. *Food Res. Int.* **2011**, *44*, 530–536. [CrossRef]
27. Yin, M.-C.; Cheng, W.-S. Inhibition of *Aspergillus niger* and *Aspergillus flavus* by some herbs and spices. *J. Food Prot.* **1998**, *61*, 123–125. [CrossRef]
28. Tapsell, L.C.; Hemphill, I.; Cobiac, L.; Sullivan, D.R.; Fenech, M.; Patch, C.S.; Roodenrys, S.; Keogh, J.B.; Clifton, P.M.; Williams, P.G. Health benefits of herbs and spices: The past, the present, the future. *Med. J. Aust.* **2006**, *185*, 4–24. [CrossRef]
29. Tantipopipat, S.; Boonpraderm, A.; Charoenkiatkul, S.; Wasantwisut, E.; Winichagoon, P. Dietary intake of spices and herbs in habitual northeast Thai diets. *Malays. J. Nutr.* **2010**, *16*, 137–148.
30. Akeem, S.; Joseph, J.; Kayode, R.; Kolawole, F. Comparative phytochemical analysis and use of some Nigerian spices. *Croat. J. Food Technol. Biotechnol. Nutr.* **2016**, *11*, 145–151.
31. Borquaye, L.S.; Darko, G.; Laryea, M.K.; Gasu, E.N.; Amponsah, N.A.A.; Appiah, E.N. Nutritional and anti-nutrient profiles of some Ghanaian spices. *Cogent Food Agric.* **2017**, *3*, 1348185. [CrossRef]
32. Nwinuka, N.M.; Ibeh, G.O.; Ekeke, G.I. Proximate composition and levels of some toxicants in four commonly consumed spices. *J. Appl. Sci. Environ. Manag.* **2005**, *9*, 150–155. [CrossRef]
33. Otunola, G.A.; Oloyede, O.B.; Oladiji, A.T.; Afolayan, A.J. Comparative analysis of the chemical composition of three spices–*Allium sativum* L. *Zingiber officinale* Rosc. and *Capsicum frutescens* L. commonly consumed in Nigeria. *Afr. J. Biotechnol.* **2010**, *9*, 6927–6931. [CrossRef]
34. Chilaka, C.A.; De Boevre, M.; Atanda, O.O.; De Saeger, S. Quantification of *Fusarium* mycotoxins in Nigerian traditional beers and spices using a multi-mycotoxin LC-MS/MS method. *Food Control* **2018**, *87*, 203–210. [CrossRef]
35. Nguegwouo, E.; Sone, L.E.; Tchuenchieu, A.; Tene, H.M.; Mounchigam, E.; Njayou, N.F.; Nama, G.M. Ochratoxin A in black pepper, white pepper and clove sold in Yaoundé (Cameroon) markets: Contamination levels and consumers' practices increasing health risk. *Int. J. Food Contam.* **2018**, *5*, 1. [CrossRef]
36. Dalhat, M.H.; Adefolake, F.A.; Musa, M. Nutritional composition and phytochemical analysis of aqueous extract of *Allium cepa* (Onion) and *Allium sativum* (Garlic). *Asian Food Sci. J.* **2018**, *3*, 1–9. [CrossRef] [PubMed]
37. FAOSTAT. Food and Agriculture Organization of the United Nations. Available online: http://www.fao.org/faostat/en/#data/QC/visualize (accessed on 25 February 2020).
38. Bennett, J.W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, *16*, 497–516. [CrossRef]
39. Bhat, R.; Rai, R.V.; Karim, A.A. Mycotoxins in food and feed: Present status and future concerns. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 57–81. [CrossRef]
40. Frisvad, J.C.; Andersen, B.; Samson, R.A. Association of moulds to foods. In *Food Mycology: A Multifaceted Approach to Fungi and Food*; Dijksterhuis, J., Samson, R.A., Eds.; CRC Press: Boca Raton, FL, USA, 2007; pp. 199–239. ISBN 978-0-8493-9818-6.
41. Ismaiel, A.; Papenbrock, J. Mycotoxins: Producing fungi and mechanisms of phytotoxicity. *Agriculture* **2015**, *5*, 492–537. [CrossRef]
42. Frisvad, J.C.; Hubka, V.; Ezekiel, C.N.; Hong, S.-B.; Nováková, A.; Chen, A.J.; Arzanlou, M.; Larsen, T.O.; Sklenář, F.; Mahakarnchanakul, W.; et al. Taxonomy of *Aspergillus* section *Flavi* and their production of aflatoxins, ochratoxins and other mycotoxins. *Stud. Mycol.* **2019**, *93*, 1–63. [CrossRef]
43. Haque, M.A.; Wang, Y.; Shen, Z.; Li, X.; Saleemi, M.K.; He, C. Mycotoxin contamination and control strategy in human domestic animal and poultry: A review. *Microb. Pathog.* **2020**, *142*, 104095. [CrossRef]

44. Ojuri, O.T.; Ezekiel, C.N.; Sulyok, M.; Ezeokoli, O.T.; Oyedele, O.A.; Ayeni, K.I.; Eskola, M.K.; Šarkanj, B.; Hajšlová, J.; Adeleke, R.A.; et al. Assessing the mycotoxicological risk from consumption of complementary foods by infants and young children in Nigeria. *Food Chem. Toxicol.* **2018**, *121*, 37–50. [CrossRef]
45. Selvaraj, J.N.; Wang, Y.; Zhou, L.; Zhao, Y.; Xing, F.; Dai, X.; Liu, Y. Recent mycotoxin survey data and advanced mycotoxin detection techniques reported from China: A review. *Food Addit. Contam. Part A* **2015**, *32*, 440–452. [CrossRef]
46. European Food Safety Authority. Dietary exposure assessment to *Alternaria* toxins in the European population. *EFSA J.* **2016**, *14*, e04654. [CrossRef]
47. PubChem. Available online: https://pubchem.ncbi.nlm.nih.gov/ (accessed on 15 April 2020).
48. Pitt, J.I.; Miller, J.D. A concise history of mycotoxin research. *J. Agric. Food Chem.* **2017**, *65*, 7021–7033. [CrossRef] [PubMed]
49. Arenas-Huertero, F.; Zaragoza-Ojeda, M.; Sánchez-Alarcón, J.; Milić, M.; Šegvić Klarić, M.; Montiel-González, J.M.; Valencia-Quintana, R. Involvement of AhR pathway in toxicity of aflatoxins and other mycotoxins. *Front. Microbiol.* **2019**, *10*, 2347. [CrossRef] [PubMed]
50. Kensler, T.W.; Roebuck, B.D.; Wogan, G.N.; Groopman, J.D. Aflatoxin: A 50-year odyssey of mechanistic and translational toxicology. *Toxicol. Sci.* **2011**, *120*, S28–S48. [CrossRef]
51. Kumar, P.; Mahato, D.K.; Kamle, M.; Mohanta, T.K.; Kang, S.G. Aflatoxins: A global concern for food safety, human health and their management. *Front. Microbiol.* **2017**, *7*, 2170. [CrossRef]
52. Ostry, V.; Malir, F.; Toman, J.; Grosse, Y. Mycotoxins as human carcinogens—The IARC monographs classification. *Mycotoxin Res.* **2017**, *33*, 65–73. [CrossRef]
53. Benkerroum, N. Aflatoxins: Producing-molds, structure, health issues and incidence in Southeast Asian and Sub-Saharan African countries. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1215. [CrossRef]
54. Medina, Á.; Valle-Algarra, F.M.; Mateo, R.; Gimeno-Adelantado, J.V.; Mateo, F.; Jiménez, M. Survey of the mycobiota of Spanish malting barley and evaluation of the mycotoxin producing potential of species of *Alternaria*, *Aspergillus* and *Fusarium*. *Int. J. Food Microbiol.* **2006**, *108*, 196–203. [CrossRef]
55. Varga, J.; Frisvad, J.C.; Samson, R.A. Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. *Stud. Mycol.* **2011**, *69*, 57–80. [CrossRef]
56. Calderari, T.O.; Iamanaka, B.T.; Frisvad, J.C.; Pitt, J.I.; Sartori, D.; Pereira, J.L.; Fungaro, M.H.P.; Taniwaki, M.H. The biodiversity of *Aspergillus* section *Flavi* in Brazil nuts: From rainforest to consumer. *Int. J. Food Microbiol.* **2013**, *160*, 267–272. [CrossRef]
57. Pfohl-Leszkowicz, A.; Manderville, R.A. Ochratoxin A: An overview on toxicity and carcinogenicity in animals and humans. *Mol. Nutr. Food Res.* **2007**, *51*, 61–99. [CrossRef] [PubMed]
58. Shin, H.S.; Lee, H.J.; Pyo, M.C.; Ryu, D.; Lee, K.-W. Ochratoxin A-induced hepatotoxicity through phase I and phase II reactions regulated by AhR in liver cells. *Toxins* **2019**, *11*, 377. [CrossRef] [PubMed]
59. Malir, F.; Ostry, V.; Novotna, E. Toxicity of the mycotoxin ochratoxin A in the light of recent data. *Toxin Rev.* **2013**, *32*, 19–33. [CrossRef]
60. European Food Safety Authority. Risk assessment of ochratoxin A in food. *EFSA J.* **2020**, *18*, 6113. [CrossRef]
61. Ostry, V.; Malir, F.; Ruprich, J. Producers and important dietary sources of ochratoxin A and citrinin. *Toxins* **2013**, *5*, 1574–1586. [CrossRef]
62. Samson, R.A.; Visagie, C.M.; Houbraken, J.; Hong, S.-B.; Hubka, V.; Klaassen, C.H.W.; Perrone, G.; Seifert, K.A.; Susca, A.; Tanney, J.B.; et al. Phylogeny, identification and nomenclature of the genus *Aspergillus*. *Stud. Mycol.* **2014**, *78*, 141–173. [CrossRef]
63. De Oliveira Filho, J.W.G.; Islam, M.T.; Ali, E.S.; Uddin, S.J.; Santos, J.V.O.; De Alencar, M.V.O.B.; Júnior, A.L.G.; Paz, M.F.C.J.; De Brito, M.R.M.; E Sousa, J.M.C.; et al. A comprehensive review on biological properties of citrinin. *Food Chem. Toxicol.* **2017**, *110*, 130–141. [CrossRef]
64. Flajs, D.; Peraica, M. Toxicological properties of citrinin. *Arch. Ind. Hyg. Toxicol.* **2009**, *60*, 457–464. [CrossRef]
65. European Food Safety Authority. Scientific Opinion on the risks for public and animal health related to the presence of citrinin in food and feed. *EFSA J.* **2012**, *10*, 2605. [CrossRef]
66. Broggi, L.E.; González, H.H.L.; Resnik, S.L.; Pacin, A.M. Mycoflora distribution in dry-milled fractions of corn in Argentina. *Cereal Chem.* **2002**, *79*, 741–744. [CrossRef]
67. Blanc, P.J.; Laussac, J.P.; Le Bars, J.; Le Bars, P.; Loret, M.O.; Pareilleux, A.; Prome, D.; Prome, J.C.; Santerre, A.L.; Goma, G. Characterization of monascidin A from *Monascus* as citrinin. *Int. J. Food Microbiol.* **1995**, *27*, 201–213. [CrossRef]

68. Escrivá, L.; Font, G.; Manyes, L. In vivo toxicity studies of *fusarium* mycotoxins in the last decade: A review. *Food Chem. Toxicol.* **2015**, *78*, 185–206. [CrossRef] [PubMed]
69. Kamle, M.; Mahato, D.K.; Devi, S.; Lee, K.E.; Kang, S.G.; Kumar, P. Fumonisins: Impact on agriculture, food, and human health and their management strategies. *Toxins* **2019**, *11*, 328. [CrossRef] [PubMed]
70. Desjardins, A.E. *Fusarium Mycotoxins: Chemistry, Genetics, and Biology*; APS Press: St. Paul, MN, USA, 2006; ISBN 0-89-54-335-6.
71. Logrieco, A.; Visconti, A. *An Overview on Toxigenic Fungi and Mycotoxins in Europe*; Springer: New York, NY, USA, 2004; ISBN 978-1-4020-2645-4.
72. Frisvad, J.C.; Smedsgaard, J.; Samson, R.A.; Larsen, T.O.; Thrane, U. Fumonisin B2 production by *Aspergillus niger*. *J. Agric. Food Chem.* **2007**, *55*, 9727–9732. [CrossRef] [PubMed]
73. Mogensen, J.M.; Frisvad, J.C.; Thrane, U.; Nielsen, K.F. Production of fumonisin B2 and B4 by *Aspergillus niger* on grapes and raisins. *J. Agric. Food Chem.* **2010**, *58*, 954–958. [CrossRef] [PubMed]
74. European Food Safety Authority. Scientific opinion on the risks for animal and public health related to the presence of T-2 and HT-2 toxin in food and feed. *EFSA J.* **2011**, *9*, 2481. [CrossRef]
75. European Food Safety Authority. Risks to human and animal health related to the presence of deoxynivalenol and its acetylated and modified forms in food and feed. *EFSA J.* **2017**, *15*, e04718. [CrossRef]
76. Ostry, V.; Dofkova, M.; Blahova, J.; Malir, F.; Kavrik, R.; Rehurkova, I.; Ruprich, J. Dietary exposure assessment of sum deoxynivalenol forms, sum T-2/HT-2 toxins and zearalenone from cereal-based foods and beer. *Food Chem. Toxicol.* **2020**, *139*, 111280. [CrossRef]
77. Desjardins, A.E.; Proctor, R.H. Molecular biology of *Fusarium* mycotoxins. *Int. J. Food Microbiol.* **2007**, *119*, 47–50. [CrossRef]
78. Frisvad, J.C.; Thrane, U.; Samson, R.A. Mycotoxin producers. In *Food Mycology: A Multifaceted Approach to Fungi and Food*; Dijksterhuis, J., Samson, R.A., Eds.; CRC Press: Boca Raton, FL, USA, 2007; pp. 135–159.
79. European Food Safety Authority. Appropriateness to set a group health-based guidance value for zearalenone and its modified forms. *EFSA J.* **2016**, *14*, 4425. [CrossRef]
80. Bertero, A.; Moretti, A.; Spicer, L.; Caloni, F. *Fusarium* molds and mycotoxins: Potential species-specific effects. *Toxins* **2018**, *10*, 244. [CrossRef] [PubMed]
81. Ostry, V. *Alternaria* mycotoxins: An overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J.* **2008**, *1*, 175–188. [CrossRef]
82. Liu, G.T.; Qian, Y.Z.; Zhang, P.E.; Dong, W.H.; Qi, Y.M.; Guo, H. Etiological role of *Alternaria alternata* in human esophageal cancer. *Chin. Med. J. (Engl.)* **1992**, *105*, 394–400.
83. Logrieco, A.; Bottalico, A.; Mulé, G.; Moretti, A.; Perrone, G. Epidemiology of toxigenic fungi and their associated mycotoxins for some mediterranean crops. *Eur. J. Plant Pathol.* **2003**, *109*, 645–667. [CrossRef]
84. Romero, S.M.; Comerio, R.M.; Larumbe, G.; Ritieni, A.; Vaamonde, G.; Fernández Pinto, V. Toxigenic fungi isolated from dried vine fruits in Argentina. *Int. J. Food Microbiol.* **2005**, *104*, 43–49. [CrossRef] [PubMed]
85. Andersen, B.; Krøger, E.; Roberts, R.G. Chemical and morphological segregation of *Alternaria arborescens*, *A. infectoria* and *A. tenuissima* species-groups. *Mycol. Res.* **2002**, *106*, 170–182. [CrossRef]
86. Andersen, B.; Hansen, M.E.; Smedsgaard, J. Automated and unbiased image analyses as tools in phenotypic classification of small-spored *Alternaria* spp. *Phytopathology* **2005**, *95*, 1021–1029. [CrossRef] [PubMed]
87. European Food Safety Authority. Scientific opinion on the risk for public and animal health related to the presence of sterigmatocystin in food and feed. *EFSA J.* **2013**, *11*, 3254. [CrossRef]
88. Chrevatidis, A. Mycotoxins|Occurrence and determination. In *Encyclopedia of Food Sciences and Nutrition*, 2nd ed.; Caballero, B., Ed.; Academic Press: Oxford, UK, 2003; pp. 4089–4096. ISBN 978-0-12-227055-0.
89. Piontek, M.; Łuszczyńska, K.; Lechów, H. Spergilli on building partitions infested with moulds in residential housing and public utility premises. *Civ. Environ. Eng. Rep.* **2017**, *27*, 91–104. [CrossRef]
90. Rank, C.; Nielsen, K.F.; Larsen, T.O.; Varga, J.; Samson, R.A.; Frisvad, J.C. Distribution of sterigmatocystin in filamentous fungi. *Fungal Biol.* **2011**, *115*, 406–420. [CrossRef]
91. Yu, J.; Chang, P.-K.; Ehrlich, K.C.; Cary, J.W.; Bhatnagar, D.; Cleveland, T.E.; Payne, G.A.; Linz, J.E.; Woloshuk, C.P.; Bennett, J.W. Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* **2004**, *70*, 1253–1262. [CrossRef] [PubMed]

92. Committee on Contaminants in Foods. Report of the 9th Session of the Codex Committee on Contaminants in Foods. 16–20 March 2015. Available online: http://www.fao.org/fao-who-codexalimentarius/sh-proxy/tr/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-735-12%252FREPORT%252520%2528FINAL%2529%252FREP18_CFe.pdf (accessed on 20 April 2020).
93. Committee on Contaminants in Foods. Report of the 12th Session of the Codex Committee on Contaminants in Foods. 12–16 March 2018. Available online: http://www.fao.org/fao-who-codexalimentarius/sh-proxy/en/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-735-12%252FWD%252Fcf12_11e.pdf (accessed on 20 April 2020).
94. Council of the European Communities. Council Regulation (EEC) No 315/93 of 8 February 1993 laying down Community procedures for contaminants in food. *J. Eur. Union* **1993**, *37*, 1–3.
95. European Comission. Commission Regulation (EU) No. 105/2010 of 5 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards ochratoxin A. *Off. J. Eur. Union* **2010**, *L35*, 7–8.
96. European Comission. Commission regulation (EU) No 594/2012 of 5 July 2012 amending Regulation (EC) 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foodstuffs. *Off. J. Eur. Union* **2012**, *176*, 43–45.
97. Ham, H.; Kim, S.; Kim, M.-H.; Lee, S.; Hong, S.K.; Ryu, J.-G.; Lee, T. Mycobiota of ground red pepper and their aflatoxigenic potential. *J. Microbiol.* **2016**, *54*, 832–837. [CrossRef]
98. Food Safety and Standards Authority of India. FSSAI Publishes Guidance Note of Aflatoxins. Available online: https://foodsafetyhelpline.com/fssai-publishes-guidance-note-of-aflatoxins/ (accessed on 20 April 2020).
99. Wu, L.; Zhu, D. *Food Safety in China: A Comprehensive Review*, 1st ed.; CRC Press: Boca Raton, FL, USA, 2014; ISBN 978-1-4822-1833-6.
100. Tao, L. China Consults on GB 2761, 2762 and 29921 for the Maximum Limits of Mycotoxins, Contaminants and Pathogenic Bacteria in Foods. Available online: https://food.chemlinked.com/news/food-news/china-consults-gb-2761-2762-and-29921-maximum-limits-mycotoxins-contaminants-and-pathogenic-bacteria-foods (accessed on 20 April 2020).
101. Taniwaki, M.H.; Pitt, J.I.; Copetti, M.V.; Teixeira, A.A.; Iamanaka, B.T. Understanding mycotoxin contamination across the food chain in Brazil: Challenges and opportunities. *Toxins* **2019**, *11*, 411. [CrossRef]
102. Kolybye, A.C., Jr. *Statement. Hearings before the Subcommittee on Science, Technology and Space of the Committee on Commerce, Science and Transportation, United States Senate, Ninety-fifth Congress, First Session on Toxic Substances, Polybrominated Biphenyls (PBB) Contamination in Michigan*; US Government Printing Office: Washington, DC, USA, 1977.
103. European Commission. Commission Regulation (EU) No. 165/2010 of 26 February 2010 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs as regards aflatoxins. *Off. J. Eur. Union* **2010**, *50*, 8–12.
104. European Comission. Commission regulation (EU) 2015/1137 of 13 July 2015 amending Regulation (EC) No 1881/2006 as regards the maximum level of Ochratoxin A in *Capsicum* spp. spices. *Off. J. Eur. Union* **2015**, *L185*, 11–12.
105. Dharmaputra, O.S.; Ambarwati, S.; Retnowati, I.N.A.; Nurfadila, N. Fungal infection and aflatoxin contamination in stored nutmeg (*Myristica fragrans*) kernels at various stages of delivery chain in North Sulawesi province. *Biotropia* **2016**, *22*, 129–139. [CrossRef]
106. Singh, P.; Cotty, P.J. Aflatoxin contamination of dried red chilies: Contrasts between the United States and Nigeria, two markets differing in regulation enforcement. *Food Control* **2017**, *80*, 374–379. [CrossRef]
107. Gambacorta, L.; Magistà, D.; Perrone, G.; Murgolo, S.; Logrieco, A.F.; Solfrizzo, M. Co-occurrence of toxigenic moulds, aflatoxins, ochratoxin A, *Fusarium* and *Alternaria* mycotoxins in fresh sweet peppers (*Capsicum annuum*) and their processed products. *World Mycotoxin J.* **2018**, *11*, 159–174. [CrossRef]
108. Zahra, N.; Khan, M.; Mehmood, Z.; Saeed, M.; Kalim, I.; Ahmad, I.; Malik, K. Determination of aflatoxins in spices and dried fruits. *J. Sci. Res.* **2018**, *10*, 315–321. [CrossRef]
109. Migahed, F.; Abdel-Gwad, M.; Mohamed, S. Aflatoxigenic fungi associated with some medicinal plants. *Annu. Res. Rev. Biol.* **2017**, *14*, 1–20. [CrossRef]

110. Khazaeli, P.; Mehrabani, M.; Heidari, M.R.; Asadikaram, G.; NAJAFI, M.L. Prevalence of aflatoxin contamination in herbs and spices in different regions of Iran. *Iran. J. Public Health* **2017**, *46*, 1540–1545. [PubMed]
111. Azzoune, N.; Mokrane, S.; Riba, A.; Bouras, N.; Verheecke-Vaessen, C.; Sabaou, N.; Mathieu, F. Contamination of common spices by aflatoxigenic fungi and aflatoxin B1 in Algeria. *Qual. Assur. Saf. Crop. Foods* **2015**, *8*, 137–144. [CrossRef]
112. Ali, N.; Hashim, N.H.; Shuib, N.S. Natural occurrence of aflatoxins and ochratoxin A in processed spices marketed in Malaysia. *Food Addit. Contam. Part Chem. Anal. Control Expo. Risk Assess.* **2015**, *32*, 518–532. [CrossRef]
113. Wikandari, R.; Mayningsih, I.C.; Sari, M.D.P.; Purwandari, F.A.; Setyaningsih, W.; Rahayu, E.S.; Taherzadeh, M.J. Assessment of microbiological quality and mycotoxin in dried chili by morphological identification, molecular detection, and chromatography analysis. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1847. [CrossRef]
114. Alsharif, A.M.A.; Choo, Y.-M.; Tan, G.-H. Detection of five mycotoxins in different food matrices in the Malaysian market by using validated liquid chromatography electrospray ionization triple quadrupole mass spectrometry. *Toxins* **2019**, *11*, 196. [CrossRef]
115. Karaaslan, M.; Arslanğray, Y. Aflatoxins B1, B2, G1, and G2 contamination in ground red peppers commercialized in Sanliurfa, Turkey. *Environ. Monit. Assess.* **2015**, *187*, 184–192. [CrossRef]
116. Jalili, M. Natural occurrence of aflatoxins contamination in commercial spices in Iran. *Iran. J. Health Saf. Environ.* **2016**, *3*, 513–517.
117. Manda, P.; Adanou, K.M.; Ardjouma, D.; Adepo, A.J.B.; Dano, D.S. Occurrence of ochratoxin A in spices commercialized in Abidjan (Côte d'Ivoire). *Mycotoxin Res.* **2016**, *32*, 137–143. [CrossRef]
118. Jacxsens, L.; Yogendrarajaha, P.; Meulenaer, B. Risk assessment of mycotoxins and predictive mycology in Sri Lankan spices: Chilli and pepper. *Procedia Food Sci.* **2016**, *6*, 326–330. [CrossRef]
119. Gherbawy, Y.A.; Shebany, Y.M. Mycobiota, total aflatoxins and ochratoxin A of cardamom pods. *Food Sci. Technol. Res.* **2018**, *24*, 87–96. [CrossRef]
120. Ostry, V.; Malir, F.; Dofkova, M.; Skarkova, J.; Pfohl-Leszkowicz, A.; Ruprich, J. Ochratoxin A dietary exposure of ten population groups in the Czech Republic: Comparison with data over the world. *Toxins* **2015**, *7*, 3608–3635. [CrossRef] [PubMed]
121. Jalili, M. Natural occurrence of ochratoxin A contamination in commercial spices in Tehran. *Nutr. Food Sci. Res.* **2016**, *3*, 25–30. [CrossRef]
122. Abd-Elhaleem, Z.A. Determination of common spices and herbs contamination with aflatoxin in Al Majmaah province. *J. Chem. Biol. Phys. Sci.* **2017**, *8*, 69–77. [CrossRef]
123. Reinholds, I.; Pugajeva, I.; Bavrins, K.; Kuckovska, G.; Bartkevics, V. Mycotoxins, pesticides and toxic metals in commercial spices and herbs. *Food Addit. Contam. Part B* **2016**, *10*, 5–14. [CrossRef] [PubMed]
124. Potortì, A.; Tropea, A.; Turco, V.; Pellizzeri, V.; Belfita, A.; Dugo, G.; Bella, G. Mycotoxins in spices and culinary herbs from Italy and Tunisia. *Nat. Prod. Res.* **2019**, *34*, 167–171. [CrossRef]
125. Naz, N.; Kashif, A.; Kanwal, K.; Khan, A.M.; Abbas, M. Quantitative scrutinization of aflatoxins in different spices from Pakistan. *Int. J. Anal. Chem.* **2016**, *2016*, 4907425. [CrossRef]
126. Jeswal, P.; Kumar, D. Natural occurrence of toxigenic mycoflora and ochratoxin A & aflatoxins in commonly used spices from Bihar state (India). *J. Environ. Sci. Toxicol. Food Technol.* **2015**, *9*, 50–55. [CrossRef]
127. Aiko, V.; Mehta, A. Prevalence of toxigenic fungi in common medicinal herbs and spices in India. *3 Biotech* **2016**, *6*, 159–168. [CrossRef] [PubMed]
128. Aye, C.; Nakagawa, H.; Kushiro, M. Occurrence of aflatoxins in processed chili pepper sold in Myanmar. *JSM Mycotoxins* **2019**, *69*, 9–13. [CrossRef]
129. Barani, A.; Nasiri, Z.; Jarrah, N. Natural occurrence of Aflatoxins in commercial pepper in Iran. *Food Agric. Immunol.* **2016**, *27*, 570–576. [CrossRef]
130. Fofana-Diomande, A.; Kuaou, K.; Narcisse, A.; Sory, T.; Dembele, A. Study of the contamination of some spices from Côte d'Ivoire by mycotoxins (AFB1 and OTA). *J. Chem. Biol. Phys. Sci.* **2019**, *9*, 389–399. [CrossRef]
131. Garcia, M.V.; Mallmann, C.A.; Copetti, M.V. Aflatoxigenic and ochratoxigenic fungi and their mycotoxins in spices marketed in Brazil. *Food Res. Int.* **2018**, *106*, 136–140. [CrossRef]

132. Gherbawy, Y.A.; Shebany, Y.M.; Hussein, M.A.; Maghraby, T.A. Molecular detection of mycobiota and aflatoxin contamination of chili. *Arch. Biol. Sci.* **2015**, *67*, 223–234. [CrossRef]
133. Motloung, L.; De Saeger, S.; De Boevre, M.; Detavernier, C.; Audenaert, K.; Adebo, O.A.; Njobeh, P.B. Study on mycotoxin contamination in South African food spices. *World Mycotoxin J.* **2018**, *11*, 401–409. [CrossRef]
134. Mozaffarinejad, A.S.; Giri, A. The measurement of aflatoxin B1 in chilli and black peppers of Qaemshahr, Iran. *J. Kerman Univ. Med. Sci.* **2015**, *22*, 185–193.
135. Pesavento, G.; Ostuni, M.; Calonico, C.; Rossi, S.; Capei, R.; Lo Nostro, A. Mycotic and aflatoxin contamination in *Myristica fragrans* seeds (nutmeg) and *Capsicum annum* (chilli), packaged in Italy and commercialized worldwide. *J. Prev. Med. Hyg.* **2016**, *57*, E102–E109.
136. Yilmaz, S. The contamination rate of aflatoxins in ground red peppers, dried figs, walnuts without shell and seedless black raisins commercialized in Sakarya City Center, Turkey. *Ital. J. Food Sci.* **2017**, *29*, 591–598. [CrossRef]
137. Kim, S.; Lee, S.; Nam, T.-G.; Seo, D.; Yoo, M. Comparison of a newly developed liquid chromatography with tandem mass spectrometry method and enzyme-linked immunosorbent assay for detection of multiple mycotoxins in red pepper powder. *J. Food Prot.* **2017**, *80*, 1347–1354. [CrossRef] [PubMed]
138. Iqbal, S.Z.; Asi, M.R.; Mehmood, Z.; Mumtaz, A.; Malik, N. Survey of aflatoxins and ochratoxin A in retail market chilies and chili sauce samples. *Food Control* **2017**, *81*, 218–223. [CrossRef]
139. Bisht, D.; Menon, K.R.K. Variation in the occurence of Aflatoxins in various processed forms of dried Ginger. *J. Microbiol. Biotechnol. Food Sci.* **2017**, *7*, 110–112. [CrossRef]
140. Lippolis, V.; Irurhe, B.; Porricelli, A.; Cortese, M.; Schena, R.; Imafidon, T.; Oluwadun, A.; Pascale, M. Natural co-occurrence of aflatoxins and ochratoxin A in ginger (*Zingiber officinale*) from Nigeria. *Food Control* **2016**, *73*, 1061–1067. [CrossRef]
141. Huang, X.; Wang, S.; Mao, D.; Miao, S.; Hu, Q.; Ji, S. Optimized QuEChERS method combined with UHPLC-MS/MS for the simultaneous determination of 15 mycotoxins in liquorice. *J. AOAC Int.* **2018**, *101*, 633–642. [CrossRef]
142. Tonti, S.; Mandrioli, M.; Nipoti, P.; Pisi, A.; Toschi, T.G.; Prodi, A. Detection of fumonisins in fresh and dehydrated commercial garlic. *J. Agric. Food Chem.* **2017**, *65*, 7000–7005. [CrossRef]
143. Gürer, Ü.; Omurtag Korkmaz, B.I.; Dumlu, M.; Omurtag, G. Occurrence of fumonisins B 1 and B 2 in homemade medicinal plants: Exposure assessment in northern Turkey. *Acta Aliment.* **2016**, *45*, 54–60. [CrossRef]
144. Makhlouf, J.; Carvajal-Campos, A.; Querin, A.; Tadrist, S.; Puel, O.; Lorber, S.; Oswald, I.P.; Hamze, M.; Bailly, J.-D.; Bailly, S. Morphologic, molecular and metabolic characterization of *Aspergillus* section *Flavi* in spices marketed in Lebanon. *Sci. Rep.* **2019**, *9*, 5263. [CrossRef]
145. Mezeal, I.A.; Alwaan, N.M. Discovery of Fungi Supplementary with Some Spices Collected from Iraqi Markets. Available online: https://www.semanticscholar.org/paper/Discovery-of-Fungi-Supplementary-with-Some-Spices-Mezeal-Alwaan/06182a58968b8ef306d3be6da96f5f066db4c769 (accessed on 30 March 2020).
146. Temu, G.E. Molecular identification of aspergillus strains and quick detection of aflatoxin from selected common spices in Tanzania. *J. Sci. Res. Rep.* **2016**, *10*, 1–8. [CrossRef]
147. Haruna, M.; Dangora, D.B.; Khan, A.U.; Saleh, A. Mycobiota and aflatoxin contaminations of some spices and condiments sold in Katsina central market, Nigeria. *UMYU J. Microbiol. Res.* **2016**, *1*, 143–151.
148. Haruna, M.; Dangora, D.B.; Khan, A.U. Natural occurrence of fungi and aflatoxin in spices and condiments sold at Kafur market, Katsina State, Nigeria. *Niger. J. Sci. Res.* **2017**, *16*, 720–724.
149. Garcia, M.V.; Parussolo, G.; Moro, C.; Bernardi, A.; Copetti, M.V. Fungi in spices and mycotoxigenic potential of some Aspergilli isolated. *Food Microbiol.* **2018**, *73*, 93–98. [CrossRef] [PubMed]
150. Lema, A.A.; Mudansiru, A.; Alexander, B.A.; Sakinatu, M.J. Evaluation of fungal species isolated from three different varieties of pepper (*Capsicum chinense, C. frutescens* and *C. annum* L.) in Dutsin-ma, Katsina State. *Ann. Biol. Sci.* **2018**, *6*, 13–17. [CrossRef]
151. Sabokbar, A.; Motevalibashi, M.; Talebi, S. Molecular identification of Aflatoxin B1 *Aspergillus flavus* in red, black and white pepper using PCR method. *Int. J. Mol. Clin. Microbiol.* **2018**, *8*, 1016–1022.
152. Dharmaputra, O.S.; Ambarwati, S.; Retnowati, I.; Nurfadila, N. Determining appropriate postharvest handling method to minimize fungal infection and aflatoxin contamination in nutmeg (*Myristica fragrans*). *Int. Food Res. J.* **2018**, *25*, 545–552.

153. Nurtjahja, K.; Dharmaputra, O.S.; Rahayu, W.P.; Syarief, R. Fungal population and aflatoxin contamination on stored gamma-irradiated nutmeg (*Myristica fragrans*) kernels. *At. Indones.* **2018**, *44*, 57–61. [CrossRef]
154. Nurtjahja, K.; Dharmaputra, O.S.; Rahayu, W.P.; Syarief, R. Fungal population of nutmeg (*Myristica fragrans*) kernels affected by water activity during storage. *Agritech* **2017**, *37*, 288–294. [CrossRef]
155. Yogendrarajah, P.; Devlieghere, F.; Njumbe Ediage, E.; Jacxsens, L.; Meulenaer, B.; Saeger, S. Toxigenic potentiality *of Aspergillus flavus* and *Aspergillus parasiticus* strains isolated from black pepper assessed by an LC-MS/MS based multi-mycotoxin method. *Food Microbiol.* **2015**, *52*, 185–196. [CrossRef]
156. RASFF. Rapid Alert System for Food and Feed Portal Database. Available online: https://webgate.ec.europa.eu/rasff-window/portal/ (accessed on 9 February 2020).
157. Basilico, M.Z.; Basilico, J.C. Inhibitory effects of some spice essential oils on *Aspergillus ochraceus* NRRL 3174 growth and ochratoxin A production. *Lett. Appl. Microbiol.* **1999**, *29*, 238–241. [CrossRef]
158. Magan, N.; Aldred, D. Post-harvest control strategies: Minimizing mycotoxins in the food chain. *Int. J. Food Microbiol.* **2007**, *119*, 131–139. [CrossRef]
159. Soliman, K.M.; Badeaa, R.I. Effect of oil extracted from some medicinal plants on different mycotoxigenic fungi. *Food Chem. Toxicol.* **2002**, *40*, 1669–1675. [CrossRef]

Supplementary Materials: A Review on Mycotoxins and Microfungi in Spices in the Light of the Last Five Years

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Table S1. Studies positivity: Natural occurrence of mycotoxins produced by *Aspergillus* and *Penicillium* species in spices in the last 5 years (since 2015).

Mycotoxin[a]/ Spice	AFB_1 Positive[b] (%)	n[c]	AFB_2 Positive (%)	n	AFG_1 Positive (%)	n	AFG_2 Positive (%)	n	AFs Positive (%)	n	OTA Positive (%)	n	CIT Positive (%)	n	Reference
Allspice	- -	0	- -	0	- -	0	- -	0	● 100	1	× 0.0	1	- -	0	[9]
Anise	● 100	2	× 0.0	1	● 100	1	× 0.0	1	◉ 75.0	4	● 100	1	- -	0	[9,109,111,122]
Basil	× 0.0	2	- -	0	- -	0	- -	0	× 0.0	1	× 0.0	2	× 0.0	1	[9,110,123]
Bay leaf	× 0.0	3	× 0.0	2	○ 50.0	2	○ 50.0	2	○ 50.0	4	× 0.0	1	- -	0	[9,110,122,124]
Caraway	○ 40.0	5	○ 50.0	4	○ 25.0	4	○ 25.0	4	○ 50.0	6	◉ 66.7	3	× 0.0	1	[8,9,120,124–126]
Cardamom	× 0.0	2	× 0.0	1	× 0.0	1	× 0.0	1	◉ 60.0	5	◉ 66.7	3	- -	0	[9,109,119,122,126,127]
Carom	● 100	1	× 0.0	1	× 0.0	1	× 0.0	1	● 100	1	- -	0	- -	0	[125]
Chili	● 95.7	23	◉ 75.0	12	○ 50.0	12	○ 45.5	11	● 96.3	27	● 85.7	14	● 100	2	[8,9,106,108,110–118,120–122,128–138,147,148]
Cinnamon	○ 50.0	8	○ 20.0	5	○ 40.0	5	○ 20.0	5	◉ 57.1	7	○ 33.3	3	- -	0	[9,110–112,116,121,122,125,127,131]
Cloves	× 0.0	2	× 0.0	1	× 0.0	1	× 0.0	1	○ 40.0	5	× 0.0	3	- -	0	[9,35,122,127,131,147,148]
Coriander	◉ 57.1	7	○ 50.0	6	○ 33.3	6	○ 33.3	6	◉ 62.5	8	◉ 75.0	4	● 100	1	[8,9,109,111,112,120,124,125]
Cumin	◉ 57.1	7	○ 40.0	5	◉ 60.0	5	○ 40.0	5	◉ 66.7	6	◉ 66.7	3	● 100	1	[8,9,109–112,122,125]
Cumin, black	◉ 66.7	3	○ 50.0	2	● 100	2	○ 50.0	2	● 100	2	- -	0	- -	0	[109,110,125]
Curry	● 100	1	● 100	1	● 100	1	● 100	1	○ 33.3	3	● 100	1	- -	0	[112,147,148]
Dawadawa	● 100	1	- -	0	- -	0	- -	0	● 100	1	● 100	1	- -	0	[130]
Fennel	○ 40.0	10	○ 28.6	7	○ 42.9	7	○ 28.6	7	◉ 60.0	10	◉ 60.0	5	× 0.0	1	[8,9,109–112,124–127,131]
Fenugreek	○ 50.0	2	○ 50.0	2	● 100	2	○ 50.0	2	● 100	3	○ 50.0	2	● 100	1	[8,9,109]
Garlic	- -	0	- -	0	- -	0	- -	0	× 0.0	3	● 100	1	- -	0	[9,147,148]
Ginger	● 100	8	◉ 60.0	5	○ 40.0	5	○ 40.0	5	● 81.8	11	● 83.3	6	● 100	1	[8,9,109–111,117,120,122,130,139,140,147,148]
Licorice	○ 50.0	2	× 0.0	2	○ 50.0	2	× 0.0	2	○ 50.0	2	● 100	2	● 100	1	[109,120,141]
Mace	- -	0	- -	0	- -	0	- -	0	● 100	1	● 100	1	- -	0	[126]
Marjoram	● 100	1	× 0.0	1	× 0.0	1	× 0.0	1	○ 50.0	2	● 100	1	- -	0	[9,109]
Mint	× 0.0	3	× 0.0	2	× 0.0	2	× 0.0	2	× 0.0	3	× 0.0	1	- -	0	[9,110,124]
Mustard	○ 50.0	2	× 0.0	1	● 100	1	● 100	1	● 100	1	● 100	1	- -	0	[109,120,127]
Nutmeg	○ 33.3	6	○ 50.0	2	○ 50.0	2	× 0.0	2	● 85.7	7	● 100	3	× 0.0	1	[9,105,109,120,123,127,135,147,148,152,153]
Onion	× 0.0	1	- -	0	× 0.0	1	- -	0	× 0.0	2	× 0.0	2	- -	0	[9,133]

Mycotoxin[a]/ Spice	AFB_1 Positive[b] (%)	n[c]	AFB_2 Positive (%)	n	AFG_1 Positive (%)	n	AFG_2 Positive (%)	n	AFs Positive (%)	n	OTA Positive (%)	n	CIT Positive (%)	n	Reference
Oregano	× 0.0	4	× 0.0	3	× 0.0	3	× 0.0	3	○ 25.0	4	× 0.0	3	× 0.0	1	[9,123,124,131]
Paprika	● 100	3	● 100	1	● 100	2	● 100	1	● 100	4	● 100	4	- -	0	[9,107,111,120,133]
Parsley	● 100	1	● 100	1	● 100	1	× 0.0	1	◌ 50.0	2	× 0.0	1	- -	0	[9,109]
Pepper, black	◉ 64.3	14	◉ 57.1	7	◌ 42.9	7	◌ 28.6	7	◉ 73.3	15	◉ 66.7	12	◉ 66.7	3	[8,9,35,108–112,116–118,120–123,125–127,129–131,134]
Pepper, white	◌ 33.3	3	◌ 33.3	3	◌ 33.3	3	◌ 33.3	3	◌ 50.0	4	○ 25.0	4	- -	0	[9,35,112,125,131]
Rosemary	◌ 50.0	4	◌ 50.0	4	○ 25.0	4	◌ 50.0	4	● 100	5	◌ 50.0	2	- -	0	[9,109,124,131]
Saffron	● 100	1	- -	0	- -	0	- -	0	◌ 50.0	2	× 0.0	1	- -	0	[9,111]
Sage	◌ 50.0	2	× 0.0	1	● 100	1	× 0.0	1	● 100	3	● 100	1	- -	0	[9,109,110]
Star anise	× 0.0	1	- -	0	- -	0	- -	0	- -	0	- -	0	- -	0	[127]
Sumac	× 0.0	1	- -	0	- -	0	- -	0	× 0.0	1	× 0.0	1	- -	0	[9,110]
Thyme	◌ 40.0	5	× 0.0	3	◌ 33.3	3	× 0.0	3	◌ 42.9	7	× 0.0	2	× 0.0	1	[9,109,110,123,124,147,148]
Turmeric	● 85.7	7	◉ 66.7	6	◉ 66.7	6	○ 16.7	6	● 77.8	9	● 100	5	× 0.0	1	[8,9,109,110,112,116,121,122,125,126]

Notes: [a] AFB_1 = Aflatoxin B_1, AFB_2 = Aflatoxin B_2, AFG_1 = Aflatoxin G_1, AFG_2 = Aflatoxin G_2, AFs = Aflatoxins, OTA = Ochratoxin A, CIT = Citrinin; [b] Positive = the percentage of studies with at least one related spice sample positive on related mycotoxin; [c] n = number of studies concerning related spice and mycotoxin; × = none occurrence (0 %); ☆ = rare occurrence (up to 5 %); ○ = low occurrence (up to 25 %); ◌ = moderate occurrence (up to 50 %); ◉ = high occurrence (up to 75 %); ● = very high occurrence (more than 75 %).

Table S2. Studies positivity: Natural occurrence of *Fusarium* mycotoxins in spices in the last 5 years (since 2015).

Mycotoxin[a]/ Spice	FB_1 Positive[b]	(%)	n[c]	FB_2 Positive	(%)	n	DON Positive	(%)	n	NIV Positive	(%)	n	T-2 Positive	(%)	n	HT-2 Positive	(%)	n	ZEA Positive	(%)	n	Reference
Basil	×	0.0	2	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	[123,143]
Bay leaf	×	0.0	3	×	0.0	1	-	-	0	-	-	0	×	0.0	2	×	0.0	2	-	-	0	[124,143]
Caraway	×	0.0	2	-	-	0	-	-	0	-	-	0	×	0.0	2	×	0.0	2	-	-	0	[124]
Chili	×	0.0	1	●	100	2	-	-	0	-	-	0	-	-	0	-	-	0	×	0.0	1	[118,133,137]
Coriander	×	0.0	3	×	0.0	1	-	-	0	-	-	0	◌	50.0	2	×	0.0	2	-	-	0	[124,143]
Dawadawa	●	100	1	●	100	1	×	0.0	1	×	0.0	1	●	100	1	●	100	1	●	100	1	[34]
Fennel	×	0.0	2	-	-	0	-	-	0	-	-	0	×	0.0	2	×	0.0	2	-	-	0	[124]
Garlic	●	100	1	×	0.0	1	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[142]
Licorice	●	100	1	×	0.0	1	●	100	1	-	-	0	×	0.0	1	-	-	0	●	100	1	[141]
Mint	◌	33.3	3	×	0.0	1	-	-	0	-	-	0	◌	50.0	2	×	0.0	2	-	-	0	[124,143]
Nutmeg	●	100	1	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	[123]
Onion	●	100	1	●	100	1	-	-	0	-	-	0	-	-	0	-	-	0	-	-	0	[133]
Oregano	×	0.0	3	-	-	0	×	0.0	1	×	0.0	1	×	0.0	3	×	0.0	3	×	0.0	1	[123,124]
Paprika	◌	50.0	2	●	100	2	●	100	1	●	100	1	●	100	1	●	100	1	●	100	1	[107,133]
Pepper, black	◌	50.0	2	-	-	0	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	×	0.0	1	[118,123]
Rosemary	×	0.0	2	-	-	0	-	-	0	-	-	0	×	0.0	2	×	0.0	2	-	-	0	[124]
Thyme	○	25.0	4	×	0.0	1	●	100	1	×	0.0	1	×	0.0	3	×	0.0	3	●	100	1	[123,124,143]

Notes: [a] FB_1 = fumonisin B_1, FB_2 = Fumonisin B_2, DON = Deoxynivalenol, NIV = Nivalenol, T-2 = T-2 toxin, HT-2 = HT-2 toxin, ZEA = Zearalenone; [b] Positive = the percentage of studies with at least one related spice sample positive on related mycotoxin; [c] n = number of studies concerning related spice and mycotoxin; × = none occurrence (0 %); ☆ = rare occurrence (up to 5 %); ○ = low occurrence (up to 25 %); ◌ = moderate occurrence (up to 50 %); ◍ = high occurrence (up to 75 %); ● = very high occurrence (more than 75 %).

Table S3. Studies positivity: Natural occurrence of *Alternaria* mycotoxins in spices in the last 5 years (since 2015).

Mycotoxin[a]/ Spice	ALT Positive[b] (%)		n[c]	AOH Positive (%)		n	TEA Positive (%)		n	Reference
Allspice	×	0.0	1	●	100	1	×	0.0	1	[10]
Anise	×	0.0	1	×	0.0	1	×	0.0	1	[10]
Basil	×	0.0	1	×	0.0	1	×	0.0	1	[10]
Bay leaf	×	0.0	1	×	0.0	1	●	100	1	[10]
Caraway	×	0.0	1	×	0.0	1	●	100	1	[10]
Cardamom	×	0.0	1	×	0.0	1	●	100	1	[10]
Chili	●	100	1	●	100	1	●	100	1	[10]
Cinnamon	●	100	1	●	100	1	●	100	1	[10]
Cloves	●	100	1	×	0.0	1	●	100	1	[10]
Coriander	×	0.0	1	×	0.0	1	●	100	1	[10]
Cumin	×	0.0	1	×	0.0	1	●	100	1	[10]
Fennel	×	0.0	1	×	0.0	1	●	100	1	[10]
Fenugreek	×	0.0	1	×	0.0	1	●	100	1	[10]
Garlic	×	0.0	1	●	100	1	●	100	1	[10]
Ginger	●	100	1	●	100	1	●	100	1	[10]
Licorice	-	-	0	●	100	1	-	-	0	[141]
Marjoram	×	0.0	1	×	0.0	1	●	100	1	[10]
Mint	×	0.0	1	●	100	1	●	100	1	[10]
Nutmeg	×	0.0	1	●	100	1	●	100	1	[10]
Onion	×	0.0	1	●	100	1	●	100	1	[10]
Oregano	×	0.0	1	●	100	1	●	100	1	[10]
Paprika	●	100	2	●	100	2	●	100	2	[10,107]
Parsley	×	0.0	1	×	0.0	1	×	0.0	1	[10]
Pepper, black	×	0.0	1	●	100	1	●	100	1	[10]
Pepper, white	×	0.0	1	●	100	1	●	100	1	[10]
Rosemary	×	0.0	1	×	0.0	1	●	100	1	[10]
Sage	×	0.0	1	●	100	1	●	100	1	[10]
Sumac	×	0.0	1	●	100	1	●	100	1	[10]
Thyme	×	0.0	1	×	0.0	1	●	100	1	[10]
Turmeric	●	100	1	×	0.0	1	●	100	1	[10]

Notes: [a] ALT = Altenuene, AOH = Alternariol, TEA = Tenuazonic acid; [b] Positive = the percentage of studies with at least one related spice sample positive on related mycotoxin; [c] n = a total number of studies concerning related spice and mycotoxin; × = none occurrence (0 %); ☆ = rare occurrence (up to 5 %); ○ = low occurrence (up to 25 %); ◔ = moderate occurrence (up to 50 %); ◕ = high occurrence (up to 75 %); ● = very high occurrence (more than 75 %).

Příloha 3

A recent overview of producers and important dietary sources of aflatoxins

toxins

MDPI

Review

A Recent Overview of Producers and Important Dietary Sources of Aflatoxins

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Abstract: Aflatoxins (AFs) are some of the most agriculturally important and harmful mycotoxins. At least 20 AFs have been identified to this date. Aflatoxin B_1 (AFB_1), the most potent fungal toxin, can cause toxicity in many species, including humans. AFs are produced by 22 species of *Aspergillus* section *Flavi*, 4 species of *A.* section *Nidulantes,* and 2 species *of A.* section *Ochraceorosei*. The most important and well-known AF-producing species of section *Flavi* are *Aspergillus flavus*, *A. parasiticus*, and *A. nomius*. AFs contaminate a wide range of crops (mainly groundnuts, pistachio nuts, dried figs, hazelnuts, spices, almonds, rice, melon seeds, Brazil nuts, and maize). Foods of animal origin (milk and animal tissues) are less likely contributors to human AF exposure. Despite the efforts to mitigate the AF concentrations in foods, and thus enhance food safety, AFs continue to be present, even at high levels. AFs thus remain a current and continuously pressing problem in the world.

Keywords: aflatoxigenic microfungi; aflatoxins; food

Key Contribution: As of 2020, 60 years have passed since the discovery of aflatoxins. A total of 22, 4, and 2 *Aspergillus* producers of section *Flavi, Nidulantes,* and *Ochracerosei* produce aflatoxins, respectively. *Aspergillus flavus*, *Aspergillus parasiticus,* and *Aspergillus nomius* are the most important aflatoxin producers of section *Flavi*. Groundnuts, pistachio nuts, dried figs, hazelnuts, spices, almonds, rice, melon seeds, Brazil nuts, and maize are the most common commodities contaminated with aflatoxins.

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1. Introduction

Aflatoxins (AFs) are some of the most important and harmful mycotoxins. As of 2020, 60 years have already passed since their discovery. AFs are one of the five agriculturally most important mycotoxins [1–4]. Chemically, the AFs are difuranocoumarin derivatives with a bifuran group attached to the coumarin nucleus and a pentanone ring (in the case of aflatoxin AFBs) or a lactone ring (in case of aflatoxin AFGs) [5]. There are more than 20 known AFs, but the most common are aflatoxin B_1 (AFB_1) (PubChem CID: 186907), aflatoxin B_2 (AFB_2) (PubChem CID: 2724360), aflatoxin G_1 (AFG_1) (PubChem CID: 14421), and aflatoxin G_2 (AFG_2) (PubChem CID: 2724362) (PubChem, 2020), from which AFB_1 is the major representative in food crops [6]. Aflatoxin M_1 (AFM_1) (PubChem CID: 15558498) and M_2 (AFM_2) (PubChem CID: 10903619) are the hydroxylated metabolites of AFB_1 and AFB_2 [7–9].

AFs are acutely toxic, hepatotoxic, immunosuppressive, mutagenic, teratogenic, and carcinogenic compounds [10–14]. The International Agency for Research on Cancer (IARC) evaluated the carcinogenicity of naturally occurring AFs (AFB_1, AFB_2, AFG_1, and AFG_2) for humans as Group 1 "carcinogenic to humans" in 1987 [10,15], and re-evaluated in 2012 [16,17]. AFM_1 is often misclassified in the literature as Group 1; however, it was classified as Group 2B "possibly carcinogenic to humans" in 1993 [1] and has not been

re-evaluated since. For these reasons, AFs need to be monitored and their concentrations in food should be kept at the lowest possible levels.

While acute exposure to a high dose can result in vomiting, abdominal pain, and even death, chronic exposure to low doses may lead to liver cancer [18,19], which is generally considered to be the most significant impact of AFs on human health [10,20]. According to the latest data from the Global Cancer Observatory, liver cancer is the sixth most common cancer for both sexes of all ages, with a total of 905,677 new cases estimated in 2020 [21]. It has been estimated that AFs contribute to 4.6% to 28.2% of all global hepatocellular carcinomas [22].

Nowadays, AFs are of great interest as they are one of the most serious contaminants that can significantly affect the food chain. Humans, at the top of the food chain, often consume contaminated foodstuffs of both plant and animal origins. Besides human health, food insecurity caused by AFs contamination can also affect humanity at the social, political, and economic levels [23].

Therefore, in this article, attention is paid to AFs in terms of AF producers and the occurrence of AFs in foods around the world.

2. Producers of Aflatoxins

To date, AFs are produced by 28 species of the genus *Aspergillus*. *Aspergillus* subgenus *Circumdati* section *Flavi* contains some of the most important species in the genus, which usually produce AFs [24–26].

The accurate identification of *Aspergillus* section *Flavi* requires a polyphasic approach that includes the morphological characters (the microscopic structures, such as the uni- or biseriate conidial heads, the production of dark-colored sclerotia by certain species, and yellow-green to brown shades of conidia), and the chemical (extrolite data) and molecular (partial sequences of calmodulin, β-tubulin, and internal transcribed spacer region) approaches, as these species are closely related and could not be easily distinguished by morphological characteristics alone [24–26].

Aspergillus section *Flavi* currently contains a total of 34 species in 8 clades: the *Aspergillus alliaceus*-, *A. avenaceus*-, *A. bertholletius*-, *A. coremiiformis*-, *A. flavus*-, *A. leporis*-, *A. nomius*-, and *A. tamarii*-clade [24–27]. The three new clades *A. texensis*-, *A. agricola*-, and *A. toxicus*-clade with three species were presented in the year 2020 [28,29].

Table 1 gives an overview of the current identity of *Aspergillus* species from *Aspergillus* section *Flavi* as AF producers focus on foodstuffs [24–30].

The most important and most well-known AF-producing species of section *Flavi* in foodstuffs are *Aspergillus flavus* [31,32], *A. parasiticus* [33–35], and *A. nomius* [36,37]. While *Aspergillus flavus* produces AFB_1 and AFB_2, *A. parasiticus* and *A. nomius* can produce AFB_1, AFB_2, AFG_1, and AFG_2.

Aspergillus minisclerotigenes and *A. parvisclerotigenes* also belong to section *Flavi*. Both have morphological and physiological similarities to *A. flavus*; however, they produce more but smaller sclerotia. In contrast to *A. flavus*, this is usually coupled with a high and consistent production of both the B and G type of AFs [24].

In addition to *Aspergillus flavus*, four other *A.* species (*A. agricola*, *A. pseudotamarii*, *A. togoensis,* and *A. toxicus*) only produce AFB_1 and AFB_2. Seventeen other *Aspergillus* species can produce AFB_1, AFB_2, AFG_1, and AFG_2. It is generally accepted that *A. flavus* is unable to produce AFs type G, but it is also reported that some Korean strains are capable of producing both AFG_1 and AFG_2 [25]. However, some *Aspergillus* species from *Aspergillus* section *Nidulantes* [38] or *Aspergillus* section *Ochraceorosei* [32,39] can also produce AFs.

Table 1. Aflatoxigenic *Aspergillus* species from *Aspergillus* section *Flavi.*

Species	AF Producer	Year of Identification	Occurrence
A. flavus	B_1, B_2	1962	Peanuts, maize, spices
A. parasiticus	B_1, B_2 G_1, G_2	1963	Maize, peanuts
A. nomius	B_1, B_2 G_1, G_2	1987	Wheat, turmeric
A. pseudonomius	B_1, B_2 G_1, G_2	1997	Brazil nut
A. pseudotamarii	B_1, B_2	2001	Brazil nut
A. parvisclerotigenes	B_1, B_2 G_1, G_2	2005	Peanuts
A. arachidicola	B_1, B_2 G_1, G_2	2008	Carob flour
A. luteovirescens [a]	B_1, B_2 G_1, G_2	2008	Brazil nut
A. minisclerotigenes	B_1, B_2 G_1, G_2	2008	Peanuts, curry, red chili
A. pseudocaelatus	B_1, B_2 G_1, G_2	2011	Peanuts, Brazil nut
A. togoensis	B_1, B_2	2011	Fruit of Landolphia spp.
A. mottae	B_1, B_2 G_1, G_2	2012	Maize
A. novoparasiticus	B_1, B_2 G_1, G_2	2012	No occurrence in food [b]
A. sergii	B_1, B_2 G_1, G_2	2012	Almond
A. transmontanensis	B_1, B_2 G_1, G_2	2012	Almond
A. texensis	B_1, B_2 G_1, G_2	2018	Maize
A. aflatoxiformans	B_1, B_2 G_1, G_2	2019	Peanuts, sesame
A. austwickii	B_1, B_2 G_1, G_2	2019	Rice, sesame
A. cerealis	B_1, B_2 G_1, G_2	2019	Rice, maize, peanut
A. pipericola	B_1, B_2 G_1, G_2	2019	Black pepper
A. agricola sp. nov.	B_1, B_2	2020	Maize
A. toxicus sp. nov.	B_1, B_2	2020	Maize

[a] Formerly named *Aspergillus bombycis*; [b] Sputum of leukemic patient.

The identification of *Aspergillus* section *Nidulantes* requires a polyphasic approach which includes the morphological characters (the microscopic structures such as the color, shape, size, and ornamentation of ascospores, the shape and size of conidia and vesicles, and growth temperatures), and the chemical (extrolite data) and molecular (internal transcribed spacer region, partial β-tubulin, calmodulin, and RNA polymerase II the second largest subunit (RPB2) gene sequences) approaches [38]. Based on this polyphasic approach, *Aspergillus* section *Nidulantes* was subdivided into 7 clades and 65 species [38]. The majority of section *Nidulantes* species can produce a sexual state, and those species were, in the dual name nomenclature system, assigned to the genus *Emericella*. Because of the adoption of the "one fungus: one name" nomenclatural system, all *Emericella* species were transferred to *Aspergillus* [40]. AFB_1 was produced by four species: *Aspergillus astellatus* [41], *A. miraensis* [42,43], *A. olivicola* [44], and *A. venezuelensis* [45]. *Aspergillus ochraceoroseus* and *A. rambellii* belong to section *Ochraceorosei* [32]. *A. ochraceoroseus* produce AFB_1 [11,39,46,47], and *A. rambellii* also produce AFB_1 [32,39].

With the development of modern molecular biological and chromatographic methods, other new AF producers will certainly be identified soon and bring new research to this area.

3. Aflatoxin Occurrence in Foods

The contamination of foods with AFs, like with other mycotoxins, has become a global problem [48]. For several years, a statement claiming that a total amount of 25% of the world's crops are affected by molds and mycotoxins, supposedly estimated by the Food and Agriculture Organization (FAO), has been circulating worldwide [12,49]. However, this estimation has been challenged in the most recent studies dealing with the background of this matter, as this statement was not possible to trace back, since even FAO experts were not able to do so [50]. On the basis of an extensive study by the BIOMIN Company in 2004–2011, 72% of samples of feed (mainly maize, wheat, barley, and silage) and feed raw materials (especially for swine, poultry, and cows) from all over the world, but mainly from Asia (40%) and Europe (38%), contained a detectable amount of at least one mycotoxin

including AFs. Moreover, a co-occurrence of two or more mycotoxins was confirmed in 38% of samples [51], and of course, AFs can interact in synergy with other mycotoxins. This fact is alarming since the major intake of mycotoxins into human organisms is usually due to dietary exposure [52], and even a low concentration of AFs is hazardous for humans [53].

In general, inappropriate storage is considered a major cause of foods contamination with mycotoxins—especially in developing countries [54,55], in which approximately 20% of the global volume of potentially highly contaminated commodities originate [56]. In some cases, contamination of crops with mycotoxins may already occur in the field due to stress factors such as insects or drought that facilitate the contamination [57]. Climate conditions, such as high temperatures, heavy rainfalls, and high relative humidity, are likely to contribute to crop contamination as well, as they make plants more susceptible to fungal, and thus mycotoxin, contamination [58,59]. Contamination during transport and processing is also possible [23]. Good agricultural, manufacturing, and hygienic practices, good plant disease management, and adequate storage conditions can limit mycotoxin levels in the food chain, yet these practices do not eliminate mycotoxins completely [60,61].

Fortunately, some contamination-reducing chemical (ammonization, hydrogen peroxide, sodium bisulfate, organic acids, ozone, and plant extracts), physical (separation, solvent extraction, mineral adsorbents, heating, extrusion, microwaving, irradiation, and UV radiation) and biological (enzymes, bacterial cells, yeast cells, and non-toxigenic strains) technologies have been developed to enhance food safety [20,23]. However, the European Union legislation, in Section 2 of the Annex "Mycotoxins", does not allow any foods contaminated with mycotoxins to be detoxified by the chemical approach [62]. Moreover, foods treated by sorting or other physical means must not be mixed with foods intended for direct human consumption nor with foods intended to be used as food ingredients [62]. Biological control, depending on the competition between non-toxigenic and toxigenic strains, is the most commonly used method, especially in countries where AFs pose a significant threat [63]. For example, a product *Aflasafe*^{TM} has begun to be applied to reduce AFs with an average efficiency of 99% (76%–100%) in maize and groundnuts [64–66]. The principle of its use lies in the contamination of crops with non-toxigenic strains before they are contaminated by toxigenic strains of *Aspergillus flavus*. *Aflasafe*^{TM} is a relatively cheap and easy-to-apply product that ensures a long-lasting reduction of AFs (up to consumption level) [64].

AFs contaminate a wide range of foods of both plant and animal origin. AFB_1, AFB_2, AFG_1, and AFG_2 are major contaminants in commodities of plant origin, mainly groundnuts, tree nuts, spices, seeds, dry fruits, and cereals [67–69]. The daily intake of AFs at the level of nanograms to micrograms per person per day is mainly achieved through the consumption of contaminated maize and groundnuts [70]. Animal products are less likely substrates for AF producers; however, the metabolites AFM_1 and AFM_2 are typical in milk, including human breast milk [71,72], and dairy products of lactating ruminants that have been fed with contaminated feed (carry-over to dairy milk) [73–76]. AFM_1 has also been detected in cheese worldwide [77–79] and AFs (in low concentrations) have been reported to occur in certain products of animal origin, such as meat and meat products, or eggs, etc. (carry-over of AFs in products of animal origin) [74].

Drought periods combined with high temperatures significantly increase AF production in the fields [80]. It has been estimated that at least 4.5 billion people worldwide are chronically exposed to AFs from foods, especially in "hot zones" in the regions situated between 40° N and 40° S latitude [81]. Climate change and the trend of global warming may lead to an increased occurrence of mycotoxins, for the production of which higher temperatures are needed, and the same goes for AFs [82]. This might be the case in Northern [82] or Western [83] Europe, for example, where AFB_1 contamination of maize was recently observed [84]. It should be emphasized that even in the current modern age, cases of acute aflatoxicosis leading to human death may occur due to climate change [85]. Climate change is dealt with in more detail in the Special Issue of Toxins entitled "Mycotoxins in Relation to Climate Change".

On the other hand, it is known that the AFs belong to the dominant mycotoxins in the African and Asian continents, as well as North and South America and the Australian continent [86]. Additionally, despite all efforts to mitigate AFs in foods, there are still cases of high AF concentrations in foods. Therefore, to enhance food safety, there is a global need for regulatory limits and food contaminant monitoring tools.

3.1. The Occurrence of Aflatoxins in Food in the African Continent

In African countries, maize and groundnuts represent the largest exposure to AFs [87,88], where maize is a staple crop for the majority of the African population [88,89]. The case of highly contaminated (1–46, 400 μg/kg) maize in Kenya in 2004, associated with 125 human deaths, is historically relevant [85].

There are still cases of concentrations exceeding the limits set in many countries. Recently, high concentrations of AFs in maize grains of up to 9091.8 μg/kg (AFB_1) were found in Kenya [89], up to 3760 μg/kg for total AFs (where AFT is the sum of AFB_1, AFB_2, AFG_1, and AFG_2) in Uganda [90], up to 2806.5 μg/kg (AFT) in the Democratic Republic of Congo [91], up to 1460 μg/kg (AFT) in Nigeria [88], up to 945 μg/kg (AFT) in Ghana [92], and up to 107.6 μg/kg (AFT) in Zambia [93].

3.2. The Occurrence of Aflatoxins in Food in the Asian Continent

Practically all tropical countries face the problem of AFs [94]. The climate of Asian countries is very favorable for AF-producing microfungi [95], especially when it comes to commodities such as cereals—mainly maize and rice, cereal products, beans, groundnuts, and other oily products—which is alarming, as cereals and groundnuts are considered major items in the Asian diet [94].

AFs were found in maize in concentrations of up to 1572 μg/kg (AFB_1) in Vietnam [96]. However, in Asia, rice is the most important crop in terms of its consumption [55,97], and especially production, as approximately 90% of the world's rice is produced in Asia, of which nearly two-thirds are produced by China, India, and Indonesia [98]. High concentrations of AFs in rice have been reported in many scientific studies. In the case of AFB_1, reported concentrations reached up to 361.0 μg/kg in India [99], up to 185.0 μg/kg in Sri Lanka [100], and up to 26.6 μg/kg in Thailand [101]. In the case of AFT concentrations, they were found to reach up to 96.3 μg/kg in Malaysia [102], up to 77.8 μg/kg in Vietnam [55], up to 21.4 μg/kg in Turkey [103], and up to 21.0 μg/kg in China [104].

3.3. The Occurrence of Aflatoxins in Food in the American Continent

America is the largest producer of maize (565 million tons in 2019; 49.2% of world production). The United States, Brazil, Argentina, and Mexico belong to the top 10 producers worldwide [98]. Alongside sub-Saharan Africa and Southeast Asia, maize is a staple food in Latin America [105], especially in Guatemala [106] and Mexico [107].

However, concentrations of AFB_1 of up to 2656 μg/kg were observed in maize in Guatemala and are potentially high throughout the rest of Central America and Mexico [106]. Lower concentrations of up to 282.5 μg/kg (AFB_1) and 303.9 μg/kg (AFT) were detected in maize kernels in South Haiti [108], and concentrations of up to 49.9 μg/kg (AFT) were found in Brazil [109]. Processed maize products are also contaminated with AFs. For example, tortillas and popcorn have been reported to be contaminated with up to 287.23 μg/kg (AFB_1) [110] and up to 120 μg/kg (AFT) [111], respectively, in Mexico.

Of course, the problem is not only maize as a staple food, as high levels of AFs are also found in other local commodities, including up to 33.3 μg/kg (AFT) in nuts, up to 176.4 μg/kg (AFT) in *Capsicum* spices in Chile [112], and up to 70.9 μg/kg (AFT) in the case of Brazilian rice [113].

3.4. The Occurrence of Aflatoxins in Food in the Australian Continent

In Australia, hot and dry conditions typical for the arid and semi-arid areas covering much of the continent are the main stress factors that allow for the contamination of

crops with AFs. This represents a major problem in Australia in terms of peanut degradation [114,115]. The occurrence of AFs is not quite as common in Australian maize [116], and when it occurs, it is in low or moderate concentrations [117] for unknown reasons [115]. Nevertheless, maize is only a small part of the human and animal diet in Australia [115].

The occurrence of AFs in Australian maize is usually in the range of 1–5 μg/kg, but can also occasionally reach higher concentrations of up to 200 μg/kg [118]. However, higher concentrations of AFT in maize (up to 311.1 μg/kg), and also in peanuts (up to 384.8 μg/kg), sorghum (up to 138.3 μg/kg), and wheat (up to 26.8 μg/kg), have been found in Australia [115,119–121].

3.5. Aflatoxin Regulations in the European Union and around the World

The discovery of AFs and their serious negative effects on human and animal health in the early 1960s led many countries in the world to establish certain regulations of mycotoxins in foods to protect consumers from the harmful effects caused by mycotoxins [122,123]. The first limit regulating mycotoxins, namely AFs, was set in the late 1960s, and by 2003 approximately 100 countries in the world had already regulated mycotoxins in foods [123]. Although the number of countries regulating mycotoxins in foods is increasing [123], most African countries and other developing countries lack regulations [92], as the compliance with the limits in developing countries would result in a shortage of food, and thus an increase in its price.

From the perspective of all mycotoxins, the regulations of AFB_1, AFT, and AFM_1 are the greatest concern of worldwide legislation [124]. The Codex Alimentarius specifies an AF maximum limit of 15 μg/kg (for almonds, hazelnuts, Brazil nuts, peanuts, and pistachio nuts for further processing) and 10 μg/kg (for almonds, Brazil nuts, hazelnuts, and pistachio nuts for direct consumption and dried figs), and AFM_1 maximum limit of 0.5 μg/kg for milk [125]. However, the maximum levels of AFs in foods vary throughout different countries depending on the type of product and also on the import/export regime [69].

The European Union (EU) has one of the most comprehensive and strictest regulations on AF levels, set by the commission regulation 1881/2006 [62], and later on by its amending supplement 165/2010 [126], that are binding upon the 27 member states of the EU. These levels are in ranges 0.1–12 μg/kg, 4–15 μg/kg, and 0.025–0.05 μg/kg for AFB_1, AFT, and AFM_1, respectively, in the case of various foods [62,126].

For comparison with other countries, the maximum limit/regulatory limit/action level (or the range) for AFB_1 has been set at 30 μg/kg in India, at 20 μg/kg in the Philippines, at 15–20 μg/kg in Indonesia [127], at 0.5–20 μg/kg in China [128], at 5–10 μg/kg in Japan, and at 0.1–10 μg/kg in Korea [127].

The maximum/action limit (or the range) for AFT has been set at 20–35 μg/kg in Indonesia [127]; at 5–15 μg/kg in Malaysia [129]; at 30 μg/kg in Sri Lanka [127]; at 20 μg/kg in the United States [130–133], Thailand, the Philippines [127], and Nigeria [134]; at 15–20 μg/kg in Hong Kong [127]; at 1–20 in Brazil μg/kg [135]; at 15 μg/kg in Canada [136], Korea [69,127], Australia [137], and Zimbabwe [134]; at 0–15 μg/kg in Taiwan, at 10 μg/kg in Japan, Vietnam [69,127], Kenya, Mozambique, South Africa, and Uganda [134]; and at 5 μg/kg in Singapore [127].

If a country has any regulation on AFM_1 in milk or dairy products, it is usually set at 0.5 μg/kg [128,135,138,139], which is in line with the Codex Alimentarius. However, in the EU legislation, the AFM_1 maximum limits (0.025–0.05 μg/kg) are 10–20 times lower compared to the Codex Alimentarius (0.5 μg/kg) [62,125].

3.6. The Occurrence of Aflatoxins Based on Data by INFOSAN (2016–2020)

The International Food Safety Authorities Network (INFOSAN) is a global information network jointly managed by the World Health Organization (WHO) and the FAO [140]. The INFOSAN has facilitated urgent international communication during food safety emergencies between more than 600 members from 188 of the 194 FAO and WHO member states since 2004. The INFOSAN aims to reduce the incidence of foodborne diseases that

have a significant impact on public health and international trade [140,141]. Regarding AFs, only two cases, both of which concerned maize in Tanzania, were reported in 2016 and 2017 [142]. There have been no reports on AFs in foods since.

3.7. The Occurrence of Aflatoxins in Food Based on Data by RASFF (2015–2020)

The Rapid Alert System for Food and Feed (RASFF) is an important warning system for food and feed safety from the perspective of the EU countries [143]. Regarding the number of notifications reported by RASFF in 2015-2020, most mycotoxin notifications were related to AFs (approximately 88%), of which most were of the food category (approximately 94%) and less were of the feed category (approximately 6%), as shown in Table 2 [144].

Table 2. The share of aflatoxin notifications in 2015-2020.

Substance/Year	2015	2016	2017	2018	2019	2020
Mycotoxins	495	549	579	655	584	423
AFs [a]	441 (89.1%)	478 (87.1%)	539 (93.0%)	567 (86.6%)	497 (85.1%)	370 (87.5%)
AFs in food	423 (95.9%)	461 (96.4%)	515 (95.5%)	510 (89.9%)	467 (94.0%)	348 (94.1%)

[a] AFs = aflatoxins; processed according to the Rapid Alert System for Food and Feed (RASFF) database [143].

Based on data from the last years (2015–2020), the vast majority of notified products contaminated with AFs belong to the "nuts, nut products, and seeds" category, followed behind by "fruits and vegetables", "herbs and spices", "cereals and bakery products", and others. Namely, the most often notified foods are, in descending order, groundnuts, pistachio nuts, dried figs, hazelnuts, spices, almonds, rice, melon seeds, Brazil nuts, and maize [144].

Throughout the years 2015–2020, cases of very high concentrations of AFs in foods were notified. Based on these "high-level" notifications, groundnuts, pistachio nuts, almonds, dried figs, hazelnuts, chilies, melon seeds, and apricot kernels appear to be highly contaminated (with the maximum concentration of AFB_1 or AFT exceeding 1000 µg/kg). Spices (other than chilies), tiger nuts, Brazil nuts, rice, pecan nuts, walnuts, and maize represent the less contaminated foods [144]. There is a concern for the development of aflatoxicosis associated with the consumption of foods with an AF concentration of at least 1000 µg/kg [145]. This implies that the group of above-mentioned highly contaminated commodities may tend to cause aflatoxicosis in humans or animals. Some of the highest values of aflatoxin contamination in 2015–2020 are shown in Table 3.

In the year 2020, groundnuts, pistachio nuts, dried figs, spices, hazelnuts, almonds, and rice were the most notified products in relation to AF contamination. The other notified products were mostly various seeds (melon, ogbono, sunflower, lotus, and sesame seeds) and flours (wheat flour, chestnut flour, and banku mix). Single notifications concerned Brazil nuts, apricot kernels, soya, milk, and date syrup. Most notifications originated in Turkey (mainly dried figs and pistachio nuts), followed far behind by the United States (mainly groundnuts) and India (mainly groundnuts and spices). A significant number of notifications originated in Argentina (groundnuts only), Iran (pistachio nuts only), Egypt (groundnuts only), China (mainly groundnuts), Pakistan (mainly spices and rice), Nigeria (mainly groundnuts), and Georgia (hazelnuts only) (see Figure 1) [144]. Fewer notifications (the number is given in brackets) originated in other countries: Spain (7); Sri Lanka (6); Brazil (5); Italy and Ghana (3); Ethiopia, United Kingdom, Germany, Ukraine, and Cameroon (2); and Angola, Vietnam, Hong Kong, South Africa, Jordan, Togo, Hungary, Nepal, Bolivia, Cambodia, Paraguay, Indonesia, Belgium, Malaysia, Tunisia, Senegal, and Azerbaijan (1). Two notifications were of unknown origin [144].

Table 3. The highest concentrations of aflatoxin B_1 and total aflatoxins in foods notified by RASFF in 2015–2020.

No.	Product	AFB_1 (µg/kg)	AFT [a] (µg/kg)	Origin	Year
1	Peanut paste	707,000	907,000	Senegal	2016
2	Peanuts	180,200	220,900	China	2015
3	Groundnuts in shell	42,100	46,800	Egypt	2019
4	Groundnuts	17,000	38,000	Turkey	2016
5	Pistachios	–	26,300	Germany	2020
6	Peanut in shell	24,000	26,000	China	2015
7	Almonds	–	24,000	US	2018
8	Dried figs	15,300	–	Turkey	2020
9	Roasted chopped hazelnuts	4000	15,200	Turkey	2015
10	Shelled nuts	12,890	14,420	Turkey	2019
11	Organic groundnut kernels	11,000	14,000	Egypt	2020
12	Dried red chilies	13,700	14,000	India	2020
13	Roasted and salted watermelon seeds	13,700	–	Turkey	2020
14	Shelled almonds	10,440	11,420	US	2019
15	Hazelnut kernels	7200	–	Georgia	2019

[a] AFT = sum of aflatoxins B1, B2, G1, and G2; processed according to the RASFF database [144].

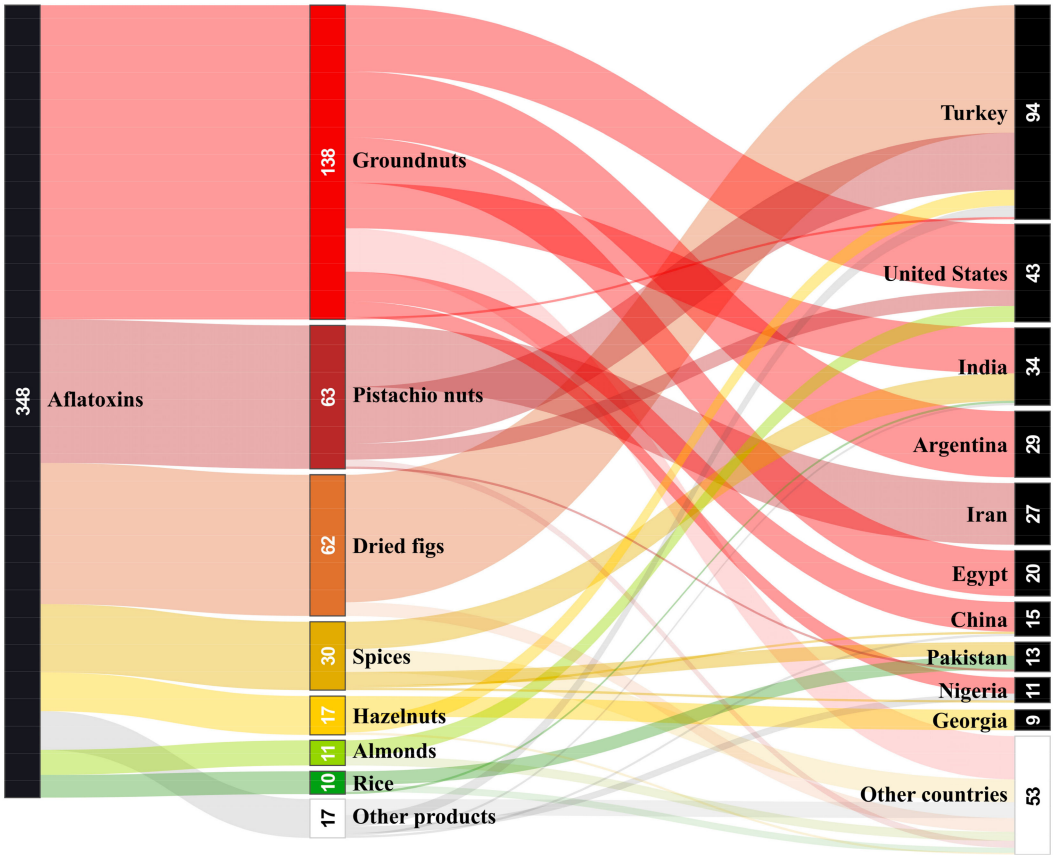

Figure 1. Aflatoxin notifications in food by the RASFF in 2020. Note: All products in the "Other products" category were notified less than four times in 2020. The category "Other countries" includes notifications from 24 countries, in which less than 9 notifications originated and 2 notifications were of unknown origin. Processed according to RASFF database [144].

The amount of the world production of these commodities should be taken into consideration as demonstrated in Table 4. Although groundnuts are the most often notified product, pistachio nuts can be labelled as the relatively most frequently notified product, with approximately one notification per 16,787 tons produced. In contrast, there is one notification per 344,886 tons of groundnuts produced [144].

Table 4. The number of RASFF aflatoxin notifications concerning certain food products in relation to their average world production.

Product	Average Annual Production (2015–2019) [a] (Tons)	Number of Notifications by RASFF (2020)	Tons Produced per RASFF Notification
Groundnuts	47,591,548	138	344,866
Pistachio nuts	1,057,587	63	16,787
Dried figs	1,185,768	62	19,125
Spices	14,541,902	30	484,730
Hazelnuts	939,927	17	55,290
Almonds	3,039,020	11	276,275
Rice	748,304,354	10	74,830,435

[a] Average annual spice production includes these categories: "Anise, badian, fennel, coriander", "Chilies and peppers, dry", "Cinnamon", "Cloves", "Ginger", "Nutmeg, mace, cardamoms", "Mustard seed", "Pepper, *Piper* pp.", "Peppermint", "Vanilla", and "Spice not elsewhere specified". Processed according to FAOSTAT and RASFF databases [98,144].

4. Conclusions

The year 2020 has already passed 60 years of AF discovery. Since then, despite the scientific progress in the knowledge on AFs and the efforts made to reduce the risk they pose to public health, developing countries still have to tolerate a high level of AF contamination of foods to not compromise the food supply. Selected research topics concerning AFs continue to draw attention worldwide, such as research on the diversity and genetic variability of AF production in *Aspergillus flavus* and other AF producers, or on the problem of using biocontrol strategies for the non-aflatoxigenic strains of *A. flavus* with the goal of the better protection of public health and the prevention of economic losses. The recent occurrence data, the recent food consumption data, and the recent toxicological data of AFs in foodstuffs are required for the assessment of the severity of AF toxicity, the estimation of human dietary exposure, and health risk assessments.

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References

1. International Agency for Research on Cancer. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occuring Substances: Food Items and Costituents, Heterocyclic Aromatic Amines and Mycotoxins*; IARC Press: Lyon, France, 1993; Volume 56, ISBN 92-832-1256-8.
2. Miller, J.D. Fungi and Mycotoxins in Grain: Implications for Stored Product Research. *J. Stored Prod. Res.* **1995**, *31*, 1–16. [CrossRef]
3. Santos, L.; Marín, S.; Sanchis, V.; Ramos, A.J. Screening of Mycotoxin Multicontamination in Medicinal and Aromatic Herbs Sampled in Spain. *J. Sci. Food Agric.* **2009**, *89*, 1802–1807. [CrossRef]
4. Winter, G.; Pereg, L. A Review on the Relation between Soil and Mycotoxins: Effect of Aflatoxin on Field, Food and Finance. *Eur. J. Soil Sci.* **2019**, *70*, 882–897. [CrossRef]
5. Schuda, P.F. Aflatoxin Chemistry and Syntheses. In *Syntheses of Natural Products*; Springer: Berlin/Heidelberg, Germany, 1980; pp. 75–111.
6. European Food Safety Authority. Effect on Public Health of a Possible Increase of the Maximum Level for 'Aflatoxin Total' from 4 to 10 μg/kg in Peanuts and Processed Products Thereof, Intended for Direct Human Consumption or Use as an Ingredient in Foodstuffs. *EFSA J.* **2018**, *16*, 1–32. [CrossRef]
7. Giray, B.; Girgin, G.; Engin, A.B.; Aydın, S.; Sahin, G. Aflatoxin Levels in Wheat Samples Consumed in Some Regions of Turkey. *Food Control* **2007**, *18*, 23–29. [CrossRef]
8. Hussain, I.; Anwar, J. A Study on Contamination of Aflatoxin M1 in Raw Milk in the Punjab Province of Pakistan. *Food Control* **2008**, *19*, 393–395. [CrossRef]
9. PubChem. Available online: https://pubchem.ncbi.nlm.nih.gov/ (accessed on 17 December 2020).
10. Peraica, M.; Radić, B.; Lucić, A.; Pavlović, M. Toxic Effects of Mycotoxins in Human. *Bull. World Health Organ.* **1999**, *77*, 754–766. [PubMed]
11. Klich, M.A.; Cary, J.W.; Beltz, S.B.; Bennett, C.A. Phylogenetic and Morphological Analysis of *Aspergillus Ochraceoroseus*. *Mycologia* **2003**, *95*, 1252–1260. [CrossRef] [PubMed]
12. Bhat, R.; Rai, R.V.; Karim, A.A. Mycotoxins in Food and Feed: Present Status and Future Concerns. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 57–81. [CrossRef]
13. Kensler, T.W.; Roebuck, B.D.; Wogan, G.N.; Groopman, J.D. Aflatoxin: A 50-Year Odyssey of Mechanistic and Translational Toxicology. *Toxicol. Sci.* **2011**, *120*, S28–S48. [CrossRef]
14. Kumar, P.; Mahato, D.K.; Kamle, M.; Mohanta, T.K.; Kang, S.G. Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. *Front. Microbiol.* **2017**, *7*, 1–10. [CrossRef]
15. International Agency for Research on Cancer. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs*; IARC Press: Lyon, France, 1987; Volume 1, ISBN 978-92-832-1411-3.
16. Ostry, V.; Malir, F.; Toman, J.; Grosse, Y. Mycotoxins as Human Carcinogens-the IARC Monographs Classification. *Mycotoxin Res.* **2017**, *33*, 65–73. [CrossRef]
17. International Agency for Research on Cancer. *Monographs on the Evaluation of Carcinogenic Risks to Humans: Chemical Agents and Related Occupations. A Review of Human Carcinogens*; IARC Press: Lyon, France, 2012; Volume 100, ISBN 978-92-832-1323-9.
18. Etzel, R. Mycotoxins. *J. Am. Med. Assoc.* **2002**, *287*, 425–427. [CrossRef] [PubMed]
19. Sherif, S.; Salama, E.; Abdel-Wahhab, P.M. Mycotoxins and Child Health: The Need for Health Risk Assessment. *Int. J. Hyg. Environ. Health* **2009**, *212*, 347–368. [CrossRef] [PubMed]
20. Ismail, A.; Gonçalves, B.L.; de Neeff, D.V.; Ponzilacqua, B.; Coppa, C.F.S.C.; Hintzsche, H.; Sajid, M.; Cruz, A.G.; Corassin, C.H.; Oliveira, C.A.F. Aflatoxin in Foodstuffs: Occurrence and Recent Advances in Decontamination. *Food Res. Int.* **2018**, *113*, 74–85. [CrossRef] [PubMed]
21. International Agency for Research on Cancer/World Health Organization. Cancer Today, Data Visualization Tools for Exploring the Global Cancer Burden in 2020. Available online: https://gco.iarc.fr/today/home (accessed on 10 January 2021).
22. Liu, Y. Wu Felicia Global Burden of Aflatoxin-Induced Hepatocellular Carcinoma: A Risk Assessment. *Environ. Health Perspect.* **2010**, *118*, 818–824. [CrossRef]
23. Udomkun, P.; Wiredu, A.N.; Nagle, M.; Müller, J.; Vanlauwe, B.; Bandyopadhyay, R. Innovative Technologies to Manage Aflatoxins in Foods and Feeds and the Profitability of Application–A Review. *Food Control* **2017**, *76*, 127–138. [CrossRef]
24. Varga, J.; Frisvad, J.C.; Samson, R.A. Two New Aflatoxin Producing Species, and an Overview of *Aspergillus* Section *Flavi*. *Stud. Mycol.* **2011**, *69*, 57–80. [CrossRef]
25. Frisvad, J.C.; Hubka, V.; Ezekiel, C.N.; Hong, S.-B.; Novakova, A.; Chen, A.J.; Arzanlou, M.; Larsen, T.O.; Sklenar, F.; Mahakarnchanakul, W.; et al. Taxonomy of *Aspergillus* Section *Flavi* and Their Production of Aflatoxins, Ochratoxins and Other Mycotoxins. *Stud. Mycol.* **2019**, *93*, 1–63. [CrossRef] [PubMed]
26. Norlia, M.; Jinap, S.; Nor-Khaizura, M.A.R.; Radu, S.; Samsudin, N.I.P.; Azri, F.A. *Aspergillus* Section *Flavi* and Aflatoxins: Occurrence, Detection, and Identification in Raw Peanuts and Peanut-Based Products along the Supply Chain. *Front. Microbiol.* **2019**, *10*, 1–17. [CrossRef]
27. Godet, M.; Munaut, F. Molecular Strategy for Identification in *Aspergillus* Section *Flavi*. *FEMS Microbiol. Lett.* **2010**, *304*, 157–168. [CrossRef]
28. Singh, P.; Orbach, M.J.; Cotty, P.J. *Aspergillus Texensis*: A Novel Aflatoxin Producer with S Morphology from the United States. *Toxins* **2018**, *10*, 513. [CrossRef]

29. Singh, P.; Callicott, K.A.; Orbach, M.J.; Cotty, P.J. Molecular Analysis of S-Morphology Aflatoxin Producers from the United States Reveals Previously Unknown Diversity and Two New Taxa. *Front. Microbiol.* **2020**, *11*, 1–16. [CrossRef] [PubMed]
30. Mom, M.P.; Romero, S.M.; Larumbe, A.G.; Iannone, L.; Comerio, R.; Smersu, C.S.S.; Simón, M.; Vaamonde, G. Microbiological Quality, Fungal Diversity and Aflatoxins Contamination in Carob Flour (*Prosopis Flexuosa*). *Int. J. Food Microbiol.* **2020**, *326*, 1–7. [CrossRef]
31. Nesbitt, B.F.; O'Kelly, J.; Sargeant, K.; Sheridan, A.N.N. *Aspergillus Flavus* and Turkey X Disease. Toxic Metabolites of *Aspergillus Flavus*. *Nature* **1962**, *195*, 1062–1063. [CrossRef] [PubMed]
32. Varga, J.; Frisvad, J.; Samson, R. A Reappraisal of Fungi Producing Aflatoxins. *World Mycotoxin J.* **2009**, *2*, 263–277. [CrossRef]
33. Codner, R.C.; Sargeant, K.; Yeo, R. Production of Aflatoxin by the Culture of Strains of *Aspergillus Flavus-Oryzae* on Sterilized Peanuts. *Biotechnol. Bioeng.* **1963**, *5*, 185–192. [CrossRef]
34. Buchanan, R.L.; Ayres, J.C. Effect of Sodium Acetate on Growth and Aflatoxin Production by *Aspergillus Parasiticus* NRRL 2999. *J. Food. Sci.* **1976**, *41*, 128–132. [CrossRef]
35. Schmidt-Heydt, M.; Rüfer, C.E.; Abdel-Hadi, A.; Magan, N.; Geisen, R. The Production of Aflatoxin B1 or G1 by *Aspergillus Parasiticus* at Various Combinations of Temperature and Water Activity Is Related to the Ratio of AflS to AflR Expression. *Mycotoxin Res.* **2010**, *26*, 241–246. [CrossRef]
36. Kurtzman, C.P.; Horn, B.W.; Hesseltine, C.W. *Aspergillus Nomius*, a New Aflatoxin-Producing Species Related to *Aspergillus Flavus* and *Aspergillus Tamarii*. *Antonie Leeuwenhoek* **1987**, *53*, 147–158. [CrossRef]
37. Yunes, N.B.S.; Oliveira, R.C.; Reis, T.A.; Baquião, A.C.; Rocha, L.O.; Correa, B. Effect of Temperature on Growth, Gene Expression, and Aflatoxin Production by *Aspergillus Nomius* Isolated from Brazil Nuts. *Mycotoxin Res.* **2020**, *36*, 173–180. [CrossRef] [PubMed]
38. Chen, A.J.; Frisvad, J.C.; Sun, B.D.; Varga, J.; Kocsubé, S.; Dijksterhuis, J.; Kim, D.H.; Hong, S.-B.; Houbraken, J.; Samson, R.A. *Aspergillus* Section *Nidulantes* (Formerly *Emericella*): Polyphasic Taxonomy, Chemistry and Biology. *Stud. Mycol.* **2016**, *84*, 1–118. [CrossRef] [PubMed]
39. Frisvad, J.C.; Skouboe, P.; Samson, R.A. Taxonomic Comparison of Three Different Groups of Aflatoxin Producers and a New Efficient Producer of Aflatoxin B1, Sterigmatocystin and 3-O-Methylsterigmatocystin, *Aspergillus Rambellii* Sp. *Nov. Syst. Appl. Microbiol.* **2005**, *28*, 442–453. [CrossRef] [PubMed]
40. Samson, R.A.; Visagie, C.M.; Houbraken, J.; Hong, S.-B.; Hubka, V.; Klaassen, C.H.W.; Perrone, G.; Seifert, K.A.; Susca, A.; Tanney, J.B.; et al. Phylogeny, Identification and Nomenclature of the Genus *Aspergillus*. *Stud. Mycol.* **2014**, *78*, 141–173. [CrossRef]
41. Frisvad, J.C.; Samson, R.A.; Smedsgaard, J. *Emericella Astellata*, a New Producer of Aflatoxin B1, B2 and Sterigmatocystin. *Lett. Appl. Microbiol.* **2004**, *38*, 440–445. [CrossRef]
42. Zhang, L.-C.; Chen, J.; Lin, W.-H.; Guo, S.-X. A New Species of Emericella from Tibet, China. *Mycotaxon* **2013**, *125*, 131–138. [CrossRef]
43. Hubka, V.; Novakova, A.; Peterson, S.W.; Frisvad, J.C.; Sklenář, F.; Matsuzawa, T.; Kubatova, A.; Kolarik, M. A Reappraisal of *Aspergillus* Section *Nidulantes* with Descriptions of Two New Sterigmatocystin-Producing Species. *Plant Syst. Evol.* **2016**, *302*, 1267–1299. [CrossRef]
44. Zalar, P.; Frisvad, J.C.; Gunde-Cimerman, N.; Varga, J.; Samson, R.A. Four New Species of *Emericella* from the Mediterranean Region of Europe. *Mycologia* **2008**, *100*, 779–795. [CrossRef] [PubMed]
45. Frisvad, J.C.; Samson, R.A. *Emericella Venezuelensis*, a New Species with Atellate Ascospores Producing Sterigmatocystin and Aflatoxin B1. *Syst. Appl. Microbiol.* **2004**, *27*, 672–680. [CrossRef]
46. Klich, M.A.; Mullaney, E.J.; Daly, C.B.; Cary, J.W. Molecular and Physiological Aspects of Aflatoxin and Sterigmatocystin Biosynthesis by *Aspergillus Tamarii* and *A. Ochraceoroseus*. *Appl. Microbiol. Biotechnol.* **2000**, *53*, 605–609. [CrossRef]
47. Cary, J.W.; Ehrlich, K.C.; Beltz, S.B.; Harris-Coward, P.; Klich, M.A. Characterization of the *Aspergillus Ochraceoroseus* Aflatoxin/Sterigmatocystin Biosynthetic Gene Cluster. *Mycologia* **2009**, *101*, 352–362. [CrossRef]
48. Hussein, H.S.; Brasel, J.M. Toxicity, Metabolism, and Impact of Mycotoxins on Humans and Animals. *Toxicology* **2001**, *167*, 101–134. [CrossRef]
49. Park, D.L.; Njapau, H.; Boutrif, E. Minimizing Risks Posed by Mycotoxins Utilizing the HACCP Concept. *Food Nutr. Agric.* **1999**, *3*, 49–54.
50. Eskola, M.; Kos, G.; Elliott, C.T.; Hajšlová, J.; Mayar, S.; Krska, R. Worldwide Contamination of Food-Crops with Mycotoxins: Validity of the Widely Cited 'FAO Estimate' of 25%. *Crit. Rev. Food Sci. Nutr.* **2020**, *60*, 2773–2789. [CrossRef] [PubMed]
51. Streit, E.; Naehrer, K.; Rodrigues, I.; Schatzmayr, G. Mycotoxin Occurrence in Feed and Feed raw Materials Worldwide: Long-Term Analysis With Special Focus on Europe and Asia. *J. Sci. Food Agric.* **2013**, *93*, 2892–2899. [CrossRef]
52. Zinedine, A.; Mañes, J. Occurrence and Legislation of Mycotoxins in Food and Feed from Morocco. *Food Control* **2009**, *20*, 334–344. [CrossRef]
53. Mahato, D.K.; Lee, K.E.; Kamle, M.; Devi, S.; Dewangan, K.; Kumar, P.; Kang, S.G. Aflatoxins in Food and Feed: An Overview on Prevalence, Detection and Control Strategies. *Front. Microbiol.* **2019**, *10*, 1–10. [CrossRef] [PubMed]
54. Heussner, A.H.; Bingle, L.E.H. Comparative Ochratoxin Toxicity: A Review of the Available Data. *Toxins* **2015**, *7*, 4253–4282. [CrossRef]
55. Huong, B.T.M.; Do, T.T.; Madsen, H.; Brimer, L.; Dalsgaard, A. Aflatoxins and Fumonisins in Rice and Maize Staple Cereals in Northern Vietnam and Dietary Exposure in Different Ethnic Groups. *Food Control* **2016**, *70*, 191–200. [CrossRef]

56. Food and Agriculture Organization. Basic Facts on the World Cereal Situation. 1996. Available online: http://www.fao.org/3/w1690e/w1690e16.htm#I1 (accessed on 13 January 2021).
57. Klaassen, C. *Casarett & Doull's Toxicology: The Basic Science of Poisons*, 7th ed.; McGraw-Hill: New York, NY, USA, 2007; ISBN 0-07-147051-4.
58. Marroquín-Cardona, A.G.; Johnson, N.M.; Phillips, T.D.; Hayes, A.W. Mycotoxins in a Changing Global Environment–a Review. *Food Chem. Toxicol.* **2014**, *69*, 220–230. [CrossRef]
59. Botana, L.M.; Sainz, M.J. (Eds.) *Climate Change and Mycotoxins*; Walter de Gruyter GmbH: Berlin, Germany, 2015; ISBN 978-3-11-033305-3.
60. Karlovsky, P.; Suman, M.; Berthiller, F.; De Meester, J.; Eisenbrand, G.; Perrin, I.; Oswald, I.P.; Speijers, G.; Chiodini, A.; Recker, T. Impact of Food Processing and Detoxification Treatments on Mycotoxin Contamination. *Mycotoxin Res.* **2016**, *32*, 179–205. [CrossRef]
61. Iha, M.H.; Trucksess, M.W. Management of Mycotoxins in Spices. *J. AOAC Int.* **2019**, *102*, 1732–1739. [CrossRef]
62. European Commission. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Off. J. Eur. Union* **2006**, *364*, 5–24.
63. Savić, Z.; Dudaš, T.; Loc, M.; Grahovac, M.; Budakov, D.; Jajić, I.; Krstović, S.; Barošević, T.; Krska, R.; Sulyok, M.; et al. Biological Control of Aflatoxin in Maize Grown in Serbia. *Toxins* **2020**, *12*, 162. [CrossRef] [PubMed]
64. Pitt, J.I. The Pros and Cons of Using Biocontrol by Competitive Exclusion as a Means for Reducing Aflatoxin in Maize in Africa. *World Mycotoxin J.* **2019**, *12*, 103–112. [CrossRef]
65. Senghor, L.A.; Ortega-Beltran, A.; Atehnkeng, J.; Callicott, K.A.; Cotty, P.J.; Bandyopadhyay, R. The Atoxigenic Biocontrol Product Aflasafe SN01 Is a Valuable Tool to Mitigate Aflatoxin Contamination of Both Maize and Groundnut Cultivated in Senegal. *Plant Dis.* **2019**, *104*, 510–520. [CrossRef]
66. Agbetiameh, D.; Ortega-Beltran, A.; Awuah, R.T.; Atehnkeng, J.; Elzein, A.; Cotty, P.J.; Bandyopadhyay, R. Field Efficacy of Two Atoxigenic Biocontrol Products for Mitigation of Aflatoxin Contamination in Maize and Groundnut in Ghana. *Biol. Control* **2020**, *150*, 1–13. [CrossRef] [PubMed]
67. Martinez-Miranda, M.M.; Rosero-Moreano, M.; Taborda-Ocampo, G. Occurrence, Dietary Exposure and Risk Assessment of Aflatoxins in Arepa, Bread and Rice. *Food Control* **2019**, *98*, 359–366. [CrossRef]
68. Pickova, D.; Ostry, V.; Malir, J.; Toman, J.; Malir, F. A Review on Mycotoxins and Microfungi in Spices in the Light of the Last Five Years. *Toxins* **2020**, *12*, 789. [CrossRef]
69. Kabak, B. Aflatoxins in Foodstuffs: Occurrence and Risk Assessment in Turkey. *J. Food Compost. Anal.* **2021**, *96*, 103734. [CrossRef]
70. International Agency for Research on Cancer. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Traditional Herbal Medicine, Some Mycotoxins, Naphthalene and Strene*; IARC Press: Lyon, France, 2002; Volume 82, ISBN 92-832-1282-7.
71. Warth, B.; Braun, D.; Ezekiel, C.N.; Turner, P.C.; Degen, G.H.; Marko, D. Biomonitoring of Mycotoxins in Human Breast Milk: Current State and Future Perspectives. *Chem. Res. Toxicol.* **2016**, *29*, 1087–1097. [CrossRef]
72. Fakhri, Y.; Rahmani, J.; Oliveira, C.A.F.; Franco, L.T.; Corassin, C.H.; Saba, S.; Rafique, J.; Khaneghah, A.M. Aflatoxin M1 in Human Breast Milk: A Global Systematic Review, Meta-Analysis, and Risk Assessment Study (Monte Carlo Simulation). *Trends Food Sci. Technol.* **2019**, *88*, 333–342. [CrossRef]
73. Fink-Gremmels, J. Mycotoxins in Cattle Feeds and Carry-over to Dairy Milk: A Review. *Food Addit. Contam.* **2008**, *25*, 172–180. [CrossRef] [PubMed]
74. Herzallah, S.M. Determination of Aflatoxins in Eggs, Milk, Meat and Meat Products Using HPLC Fluorescent and UV Detectors. *Food Chem.* **2009**, *114*, 1141–1146. [CrossRef]
75. Hassan, H.F.; Kassaify, Z. The Risks Associated with Aflatoxins M1 Occurrence in Lebanese Dairy Products. *Food Control* **2014**, *37*, 68–72. [CrossRef]
76. Benkerroum, N. Mycotoxins in Dairy Products: A Review. *Int. Dairy J.* **2016**, *62*, 63–75. [CrossRef]
77. Mulunda, M.; Ngoma, L.; Nyirenda, M.; Motsei, L.; Bakunzi, F. A Decade of Aflatoxin M1 Surveillance in Milk and Dairy Products in Developing Countries (2001-2011): A Review. In *Mycotoxin and Food Safety in Developing Countries*; Hussaini, A.M., Ed.; IntechOpen: Rijeka, Croatia, 2013; pp. 39–60. ISBN 978-953-51-1096-5.
78. Rojas-Marín, V.; Carvajal-Moreno, M.; González-Villaseñor, M.C.; García-Hernández, E.A.; González–Mendoza, A. Presence of Aflatoxin Carcinogens in Fresh and Mature Cheeses. *Pharm. Anal. Acta* **2018**, *9*, 1–6. [CrossRef]
79. Sengun, I.; Yaman, D.; Gonul, S. Mycotoxins and Mould Contamination in Cheese: A Review. *World Mycotoxin J.* **2008**, *1*, 291–298. [CrossRef]
80. Sanders, T.H.; Cole, R.J.; Blankenship, P.D.; Dorner, J.W. Aflatoxin Contamination of Peanuts from Plants Drought Stressed in Pod or Root Zones. *Peanut Sci.* **1993**, *20*, 5–8. [CrossRef]
81. Williams, J.H.; Phillips, T.D.; Jolly, P.E.; Stiles, J.K.; Jolly, C.M.; Aggarwal, D. Human Aflatoxicosis in Developing Countries: A Review of Toxicology, Exposure, Potential Health Consequences, and Interventions. *Am. J. Clin. Nutr.* **2004**, *80*, 1106–1122. [CrossRef] [PubMed]
82. Paterson, R. Further Mycotoxin Effects from Climate Change. *Food Res. Int.* **2011**, *44*, 2555–2566. [CrossRef]
83. Miraglia, M.; Marvin, H.J.P.; Kleter, G.A.; Battilani, P.; Brera, C.; Coni, E.; Cubadda, F.; Croci, L.; De Santis, B.; Dekkers, S.; et al. Climate Change and Food Safety: An Emerging Issue with Special Focus on Europe. *Food Chem. Toxicol.* **2009**, *47*, 1009–1021. [CrossRef]

84. Battilani, P.; Toscano, P.; Van der Fels-Klerx, H.J.; Moretti, A.; Leggieri, M.C.; Brera, C.; Rortais, A.; Goumperis, T.; Robinson, T. Aflatoxin B1 Contamination in Maize in Europe Increases Due to Climate Change. *Sci. Rep.* **2016**, *6*, 1–7. [CrossRef]
85. Lewis, L.; Onsongo, M.; Njapau, H.; Schurz-Rogers, H.; Luber, G.; Kieszak, S.; Nyamongo, J.; Backer, L.; Dahiye, A.M.; Misore, A. Aflatoxin Contamination of Commercial Maize Products during an Outbreak of Acute Aflatoxicosis in Eastern and Central Kenya. *Environ. Health Perspect.* **2005**, *113*, 1763–1767. [CrossRef] [PubMed]
86. Devegowda, G.; Raju, M.V.L.N.; Afzali, N.; Swamy, H.V.L.N. Mycotoxin Picture Worldwide: Novel Solutions for Their Counteraction. In *Biotechnology in Feed Industry Proceedings of the Alltech's 14th Annual Symposium*; Lyons, T.P., Jacques, K.A., Eds.; Nottingham University Press: Nottingham, UK, 1998; ISBN 978-1-897676-66-0.
87. Wild, C.P.; Miller, J.D.; Groopman, J.D. *Mycotoxin Control in Low-and Middle-Income Countries*; International Agency for Research on Cancer: Lyon, France, 2015; Volume 9, ISBN 978-92-832-2510-2.
88. Liverpool-Tasie, L.S.O.; Turna, N.S.; Ademola, O.; Obadina, A.; Wu, F. The Occurrence and Co-occurrence of Aflatoxin and Fumonisin along the Maize Value Chain in Southwest Nigeria. *Food Chem. Toxicol.* **2019**, *129*, 458–465. [CrossRef] [PubMed]
89. Mahuku, G.; Nzioki, H.S.; Mutegi, C.; Kanampiu, F.; Narrod, C.; Makumbi, D. Pre-Harvest Management Is a Critical Practice for Minimizing Aflatoxin Contamination of Maize. *Food Control* **2019**, *96*, 219–226. [CrossRef]
90. Sserumaga, J.P.; Ortega-Beltran, A.; Wagacha, J.M.; Mutegi, C.K.; Bandyopadhyay, R. Aflatoxin-Producing Fungi Associated with Pre-Harvest Maize Contamination in Uganda. *Int. J. Food Microbiol.* **2020**, *313*, 1–8. [CrossRef]
91. Kamika, I.; Ngboula, K.-T.-N.; Tekere, M. Occurrence of Aflatoxin Contamination in Maize throughout the Supply Chain in the Democratic Republic of Congo. *Food Control* **2016**, *69*, 292–296. [CrossRef]
92. Dadzie, M.A.; Oppong, A.; Ofori, K.; Eleblu, J.S.; Ifie, E.B.; Blay, E.; Obeng–Bio, E.; Appiah-Kubi, Z.; Warburton, M.L. Distribution of *Aspergillus Flavus* and Aflatoxin Accumulation in Stored Maize Grains across Three Agro-Ecologies in Ghana. *Food Control* **2019**, *104*, 91–98. [CrossRef]
93. Kachapulula, P.W.; Akello, J.; Bandyopadhyay, R.; Cotty, P.J. Aflatoxin Contamination of Groundnut and Maize in Zambia: Observed and Potential Concentrations. *J. Appl. Microbiol.* **2017**, *122*, 1471–1482. [CrossRef] [PubMed]
94. Cardona, T.; Ilangantileke, S.; Noomhorm, A. Aflatoxin research on grain in Asia: Its problems and possible solutions. In *Mycotoxin Prevention and Control in Foodgrains*; Semple, R.L., Frio, A.S., Hicks, P.A., Lozare, J.V., Eds.; FAO: Bankok, Thailand, 1991; pp. 309–322.
95. Bilgrami, K.S.; Sinha, K.K. Aflatoxin in India I. In *Aflatoxin in Maize*; Zuber, M.S., Lillehoj, E.B., Renfro, B.L., Eds.; CIMMYT, UNDP and USAID: El Batan, Mexico, 1986; pp. 349–358. ISBN 968-6127-12-7.
96. Do, T.H.; Tran, S.C.; Le, C.D.; Nguyen, H.-B.T.; Le, P.-T.T.; Le, H.-H.T.; Le, T.D.; Thai-Nguyen, H.-T. Dietary Exposure and Health Risk Characterization of Aflatoxin B1, Ochratoxin A, Fumonisin B1, and Zearalenone in Food from Different Provinces in Northern Vietnam. *Food Control* **2020**, *112*, 107108. [CrossRef]
97. Ali, N. Aflatoxins in Rice: Worldwide Occurrence and Public Health Perspectives. *Toxicol. Rep.* **2019**, *6*, 1188–1197. [CrossRef] [PubMed]
98. FAOSTAT. Food and Agriculture Organization of the United Nations. Available online: http://www.fao.org/faostat/en/#data/QC/visualize (accessed on 25 February 2020).
99. Toteja, G.S.; Mukherjee, A.; Diwakar, S.; Singh, P.; Saxena, B.N.; Sinha, K.K.; Sinha, A.K.; Kumar, N.; Nagaraja, K.V.; Bai, G. Aflatoxin B1 Contamination of Parboiled Rice Samples Collected from Different States of India: A Multi-Centre Study. *Food Addit. Contam.* **2006**, *23*, 411–414. [CrossRef]
100. Bandara, J.; Vithanege, A.K.; Bean, G.A. Occurrence of Aflatoxins in Parboiled Rice in Sri Lanka. *Mycopathologia* **1991**, *116*, 65–70. [CrossRef]
101. Panrapee, I.; Phakpoom, K.; Thanapoom, M.; Nampeung, A.; Warapa, M. Exposure to Aflatoxin B1 in Thailand by Consumption of Brown and Color Rice. *Mycotoxin Res.* **2016**, *32*, 19–25. [CrossRef] [PubMed]
102. Abdullah, N.; Nawawi, A.; Othman, I. Survey of Fungal Counts and Natural Occurrence of Aflatoxins in Malaysian Starch-Based Foods. *Mycopathologia* **1998**, *143*, 53–58. [CrossRef] [PubMed]
103. Aydin, A.; Aksu, H.; Gunsen, U. Mycotoxin Levels and Incidence of Mould in Turkish Rice. *Environ. Monit. Assess.* **2011**, *178*, 271–280. [CrossRef] [PubMed]
104. Lai, X.; Liu, R.; Ruan, C.; Zhang, H.; Liu, C. Occurrence of Aflatoxins and Ochratoxin A in Rice Samples from Six Provinces in China. *Food Control* **2015**, *50*, 401–404. [CrossRef]
105. Nuss, E.T.; Tanumihardjo, S.A. Maize: A Paramount Staple Crop in the Context of Global Nutrition. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 417–436. [CrossRef]
106. Torres, O.; Matute, J.; Gelineau-van Waes, J.; Maddox, J.R.; Gregory, S.G.; Ashley-Koch, A.E.; Showker, J.L.; Voss, K.A.; Riley, R.T. Human Health Implications from Co-exposure to Aflatoxins and Fumonisins in Maize-Based Foods in Latin America: Guatemala as a Case Study. *World Mycotoxin J.* **2015**, *8*, 143–159. [CrossRef]
107. Sandoval, I.G.; Wesseling, S.; Rietjens, I.M. Aflatoxin B1 in Nixtamalized Maize in Mexico; Occurrence and Accompanying Risk Assessment. *Toxicol. Rep.* **2019**, *6*, 1135–1142. [CrossRef]
108. Aristil, J.; Venturini, G.; Maddalena, G.; Toffolatti, S.L.; Spada, A. Fungal Contamination and Aflatoxin Content of Maize, Moringa and Peanut Foods from Rural Subsistence Farms in South Haiti. *J. Stored Prod. Res.* **2020**, *85*, 1–8. [CrossRef]
109. Oliveira, M.S.; Rocha, A.; Sulyok, M.; Krska, R.; Mallmann, C.A. Natural Mycotoxin Contamination of Maize (*Zea Mays* L.) in the South Region of Brazil. *Food Control* **2017**, *73*, 127–132. [CrossRef]

110. Zuki-Orozco, B.A.; Batres-Esquivel, L.E.; Ortiz-Pérez, M.D.; Juárez-Flores, B.I.; Díaz-Barriga, F. Aflatoxins Contamination in Maize Products from Rural Communities in San Luis Potosi, Mexico. *Ann. Glob. Health* **2018**, *84*, 300–305. [CrossRef] [PubMed]
111. Morales-Moo, T.; Hernández-Camarillo, E.; Carvajal-Moreno, M.; Vargas-Ortiz, M.; Robles-Olvera, V.; Salgado-Cervantes, M.A. Human Health Risk Associated with the Consumption of Aflatoxins in Popcorn. *Risk Manag. Healthc. Policy* **2020**, *13*, 2583–2591. [CrossRef]
112. Foerster, C.; Muñoz, K.; Delgado-Rivera, L.; Rivera, A.; Cortés, S.; Müller, A.; Arriagada, G.; Ferreccio, C.; Rios, G. Occurrence of Relevant Mycotoxins in Food Commodities Consumed in Chile. *Mycotoxin Res.* **2020**, *36*, 63–72. [CrossRef]
113. Katsurayama, A.M.; Martins, L.M.; Iamanaka, B.T.; Fungaro, M.H.P.; Silva, J.J.; Frisvad, J.C.; Pitt, J.I.; Taniwaki, M.H. Occurrence of *Aspergillus* Section *Flavi* and Aflatoxins in Brazilian Rice: From Field to Market. *Int. J. Food Microbiol.* **2018**, *266*, 213–221. [CrossRef] [PubMed]
114. Blaney, B.J. Mycotoxin Surveillance in Australia. *J. Appl. Toxicol.* **1982**, *2*, 83–87. [CrossRef]
115. Pitt, J.I.; Hocking, A.D. Mycotoxins in Australia: Biocontrol of Aflatoxin in Peanuts. *Mycopathologia* **2006**, *162*, 233–243. [CrossRef] [PubMed]
116. Blaney, B.J.; Ramsey, M.D.; Tyler, A.L. Mycotoxins and Toxigenic Fungi in Insect-Damaged Maize Harvested during 1983 in Far North Queensland. *Aust. J. Agric. Res.* **1986**, *37*, 235–244. [CrossRef]
117. Blaney, B.J.; K'Keeffe, K.; Bricknell, L.K. Managing Mycotoxins in Maize: Case Studies. *Aust. J. Exp. Agric.* **2008**, *48*, 351–357. [CrossRef]
118. Blaney, B.J. Aflatoxin Survey of Maize from the 1978 Crop in the South Burnett Region of Queensland [*Aspergillus* Fungal Infection; Stock Feed]. *Qld. J. Agric. Animal. Sci.* **1981**, *38*, 7–12.
119. Blaney, B.J. Mycotoxins in crops grown in different climatic regions of Queensland. In *Trichothecenes and Other Mycotoxins*; Lacey, J., Ed.; John Wiley and Sons Ltd.: Chichester, UK, 1985; pp. 97–108. ISBN 978-0471907510.
120. Chauhan, Y.; Wright, G.; Rachaputi, N.C. Modelling Climatic Risks of Aflatoxin Contamination in Maize. *Aust. J. Exp. Agric.* **2008**, *48*, 358–366. [CrossRef]
121. Kennedy, I.R.; (Univesrity of Sydney, Sydney, Australia). Personal communication, 2017.
122. Mazumder, P.M.; Sasmal, D. Mycotoxins–Limits and Regulations. *Anc. Sci. Life* **2001**, *20*, 1–19. [PubMed]
123. Food and Agriculture Organization. *Worldwide Regulations for Mycotoxins in Food and Feed in 2003*; Food and Agriculture Organization of the United Nations: Roma, Italy, 2004; ISBN 92-5-105162-3.
124. Blanc, M. Sampling: The Weak Link in the Sanitary Quality Control System of Agricultural Products. *Mol. Nutr. Food Res.* **2006**, *50*, 473–479. [CrossRef]
125. Codex Alimentarius Commission. Mycotoxins. In *Codex Alimentarius: General Standard for Contaminants and Toxins in Food and Feed (CXS 193-1995)*; Food and Agriculture Organization/World Health Organization: Geneva, Switzerland, 2019; pp. 13–44.
126. European Commission. Commission Regulation (EU) No. 165/2010 of 26 February 2010 Amending Regulation (EC) No 1881/2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs as Regards Aflatoxins. *Off. J. Eur. Union* **2010**, *50*, 8–12.
127. Anukul, N.; Vangnai, K.; Mahakarnchanakul, W. Significance of Regulation Limits in Mycotoxin Contamination in Asia and Risk Management Programs at the National Level. *J. Food Drug. Anal.* **2013**, *21*, 227–241. [CrossRef]
128. United States Department of Agriculture. China Releases Standard for Maximum Levels of Mycotoxins in Foods. Available online: https://gain.fas.usda.gov/Recent%20GAIN%20Publications/China%20Releases%20Standard%20for%20Maximum%20Levels%20of%20Mycotoxins%20in%20Foods%20_Beijing_China%20-%20Peoples%20Republic%20of_5-9-2018.pdf (accessed on 10 February 2020).
129. Mohd-Redzwan, S.; Jamaluddin, R.; Abd-Mutalib, M.S.; Ahmad, Z. A Mini Review on Aflatoxin Exposure in Malaysia: Past, Present and Future. *Front. Microbiol.* **2013**, *4*, 1–8. [CrossRef]
130. US Food and Drug Administration. CPG Sec. 570.200 Brazil Nuts-Adulteration with Aflatoxin. Available online: https://www.fda.gov/media/72053/download (accessed on 9 February 2020).
131. US Food and Drug Administration. CPG Sec. 555.400 Foods-Adulteration with Aflatoxin. Available online: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-555400-foods-adulteration-aflatoxin (accessed on 9 February 2020).
132. US Food and Drug Administration. CPG Sec. 570.375 Aflatoxin in Peanuts and Peanut Products. Available online: https://www.fda.gov/media/72073/download (accessed on 9 February 2020).
133. US Food and Drug Administration. CPG Sec. 570.500 Pistachio Nuts-Aflatoxin Adulteration. Available online: https://www.fda.gov/media/72084/download (accessed on 9 February 2020).
134. Ncube, J.; Maphosa, M. Current State of Knowledge on Groundnut Aflatoxins and Their Management from a Plant Breeding Perspective: Lessons for Africa. *Sci. Afr.* **2020**, *7*, 1–7. [CrossRef]
135. Ministério da Saúde/Agência Nacional de Vigilância Sanitária. Resolução RDC nº 7, de 18 de fevereiro de 2011. Available online: http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2011/res0007_18_02_2011_rep.html (accessed on 25 February 2020).
136. Government of Canada. List of Contaminants and Other Adulterating Substances in Foods. Available online: https://www.canada.ca/en/health-canada/services/food-nutrition/food-safety/chemical-contaminants/contaminants-adulterating-substances-foods.html (accessed on 25 February 2020).
137. Australia Government New Zealand Food Standards Code–Schedule 19–Maximum Levels of Contaminants and Natural Toxicants. Available online: https://www.legislation.gov.au/Details/F2017C00333 (accessed on 25 February 2020).

138. US Food and Drug Administration. CPG Sec. 527.400 Whole Milk, Lowfat Milk, Skim Milk-Aflatoxin M1. Available online: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-527400-whole-milk-lowfat-milk-skim-milk-aflatoxin-m1 (accessed on 9 February 2020).
139. Ministry of Food and Drug Safety. Food Code. Available online: https://www.mfds.go.kr/eng/brd/m_15/view.do?seq=69982 (accessed on 10 February 2020).
140. Food and Agriculture Organization. Preventing Food Safety Emergencies: INFOSAN, FAO/WHO International Food Safety Authorities Network. Available online: http://www.fao.org/3/a-i8024e.pdf (accessed on 29 May 2020).
141. Savelli, C.J.; Bradshaw, A.; Ben Embarek, P.; Mateus, C. The FAO/WHO International Food Safety Authorities Network in Review, 2004-2018: Learning from the Past and Looking to the Future. *Foodborne Pathog. Dis.* **2019**, *16*, 480–488. [CrossRef]
142. International Food Safety Authorities Network. *INFOSAN Activity Report 2016/2017*; World Health Organization and Food and Agriculture Organization of the United Nations: Geneva, Switzerland, 2018; ISBN 978-92-4-151464-4.
143. Pigłowski, M. Food Hazards on the European Union Market: The Data Analysis of the Rapid Alert System for Food and Feed. *Food Sci. Nutr.* **2020**, *8*, 1603–1627. [CrossRef]
144. Rapid Alert System for Food and Feed. Portal Database. Available online: https://webgate.ec.europa.eu/rasff-window/portal/ (accessed on 9 February 2020).
145. World Health Organization. Aflatoxins. Available online: https://www.who.int/foodsafety/FSDigest_Aflatoxins_EN.pdf (accessed on 9 February 2020).

Příloha 4

Aflatoxins: History, significant milestones, recent data on their toxicity and ways to mitigation

toxins

MDPI

Review

Aflatoxins: History, Significant Milestones, Recent Data on Their Toxicity and Ways to Mitigation

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Abstract: In the early 1960s the discovery of aflatoxins began when a total of 100,000 turkey poults died by hitherto unknown turkey "X" disease in England. The disease was associated with Brazilian groundnut meal affected by *Aspergillus flavus*. The toxin was named *Aspergillus flavus* toxin—aflatoxin. From the point of view of agriculture, aflatoxins show the utmost importance. Until now, a total of 20 aflatoxins have been described, with B_1, B_2, G_1, and G_2 aflatoxins being the most significant. Contamination by aflatoxins is a global health problem. Aflatoxins pose acutely toxic, teratogenic, immunosuppressive, carcinogenic, and teratogenic effects. Besides food insecurity and human health, aflatoxins affect humanity at different levels, such as social, economical, and political. Great emphasis is placed on aflatoxin mitigation using biocontrol methods. Thus, this review is focused on aflatoxins in terms of historical development, the principal milestones of aflatoxin research, and recent data on their toxicity and different ways of mitigation.

Keywords: turkey "X" disease; aflatoxin; milestones; toxicity; mitigation

Key Contribution: In the year 2020 it was exactly 60 years since aflatoxins were discovered. For humans, aflatoxins are considered the most important and deleterious mycotoxins. Aflatoxins are among the five most important mycotoxins in agriculture. The numerous effective pre-harvest and post-harvest biocontrol methods to aflatoxin mitigation have been applied. Therefore, worldwide, aflatoxins pose an ongoing and still unsolved problem.

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1. Introduction

In 2020, it was 60 years since the discovery of aflatoxins (AFs). AFs began the "second mycotoxicology era" that built on the "previous mycotoxicology era", e.g., ergotism, acute cardiac beriberi, alimentary toxic aleukia, stachybotriotoxicosis, "mouldy corn toxicosis"—equine leucoencephalomalacia [1–4].

2. A History of Aflatoxin Discovery

In the late 1950s and early 1960s, a new so far unknown turkey disease, characterized by heavy mortality, was identified in England. After the turkey disease outbreak of unknown nature and aetiology (the turkey "X" disease), the discovery of AFs began. A total of 100,000 turkeys died of so-called turkey "X" disease after being fed with contaminated Brazilian groundnut meal on a poultry farm in London [5].

William Percy Blount (Figure 1) was a veterinary scientist and consultant in poultry husbandry and developed a highly effective poultry disease diagnostic service for the customers of a major feed compounding company in England.

Figure 1. William Percy Blount (*1905–$^{+}$1968). Photo: World's Poultry Science Association.

Chief poultry advisor W.P. Blount carried out intensive field and laboratory research, the findings and conclusions of which were published the following year [5]. Many affected turkeys were about 4–6 weeks old, but others were aged 10–16 weeks. Affected turkeys naturally sought the heat of their brooders and then, through weakness, they would sink to the floor and become somnolent, death taking place within 24–48 h. Another characteristic of the disease was the position or the posture adopted by the poults when they died. Mortality varied but was usually high, often with death rates of 50–90% [5]. By the time the disease had subsided, about 500 outbreaks had been reported involving an estimated loss of over 200,000 turkeys [6].

A causal relationship between feed toxicity, Brazilian groundnut meal, and disease has been demonstrated by W.P. Blount, who accurately described the symptoms, especially liver lesions, and subsequently excluded that the disease could be the cause of the infectious agents. The turkey "X" disease had to be differentiated from Non-specific enteritis, Transmissible enteritis, Infectious hepatitis, Newcastle disease, New "virus" infection, and "poisoning" (bacterial, fungal, mineral, vegetable, etc.). Because veterinary examinations for pathogenic microorganisms were generally negative, and as the epidemiological picture was not that which might be associated with an "infection", the final possibility that the turkeys were "poisoned" remained. The histological findings, post mortem in young turkeys that had died suddenly were described in detail in other studies. They also suggested that the disorder was caused by poisoning, but the toxic substance had not been identified [7,8].

W.P. Blount had also excluded the participation of chemical agents and potentially toxic chemicals that are commonly found in poultry feed, either as contaminants (e.g., toxic elements, pesticides, glycosides, alkaloids, natural phytotoxins, etc.), ingredients (a toxic compound in Brazilian groundnut meal), or as a result of dishonest practices. Although his efforts did not lead to the identification of a causative factor, he provided a solid basis for peer scientists to make relatively rapid progress towards the goal [5].

The disease continued in the same area of London and induced deaths among turkeys on farms as a result of previous feeding with Brazilian groundnut from mills carried by the same company, which included groundnut in the composition of the feed. Groundnut meal was later proved as the main suspect [6,9–11]. Similar liver lesions, considered to be the most serious damage, were found during post mortem examination tests on ducklings, chicken, young pheasants, cattle, rats, and pigs fed with Brazilian groundnut meal [6,9,10,12,13].

The rats fed on groundnut meal showed toxic effects with the development of liver cancer [14,15], which was later confirmed by several studies [16–19]. An investigation of the peanut meal determined that it was highly toxic, with a naturally occurring toxic metabolite produced by mold infestations that caused the acute toxic effects in the animals [9]. Although it had been suspected since the end of 1960 that the cause might be a toxin [10], this was not finally established until the end of the following year when it

was demonstrated that metabolites synthesized by some strains of *Aspergillus flavus* Link ex Fries were responsible [20]. Mold contaminant of *Aspergillus flavus* Link ex Fries was identified in the Central Veterinary Laboratory at Weybridge in England. A replica of *A. flavus* identification is shown in Figures 2 and 3.

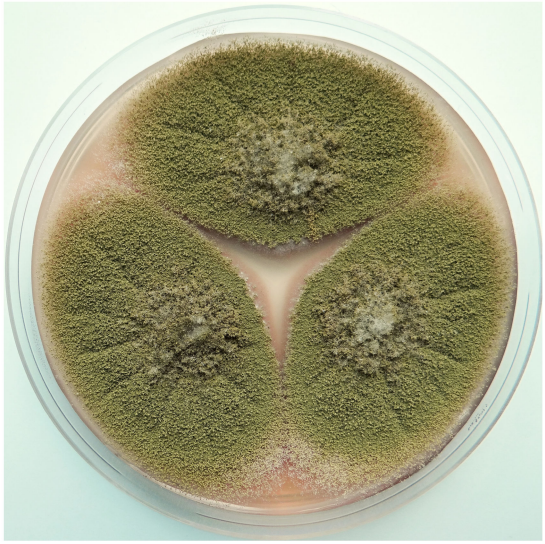

Figure 2. Macroscopic view of *Aspergillus flavus* on Czapek yeast extract agar (25 °C, 7 days). Photo: Vladimir Ostry.

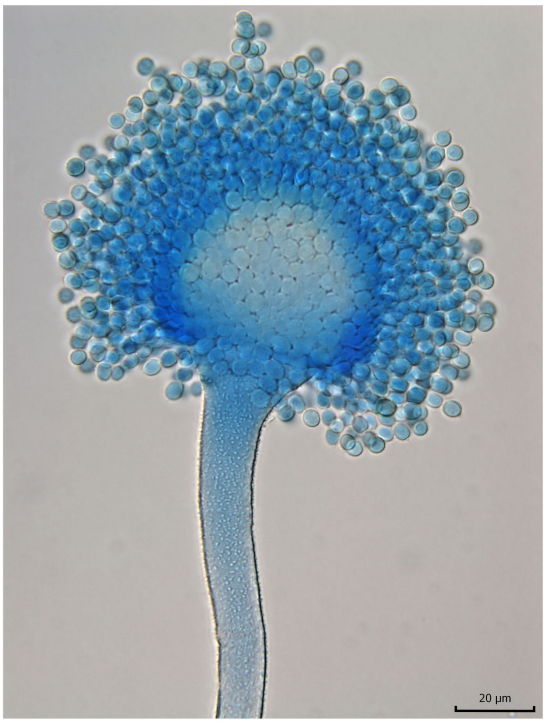

Figure 3. Light microscopy of *Aspergillus flavus* with lactophenol cotton blue. Photo: Vladimir Ostry.

Selected isolates of *A. flavus* were cultivated on mycological media under laboratory conditions. The cultures with overgrown mycelium of *A. flavus* were extracted with $CHCl_3$. Paper chromatography was used in this experiment with the mobile phase butan-1-ol: CH_3COOH. A total of 12.5% of tested extracts emitted a blue fluorescence spot (retention factor (Rf) value of 0.7) under UV light. After oral administration of the corresponding extract to one-day-old ducklings, death was observed within 24 h due to typical symptoms of turkey "X" disease, especially liver damage [10].

The isolation of the toxin crystalline form responsible for the turkey "X" disease has been performed [21]. Because the extract was poorly pure, the blue fluorescent spot probably contained more toxic metabolites. After separation and quantification by thin layer chromatography, two different fluorescent spots were detected, the former emitting blue (Rf 0.6) and the latter emitting green (at slightly lower Rf) fluorescence. *Aspergillus flavus* toxin gave rise to the name aflatoxin. The name aflatoxin was given to the toxic substance, which has since been found to contain several closely related toxic components.

AF discovery was a collective effort, involving a number of experts from various fields of research in veterinary medicine, animal nutrition, toxicology, chemistry, and mycology, and etc.

3. The Milestones in Aflatoxin Research

Research on AFs in all areas of interest is very extensive. Several valuable reviews on AFs have been published in the last decade [1,4,22–30].

These primary and valuable articles served to prepare the principal milestones of AF research. The older data from the years 1960 to 1990 were independently confirmed. Studying the principal milestones allows us to present an overview from several fields of research on AFs from their discovery to 2021.

The principal milestones are summarized in Figures 4–7.

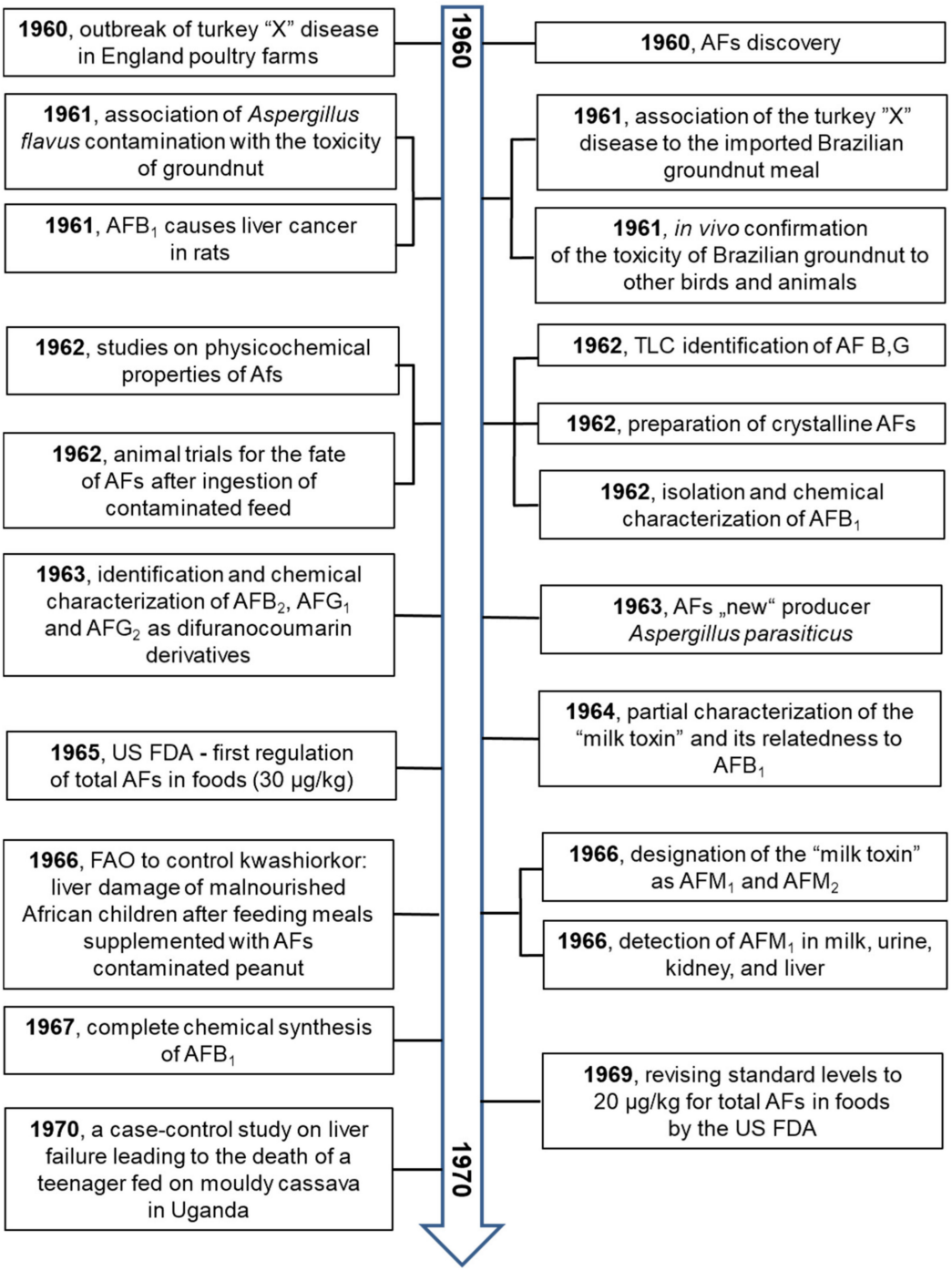

Figure 4. The milestones in aflatoxin research over the years 1960–1970 [5,9,11–15,17,19–21,31–48].

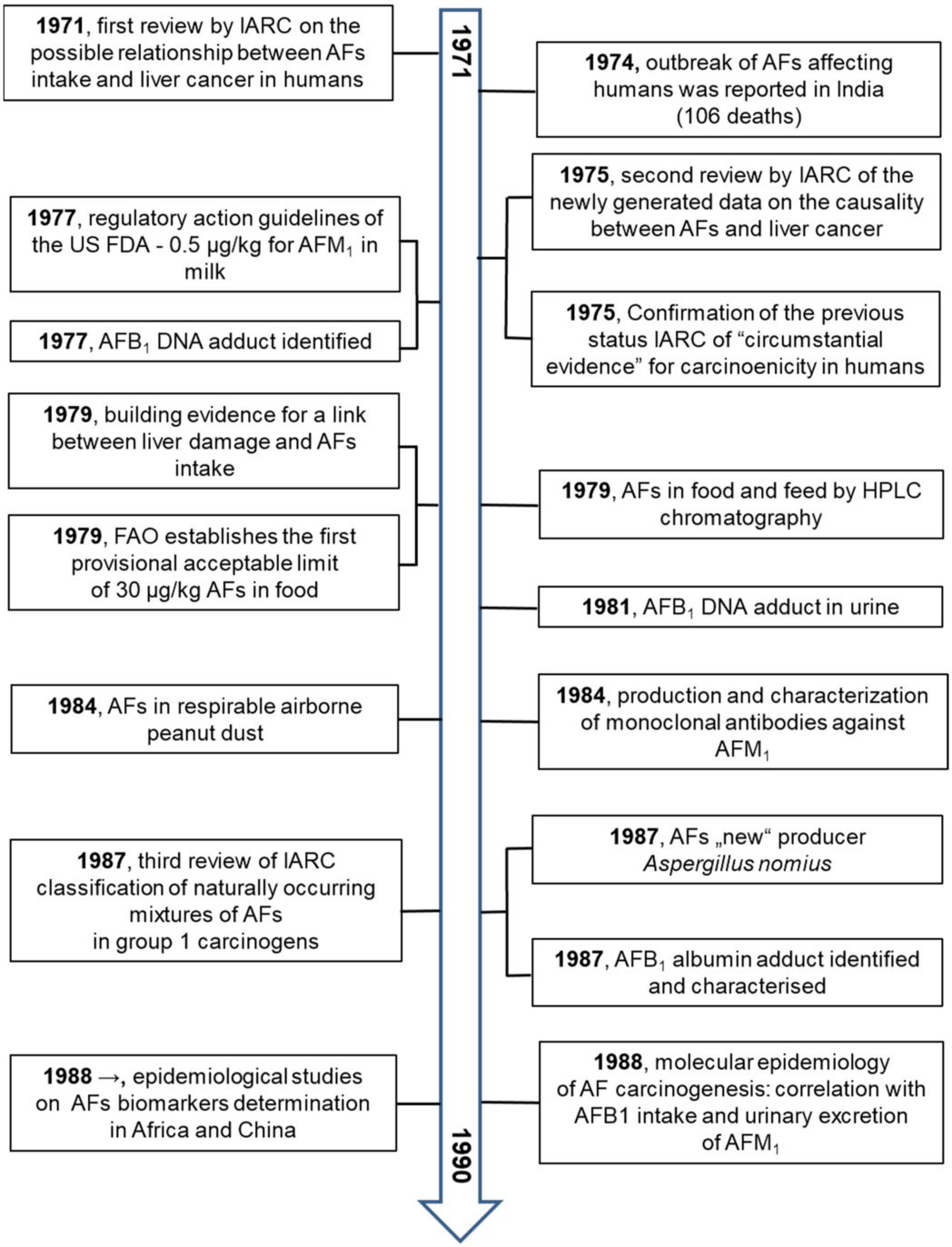

Figure 5. The milestones in aflatoxin research over the years 1971–1990 [49–65].

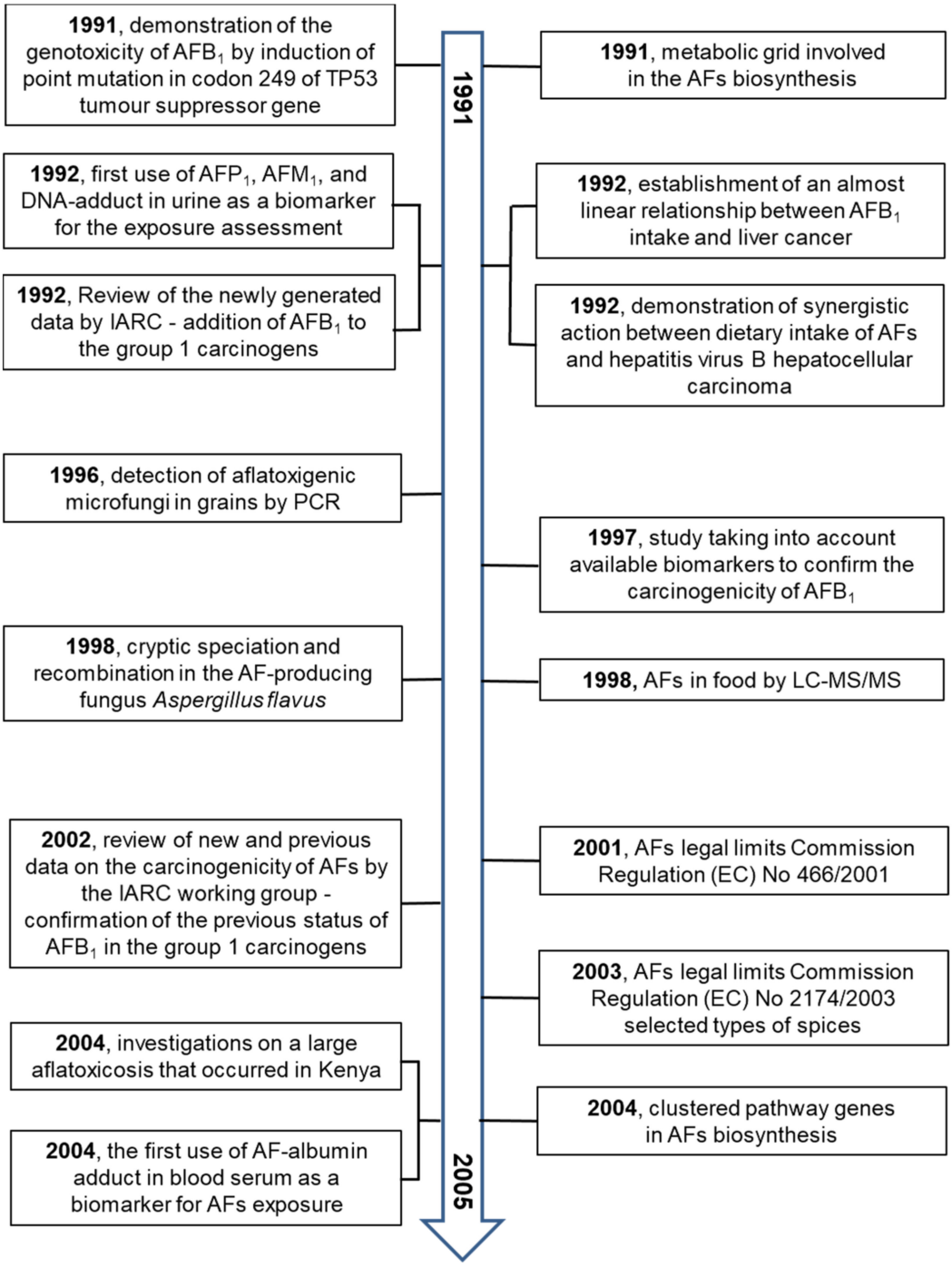

Figure 6. The milestones in aflatoxin research over the years 1991–2005 [66–78].

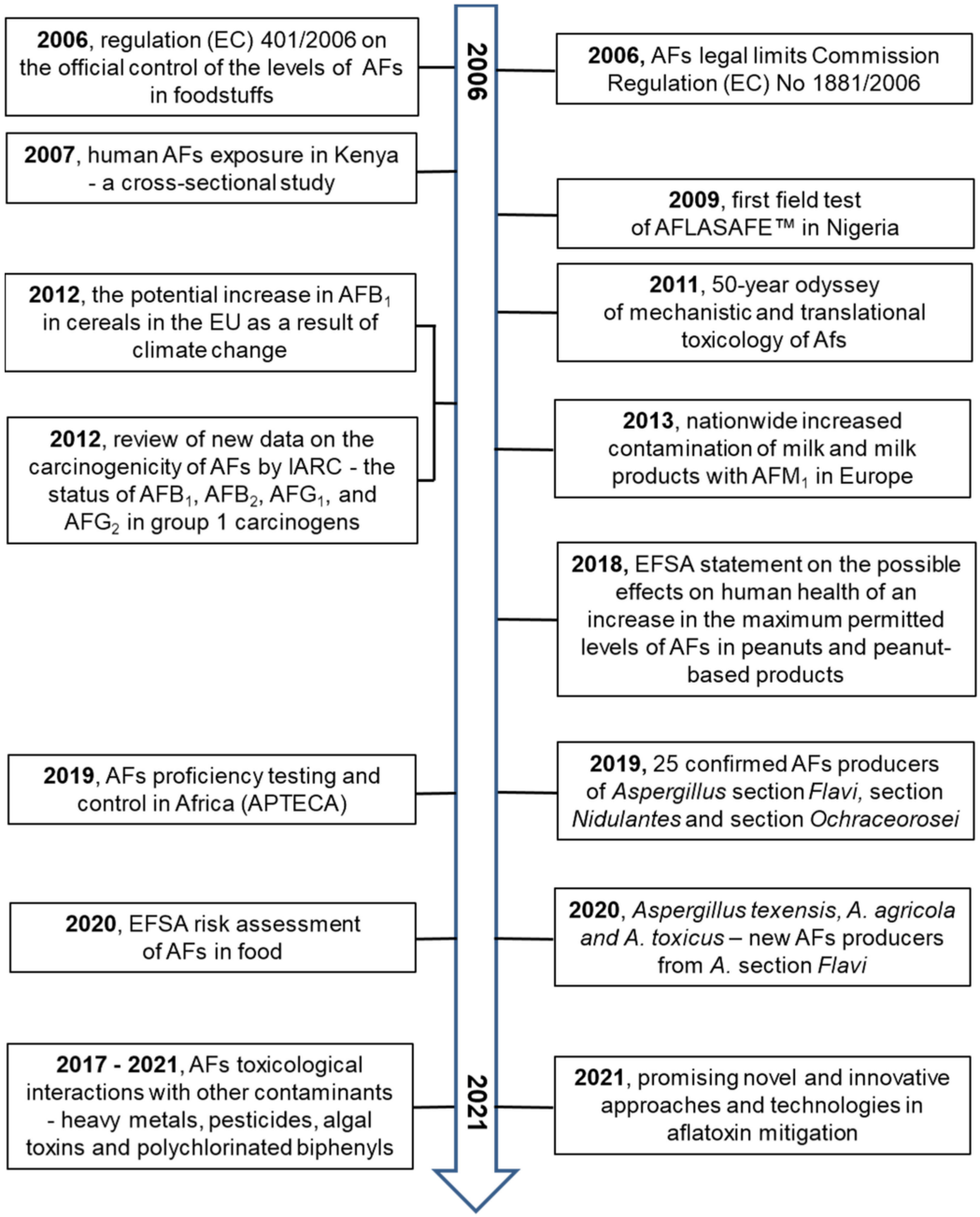

Figure 7. The milestones in aflatoxin research over the years 2006–2021 [26,79–98].

4. Recent Data on Aflatoxin Toxicity

The human population is often exposed to low AF levels due to the daily intake of various AF-contaminated products [99]. Approximately 4.5 billion people worldwide, mainly in developing countries, have been estimated to be chronically exposed to AFs via contaminated food [100,101]. Moreover, due to the difficulties in food management and the socio-economic difficulties caused by the ongoing coronavirus pandemic (COVID-19), an increase in the consumption of AF-contaminated foods can be expected [102]. In 2020 according to the RASFF (Rapid Alert System for Food and Feed) database, AFs were the most often notified in peanuts; dried figs; spices; rice; and various nuts such as hazelnuts, almonds, and pistachios [103]. However, in recent years, some of these food products have shown a relatively high concentration exceeding 1000 µg/kg [103], which may be related to

the development of aflatoxicosis, which can lead to serious health problems, in particular damage to the liver and other organs, primary liver cancer, and even death [104].

AFs are infamous for their high toxicity, therefore their presence in food (and feed) is highly feared. Naturally occurring AFs (AFB_1, AFB_2, AFG_1, and AFG_2) act as strong carcinogens, thus they are assigned into Group 1 "carcinogenic to humans" by the International Agency for Research on Cancer (IARC) [84,105]. Apart from their carcinogenicity, they have been reported to have mainly hepatotoxic, genotoxic, mutagenic, teratogenic, immunosuppressive, nephrotoxic, and cytotoxic effects [106–109].

Some toxic effects of AFs have been observed in the most recent literature. Hepatotoxic effect of AFB_1 has been demonstrated in vivo on mice [110,111], rats [112], rabbits [113], and broiler chickens [114]. The nephrotoxic effect of AFB_1 has been reported in vivo on broiler chickens [114] and rats [112]. The neurotoxicity of AFB_1 has been observed in vitro on human astrocytes and in vivo on a glial cell in zebrafish [115]. The immunosuppression of AFB_1 has been demonstrated in vitro on swine alveolar macrophages [116]. Reproductive toxicity of AFB_1 has been demonstrated in vivo on mice [110]. Pulmonary toxicity of AFB_1 has been observed in vivo on male albino rats [117]. Gastrointestinal toxicity of AFB_1 has been described in vivo on rats [118], pigs [119], and chickens [120]. The genotoxic effect has been observed in vivo on mice [110]. Cytotoxic and/or genotoxic effects of AFB_1 have been reported in vitro on the leghorn male hepatoma (LMH) cell line [121], the liver hepatocellular carcinoma (HepG2) cell line [122], buffalo rat liver (BRL-3A) cells [123], bovine mammary epithelial (BME) cells [124], and the human keratinocyte (HaCaT) cell line [125]. Cytotoxicity on BME cells has also been observed in vitro in the case of AFM_1 [124]. The embryotoxicity of AFB_1 has been reported in vitro on bovine embryos [126].

It should be emphasized that the impact of AFs, as well as other mycotoxins or contaminants in general, on human health always depends on the toxicological properties of the agent, the individual properties of the consumer, and duration of exposure to the agent, and also the presence of other contaminants with which an interaction (e.g., synergistic) could occur [127].

4.1. Toxicological Interactions of Aflatoxins and Other Mycotoxins

Worldwide, food (and also feed) may be infested by more than one type of mold. Moreover, most molds can produce several mycotoxins simultaneously, as a result of which humans and animals may be exposed to a "cocktail of mycotoxins" in their diet [95,128,129]. However, the regulations worldwide do not take into account the combined effects of co-occurred mycotoxins [130]. Various interactions (synergistic, additive, antagonistic) have been reported, mainly between AFs and ochratoxin A (OTA), fumonisins, and trichothecenes [128]. AFs with fumonisins and AFs with OTA are among the most common mycotoxin combinations in cereals and cereal products [130].

For example, in the recent literature, the antagonistic effect on inducing cytotoxicity on the LMH cell line has been observed between AFB_1 and OTA [121]. On the contrary, these two mycotoxins, also in combination with zearalenone (ZEA), acted synergistically in negatively affecting the milk production, blood metabolism, and immune function of Laoshan goats [131]. Synergistic interaction between AFB_1 and OTA has also been observed in vitro on swine alveolar macrophages (3D4/21) [132]. A synergistic effect has also been reported between AFB_1, deoxynivalenol (DON), and ZEA on human epithelial (Caco-2) cells [133]. However, reduced cytotoxicity of DON on the viability of MA-10 Leydig cells in vitro has been observed when combined with AFB_1 [134]. A synergistic effect has also been observed within the AF group between AFB_1 and AFM_1 in compromising intestinal integrity in vivo on mice and in vitro on Caco-2 cells [135].

4.2. Toxicological Interactions of Aflatoxins with Other Contaminants

Humans (but also animals) can be exposed to many other environmental toxins along with mycotoxins, such as heavy metals and pesticides, but also algal toxins [95]. Considering that some mycotoxins and other environmental toxins may share the same target organ

or tissue, the monitoring of their common combined effects pose a challenge for further research as these agents are usually studied individually rather than in combination [95]. However, there are several recent studies that address the interaction of AFs with other contaminants.

Heavy metals such as nickel, arsenic, lead, chromium, mercury, and cadmium share the main target organ, liver, with AFs [96], and it is therefore important to research their interactions. In an in vivo study, AFB_1 and cadmium chloride showed an additive interaction in inducing acute oral toxicity in kunming mice [136]. A synergistic effect of AFB_1 and sodium arsenite has been observed, including cytotoxicity in vitro in urinary bladder (HUC-PC) cells [137].

The co-contamination of agricultural crops with AFs (and also other mycotoxins) and pesticides (insecticides, fungicides, herbicides) has been reported [95]. Therefore, due to their co-occurrence, it is necessary to consider their combined toxic effects. Recently, an antagonistic interaction between AFB_1 and the insecticide chlorpyrifos has been observed on HepG2 cells for cytotoxicity and genotoxicity [122]. In the case of AFB_1 and insecticide *p,p′*-DDT, a dose-dependent interaction has been observed on MA-10 Leydig cells in vitro—additive at doses of 16 µM (5671.84 µg/L and 4996.36 µg/L of *p,p′*-DDT and AFB_1, respectively) and 32 µM (11,343.68 µg/L and 9992.64 µg/L of *p,p′*-DDT and AFB_1, respectively) and antagonistic at 64 µM (22,687.36 µg/L and 19,985.28 µg/L of *p,p′*-DDT and AFB_1, respectively) [134].

Microcystin-LR (MC-LR), a toxin that is produced by cyanobacteria *Microcystis aeruginosa*, has been observed to possibly increase the liver damage risk in people suffering from hepatitis B simultaneously exposed to AFB_1 [94]. Enhanced effects of AFB_1 and MC-LR interaction on genotoxicity and cytotoxicity have been observed on the human liver cell line (HL7702) in vitro [138]. In contrast, a recent study points to the possibility of an antagonistic effect of low levels of MC-LR on AFB_1-induced hepatocarcinogenicity on HL7702 cells in vitro through decreasing CYPA1A2 expression and AFB_1-DNA adduct generation, while demonstrating that exposure to a combination of MC-LR and AFB_1 may not worsen liver damage compared to exposure to AFB_1 alone on six-week old male Sprague-Dawley rats in vivo [139]. Additionally, the combination of AFB_1 and MC-LR predominantly exerted antagonistic effects in cytotoxicity to HepG2 and Madin-Darby bovine kidney epithelial (MDBK) cell lines in vitro [140].

Apart from the above-mentioned contaminants, AFs can interact with many other toxic substances from the environment. For example, polychlorinated biphenyls (PCB) may potentiate/enhance the genotoxicity effect of AFB_1 in the human hepatocyte line (L-02 cell line) by enhancing CYP1A1, CYP1A2, and CYP3A4 expression [141].

4.3. Toxicological Interactions of Aflatoxins with Hepatitis B and C Virus in Relation to Carcinogenicity

AFs may induce a number of cancer types (liver, breast, lung, gallbladder, esophageal), the best known of which is liver cancer [142]. Liver injury and hepatocellular carcinoma (HCC), one of the major types of liver cancer, are considered the main toxic impact of AFB_1 [99,107,143,144]. Worldwide, approximately 5–28% of HCC occurrences are attributed to AF exposure [145]. Globally, a total of 905,677 new cases (corresponding to a crude rate of 11.6 cases per 100,000 people) and 830,180 deaths (corresponding to a crude rate of 10.7 cases per 100,000 people) due to liver cancer in both sexes and all ages were estimated in 2020. Based on the total number of cases, liver cancer is ranked the 6th and 3rd cancer type in incidence and mortality, respectively, worldwide [146]. More than 80% of HCC cases come from developing countries [147]. HCC has become a serious health problem, especially in sub-Saharan African countries and countries of southeast Asia, and it is also increasing in Europe and the United States [148,149].

Dietary exposure to AFs is considered the second largest environmental risk factor for liver cancer development [148] after viral hepatitis B or C infections [150], which act synergistically with AFs [149]. Especially in developing countries, hepatitis B is considered the main risk factor for HCC; however, dietary exposure to AFs also plays a significant

role in HCC etiology [151]. A recent study on Iranian patients suffering from hepatitis B or C has demonstrated the potential involvement of AFB_1 exposure as a mean risk factor in the HCC etiology in these patients [152]. Chronic hepatitis B virus infection may induce cytochrome P450s, which is responsible for the metabolism of non-toxic AFB_1 to AFB_1-8,9-epoxide (AFBO) metabolite, with highly toxic and mutagenic effects [153]. AFBO attacks DNA through binding to the N7 position of guanine residues to produce a pro-mutagenic unstable DNA adduct AFB_1-N7-Guanin. This DNA-adduct induces a specific transverse mutation G:C to T:A at codon 249 of the tumor suppressor gene p53, which is involved in cell cycle regulation [149,154–156]. This mutation is typical for HCC patients from regions of high AF-exposure [149,155].

5. Recent Data on Aflatoxin Mitigation

Several valuable original research articles [157–159] and reviews [97,98,160–163] on AF mitigation have been published over the last three years.

AF mitigation means reducing the health risk from the occurrence of AFs in foodstuffs. AF mitigation is key to food safety and nutrition and is any process used to reduce AF concentrations in foodstuffs [164].

AFs represent a threat to food safety worldwide because they are considered to be among the most prominent and dangerous toxins that can affect any part of the food chain from pre-harvest to food processing. Prevention and mitigation of AF contamination is critical to protect consumers from the adverse health effects associated with AFs [161,165].

AF contamination arises at multiple points in the food system, from the field to the home (where pest attack or poor drying techniques and inadequate crop storage allow the *A. flavus* to grow), and to the marketplace (where lack of quality control allows contaminated food to be sold). It is important to equip producers, traders, and consumers with knowledge that can help them manage this issue [157,165].

The weather conditions prior to harvest play a cardinal role in the risk of AF production, and the globalization of trade flows, as well as climate change, lead to the occurrence of unexpected AFs in unusual products [166,167].

AFs present a significant health hazard to consumers. With the potential to contaminate a range of common foods and feeds, such as grains (wheat, corn, barley, rice, and oats), nuts, cocoa, and milk, AFs present an ongoing challenge to food safety all along the food chain. The ideal way to mitigate their risk to food safety is to prevent these toxins from entering the food chain at all, and a number of pre-harvest strategies based on good agricultural practices can help [103,164,165].

Even with the best prevention strategies, however, AFs can end up in the food chain given that they are ubiquitous worldwide and that ever-changing environmental conditions preclude strict elimination [157,163].

At the present time, to avoid unfavorable AFs effects on public health, great attention is being given to prevention as well as to pre-harvest methods intended for *A. flavus* contamination reduction [157,162,163,166].

Numerous post-harvest methods to combat AFs are also required, such as emerging physical methods (e.g., non-thermal treatments as pulsed electric fields), interventions with chemical agents (e.g., adsorbents, acids, enzymes, and gases), interventions with microbiological agents (e.g., bacteria, yeast and microfungi), and genetic engineering technologies. These methods have been reported to be effective in mycotoxin diminution in food and feed [97,98,160–162].

5.1. The Selected Effective Pre-Harvest Method for Aflatoxin Mitigation

A total of 22, 4, and 2 species of the genus *Aspergillus* from the *Flavi*, *Nidulantes*, and *Ochraceorosei* sections produce AFs, respectively, with *A. flavus* of section *Flavi* being the most important and best known species [103].

A. flavus may be divided to the L and S morphotypes. The S morphotype produces a lot of small sclerotia (average diameter <400 μm), few conidia, and a regularly high amount

of AFBs [168]. In contrast, the L morphotype can produce less numerous, larger sclerotia (average diameter >400 µm), a lot of conidia, and mutable amount of AFBs. There are also L morphotype genotypes that are not able to produce AFs (i.e., non-aflatoxigenic) due to inversions, deletions, or defects in at least one of the AF biosynthesis genes (a single mutation in the *pksA* (*aflC*) gene of its AF pathway), which bring in a premature stop codon and cause it to be defective [169,170]. As a potential biocontrol agent, found in a peanut fields in Georgia, there is another non-aflatoxigenic *A. flavus* strain (NRRL 21882), which demonstrated an important effect against native aflatoxigenic strains in laboratory tests and is commercially known as Afla-Guard®. The complete absence of the AF gene cluster is responsible for its inability to produce AFs [170]. Further, in Italy, the biopesticide (AF-X1TM) was evolved for protection of fodder maize crops [171]. The biopesticide formulation (A2085) original used strain is comparable to NRRL 21882 due to AF gene cluster lacking. Mytoolbox Af01 is another commercial product with the biocontrol strain, with a partial-cluster strain, which successfully reduces AFs in Serbia maize, due to the lack of AF cluster genes from *aflT* to *aflN* [172].

All new findings of these defected non-aflatoxigenic *A. flavus* isolates support an AF biocontrol strategy development to mitigate the AF content in crop. This new effective technology was first used widely in the US. Nowadays, this environmentally friendly and safe technology is annually employed over hundreds of thousands of hectares of susceptible crops [157,159,166,170].

Under the commercial name AflasafeTH (sorghum seed as a carrier of the non-aflatoxigenic strain of *A. flavus*) this improved biocontrol technology has been used in sub-Saharan Africa for more than 13 nations (Burkina Faso, Burundi, Gambia, Ghana, Kenya, Malawi, Mozambique, Nigeria, Rwanda, Senegal, Tanzania, Uganda, and Zambia), where effort to evolve biocontrol products is relevant, and it can be assumed that the number of participating nations will increase. Especially in African countries, modern technologies have been used to produce low-cost AflasafeTH products via mass production. Registered experimentally, AflasafeTH products have been proven to reduce AF levels in treated crops (e.g., maize and groundnut) by more than 80% compared to untreated crops with the same storage and field conditions [157,159,166,170].

5.2. The Selected Effective Post-Harvest Methods for Aflatoxin Mitigation

5.2.1. Physical Post-Harvest Methods

Sorting

Most often, AFs contaminated grain is broken or damaged, which leads to inhomogeneous contamination of the entire volume of the stored crop; therefore, separation methods are suitable for decontamination [173]. Thus, sorting machines using the weight and size of particles as a parameter have been used for a long time. Airflow flotation and centrifugation used to be employed for sorting high volumes of grain, but sorting based on the optical principle was established in the 1960s. Due to the higher efficiency, this method is still used and is based on the principle of optical control when grains or peanuts are passing along the sensors. If a grain of a different color is detected, the magnetic valve opens and a thin stream of compressed air removes the grain [165]. For improvement of this currently used sorting method, for reduction of the risk of contamination, the single kernel sorting tool could be used for detection of multiple types of mycotoxins, including AFs, in peanuts and maize [174].

Dehulling

The precondition for the successful elimination of AF content is the restriction of colonization by AF-producing fungi on the surface layers of grains [165]. The outer layers of the grain are removed by dehulling techniques, which can remove up to 93% of the AFs [175].

Steeping

The first part of wet milling of maize grains, the steeping, consist of soaking the grains for 36–50 h at 50 °C in 0.1–0.2% SO_2 water solution to disrupt protein matrix and improve germ separation and also induce production of lactic acid, which can be considered chemical treatment. The result is that the steeping liquor commonly obtains around half of the AF content [165,176]. Additionally, the level of AFs from sorghum grains could be decreased by steeping in 0.2% NaOH solution under levels of detection [177].

Wet Milling

Up to 40–50% of AFs could be eliminated from maize into the solution in wet milling; the remaining levels of AFs could be determined in the fiber fraction (28–38%), the gluten fraction (11–17%), germ (6–11%), and starch (1%) [165].

Dry Milling

Additional dry milling leads to the reduction of AF concentration in the germ fraction of the maize grain [165].

Heat Treatment

Temperatures above 160 °C have been shown to be effective in destroying pure AFB_1, with soybean matrix accelerating the destruction process [178]. While temperatures up to 100 °C used for common food preparation have little effect on AFs, the higher temperatures used in frying, baking, roasting, and extruding may be more effective in reducing AF contamination [165].

AF levels can be decreased by extrusion by up to 50–80%, depending on the temperature and humidity of the grain, while the efficiency of the whole process can be increased by alkaline treatment. Additionally, in the case of peanut meal, extrusion alone leads to AF reduction by 23–66%, but coupled with ammonium hydroxide it can be up to 87%. Another heat treatment, roasting, leads to the reduction of AF amount in pecans and peanuts (50–70%) and maize (40–80%) [165].

Irradiation

Elimination of pathogenic organisms, and also, partially, AFs in food can be achieved by ionizing (gamma) or non-ionizing (solar, UV, microwave) radiation [165].

Compared with gamma-irradiation at 25 kGy (43% reduction) or microwave heating for 10 min (32% reduction), sunlight has been found to be more effective. Degradation of AFs by sunlight in cereals leads to reduction by 40% and up to 75% after 3 h and 30 h, respectively [179].

In other studies, gamma radiation has been used to irradiate maize, pistachio nuts, rice, and peanuts. The irradiation at 10 kGy induced 59–88% AF reduction. However, in another study, where irradiation at 15 kGy was employed, only 11–21% AF reduction has been proved [180,181].

The emission of UV-A (in dose 1200 mJ/cm^2) has been shown to have a significant reduction effect on AFB_1 and AFM_1 in pure water by 70% and 84%, respectively. In cell culture studies, the increased dosage of UV- A emission has been shown to decreased or even suppress AF-induced cytotoxicity in HepG2 cells [182].

Pulsed Electric Fields

Due to AF thermostability, a pulsed electric fields (PEF) has been applied for its effective destruction. AFB_1 and AF levels have been decreased by 77% and 97%, depending on the combination of different parameters as output voltage, pulse width, and pH, so the output voltage (20–65%), pH (4–10), and pulse width (10–26 μs), coupled with 2FI and quadratic models, result in PEF process optimization, which leads to AFB_1 and total AF level reduction [183].

PEF treatment could be used for *Aspergillus parasiticus* inactivation and AF disintegration with alleviated mutagenic effects to preserve sesame seeds and their physicochemical

properties. Levels of AFs B_1, B_2, G_1, and G_2, have been reduced by 86.9%, 98.7%, 94.7%, and, 92.7%, respectively with PEF energy in the range of 0.97 to 17.28 J, while the maximum PEF energy caused a 60% reduction of *A. parasiticus* [184].

5.2.2. Chemical Post-Harvest Methods

Chemical post-harvest methods for AF mitigation are based on intervention with chemical agents, e.g., adsorbents, acids, and bases.

Adsorbents

Clay-based adsorbents have been proposed for use as a new technique for removing AFs from contaminated liquids [185]. A potential adsorbent may be, *inter alia*, bentonite [186], which is listed by United States regulations as a safe ingredient that can be used as a direct food ingredient for human [187]. The effect of bentonite in reducing AFM_1 in milk has been demonstrated by several studies. AFM_1 reduction has been observed when bentonite was added directly to naturally contaminated milk [188,189]. However, bentonite, when added to feed for dairy cattle, has also been found to be effective in reducing AFM_1 levels in milk indirectly [190,191] via adsorption of AFB_1 in the gastrointestinal tract, leading to reducing its carry-over as AFM_1 into milk [192].

Acids

Another type of AF treatment using strong acids has been proven to be effective for the conversion of AFB_1 and AFG_1 to their hemiacetal forms, demonstrated in the case of HCl (pH 2), which decreased AFB_1 concentration by 19% in 24 h [188]. Among other tested acids, e.g., citric, acetic, and lactic acids under simulating cooking conditions, the last one turned out to be the most effective in transformation of AFB_1 and AFB_2 [193].

Bases

AFs are unstable under alkaline conditions. Ammonization can reduce AF concentration by more than 99%. AF degradation by ammonia has been widely studied and has been shown to be effective in both laboratory and field experiments [165,194].

5.2.3. Microbiological Post-Harvest Methods

Microbiological post-harvest methods for AF mitigation are based on intervention with microbiological agents as bacteria, and yeasts.

Bacteria

Lactic acid bacteria (e.g., *Bifidobacterium animalis* B subsp. *lactis*, *Enterococcus avium*, *Lactobacillus acidophilus*, *L. selangorensis*, *Lactococcus lactis* subsp. *lactis*, *Pediococcus acidilactici*, *Streptococcus thermophilus*, and *Weissella confuse*) have inhibitory effects on the AF production or cause the removal of AFs from foodstuffs and feedstuffs. The quality of AF binding by lactic acid bacteria strains depends on pH, temperature, the matrix itself, the incubation time, and also the inherent properties of the strain. AF elimination ranges for AFB_1 from 16.3–98% and for AFM_1 from 5.6–99.9%, and depends on the strain of lactic acid bacteria [163].

The degradation of AFs by probiotic bacteria to less or even non-toxic products has been shown to be an effective, safe, cheap, and environmentally friendly strategy of detoxification, with an approximately detoxification rate of 19–95% (for AFB_1) and 12–100% (for AFM_1) [106].

AF elimination by non-lactic acid bacteria (e.g., *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Brachybacterium* spp., *Brevundimonas* spp., *Cellulosimicrobium funkei*, *Enterobacter* spp., *Escherichia coli*, *Mycolicibacterium fluoranthenivorans*, *Mycolicibacterium smegmatis*, *Myxococcus fulvus*, *Nocardia corynebacterioides*, *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, *Rhodococcus erythropolis*, *Streptomyces aureofaciens*, *Streptomyces lividans*, and *Stenotrophomonas maltophilia*) have an inhibitory effects on AF production or on the

removal of AFs from milk at 4 °C. AF elimination ranges for AFB_1 from 18–97% and for AFM_1 from 32–64%, and depends on the strain of bacteria [163].

Yeast

In aflatoxigenic microfungi, the production of AFs has been significantly supressed by yeasts, e.g., *Candida*, *Debaryomyces*, *Pichia*, *Saccharomyces*, *Saccharomycopsis*, *Saccharomycodes*, *Schizosaccharomyces*, *Aureobasidium pullulans*, *Trichosporon*, and *Zygosaccharomyces*. AF elimination ranges for AFB_1 from 15–100% and for AFM_1 from 60–90.3%, and depends on the strain of yeasts [163].

It is known that yeast supplementation (e.g., *Pichia kudriavzevii* and *Kluyveromyces marxianus*) is able to detoxicate the AFB_1 in rumina and reduce AFM_1 levels in milk, which improves the dairy cattle performances [195].

5.2.4. Genetic Engineering Post-Harvest Methods

Firstly, genetic engineering technologies are based on the regulation mechanism of AF biosynthesis in *A. flavus* that lack the ability to produce AFs. Only precise genomic integration of mutant allele methods is required for accurate understanding of the mechanism for regulation of AF biosynthesis produced by *A. flavus*. The new strategy for the foreign DNA site-specific integration in the *A. flavus sdh2* gene locus was evolved to prevent the disadvantage of ectopic or non-homologous recombination within integration of DNA into the genome [196].

Single substitution of amino acid (His 249 Leu) is involved in the mutant $sdh2^R$ allele on the pFC-eGFP vector, which has been established through cloning based on the yeast recombination for the transformation of fungi. *A. flavus* obtains systemic fungicide carboxin resistance as a result of a substitution of original *sdh2* allele with $sdh2^R$. Proper integration of the *A. flavus* NRRL 3357 genome into the locus *sdh2* resulted in the highly efficient generation (>96%) of transformants [196]. The rapidity and effectiveness of this method consist of the locus *sdh2* with inserted eGFP expression cassette, which leads to the alleviation of virulence and the growth of fungi. This process would be a helpful instrument for genetic manipulation of *A. flavus* [196].

On the other hand, genetic engineering technologies are based on transforming maize plants to a transgenic AF-free cultivar employing host-induced gene silencing [197]. The evolved transgenic maize with a hairpin construct focused on transcription factor *aflR* of the AF biosynthesis was exposed to an aflatoxigenic strain of *A. flavus* originating from an endemic AF outbreaks in eastern Kenya. The results demonstrated that *A. flavus aflR* transcription factor colonizing transgenic maize was downregulated. Besides, transgenic maize kernels concentrated 14-fold lower AF levels in comparison with wild maize kernels. In the transgenic maize, the silencing cassette induced reduced kernel placement and its stunting, which probable led to "off-target" silencing of unintended genes by *aflR siRNAs* in transformed plants [197].

Another study revealed that host-induced gene silencing significantly eliminates the AF toxin from transgenic maize. The maize plants were transformed by using the gene cassette, with the kernel-specific RNA interference (RNAi), targeting the *aflC* gene, encoding the enzyme at the *Aspergillus* biosynthetic pathway of the AFs. In these kernels of transgenic maize, AFs have not been detected, compared to non-transgenic maize kernels, in which the levels of AF were in thousands of ppb after pathogen infection. Meanwhile, the same similarity between transcript developing groups of transgenic and non-transgenic kernels has been observed. It was proved that small interfering RNA molecules could be employed in maize AF biosynthesis silence, which could lead to its use as an attractive strategy, and in the view of food safety improvement, may be implemented in other crops [198].

Finally, there are genetic engineering technologies based on transformed peanut with genetic *A. flavus* infection resistance and AF production using host-induced gene silencing.

In the case of peanuts, the significant resistance method could be used through biosynthetic AF pathway genes (*aflM* and *aflP*) by host-induced gene silencing and by overexpressed plant defensins *MsDef1* and *MtDef4.2* with antifungal ability. The first method, in the case of AF infection, suppresses AF production to provide permanent resistance against various morphotypes of *A. flavus*, which results in insignificant levels of AFs in peanuts; the second one improves *A. flavus* infection genetic resistance. The significant relation between the accumulation of AFs and biosynthetic AF pathway gene transcription decrease has been confirmed in the case of overexpressed defensins as well as in host-induced gene silencing lines [199].

6. Summary

In 2020, it was 60 years since the discovery of AFs, which are, among all mycotoxins, considered to be the most agriculturally important and harmful. Some toxic effects of AFs have been observed, including carcinogenicity. The numerous effective pre-harvest and post-harvest biocontrol methods for AF mitigation have been applied. Research focused on AF genetic variability and the diversity of *A. flavus* and other producers of AFs is very important and solves biocontrol strategy problematics of non-aflatoxigenic A. *flavus* strains with a view toward better public health protection and to prevent economic losses. At Present, biocontrol strategies are sufficient; however, they should be further improved due to developing knowledge about recombination using transgenic A. flavus strains and the use of genome editing methods. Future research should be focused on elaborating these novel biocontrol strategies and their wide testing possibilities in ordinary foodstuffs and feedstuffs.

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References

1. Benkerroum, N. Retrospective and Prospective Look at Aflatoxin Research and Development from a Practical Standpoint. *Int. J. Environ. Res. Public Health* **2019**, *16*, 3633. [CrossRef] [PubMed]
2. Bennett, J.W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, *16*, 497–516. [CrossRef] [PubMed]
3. Bhat, R.; Rai, R.V.; Karim, A.A. Mycotoxins in Food and Feed: Present Status and Future Concerns. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 57–81. [CrossRef] [PubMed]
4. Smith, J.S.; Paul Williams, W.; Windham, G.L. Aflatoxin in Maize: A Review of the Early Literature from "Moldy-Corn Toxicosis" to the Genetics of Aflatoxin Accumulation Resistance. *Mycotoxin Res.* **2019**, *35*, 111–128. [CrossRef]
5. Blount, W.P. Turkey "X" Disease. *J. Br. Turkey* **1961**, *9*, 55–58.
6. Wannop, C.C. Groundnut Toxicity in Poultry: Turkey X Disease. *Br. Vet. J.* **1963**, *119*, 174–177. [CrossRef]
7. Siller, W.G.; Ostler, D.C. The Histopathology of an Enterohepatic Syndrome of Turkey Poults. *Vet. Rec.* **1961**, *73*, 134–138.
8. Wannop, C.C. The Histopathology of Turkey "X" Disease in Great Britain. *Avian. Dis.* **1961**, *5*, 371–381. [CrossRef]
9. Asplin, F.D.; Carnaghan, R.B.A. The Toxicity of Certain Groundnut Meals for Poultry with Special Reference to Their Effect on Ducklings and Chickens. *Vet. Rec.* **1961**, *73*, 1215–1219.

10. Sargeant, K.; O'Kelly, J.; Carnaghan, R.B.A.; Allcroft, R. The Assay of a Toxic Principle in Certain Groundnut Meals. *Vet. Rec.* **1961**, *73*, 1219–1223.
11. Sargeant, K.; Sheridan, A.; O'Kelly, J.; Carnaghan, R.B.A. Toxicity Associated with Certain Samples of Groundnuts. *Nature* **1961**, *192*, 1096–1097. [CrossRef]
12. Loosmore, R.M.; Harding, J.D.J. A Toxic Factor in Brazilian Groundnut Causing Liver Damage in Pigs. *Vet. Rec.* **1961**, *73*, 1362–1364.
13. Loosmore, R.M.; Markson, L.M. Poisoning of Cattle by Brazilian Groundnut Meal. *Vet. Rec.* **1961**, *73*, 813–814.
14. Lancaster, M.C.; Jenkins, F.P.; Philp, J.M. Toxicity Associated with Certain Samples of Groundnuts. *Nature* **1961**, *192*, 1095–1096. [CrossRef]
15. Schoental, R. Liver Changes and Primary Liver Tumours in Rats given Toxic Guinea Pig Diet (M.R.C. Diet 18). *Br. J. Cancer* **1961**, *15*, 812–815. [CrossRef] [PubMed]
16. Allcroft, R.; Carnaghan, R.B.A. Toxic Products in Groundnuts. Biological Effects. *Chem. Ind.* **1963**, *2*, 50–53.
17. Barnes, J.M.; Butler, W.H. Carcinogenic Activity of Aflatoxin to Rats. *Nature* **1964**, *202*, 1016. [CrossRef]
18. Butler, W.H.; Barnes, J.M. Toxic Effects of Groundnut Meal Containing Aflatoxin to Rats and Guinea-Pigs. *Br. J. Cancer* **1963**, *17*, 699–710. [CrossRef] [PubMed]
19. Le Breton, E.; Frayssient, C.; Boy, J. Sur l'apparition d'hépatomes "Spontanés" Chez Le Rat Wistar. Rôle de La Toxine d'*Aspergillus Flavus*. Intérêt En Pathologie Humaine et Cancérologie Expérimentale. *C.R. Acad. Sci.* **1962**, *255*, 784–786.
20. Nesbitt, B.F.; O'Kelly, J.; Sargeant, K.; Sheridan, A.N.N. *Aspergillus Flavus* and Turkey X Disease. Toxic Metabolites of *Aspergillus Flavus*. *Nature* **1962**, *195*, 1062–1063. [CrossRef]
21. Van der Zijden, A.S.M.; Koelensmid, W.; Boldingh, J.; Barrett, C.B.; Ord, W.O.; Philip, J. *Aspergillus Flavus* and Turkey X Disease: Isolation in Crystalline Form of a Toxin Responsible for Turkey X-Disease. *Nature* **1962**, *195*, 1060–1062. [CrossRef]
22. Benkerroum, N. Aflatoxins: A Comprehensive Overview. *Preprints* **2019**, 1–80. [CrossRef]
23. Benkerroum, N. Aflatoxins: Producing-Molds, Structure, Health Issues and Incidence in Southeast Asian and Sub-Saharan African Countries. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1215. [CrossRef] [PubMed]
24. Fouché, T.; Claassens, S.; Maboeta, M. Aflatoxins in the Soil Ecosystem: An Overview of Its Occurrence, Fate, Effects and Future Perspectives. *Mycotoxin Res.* **2020**, *36*, 303–309. [CrossRef]
25. Gnonlonfin, G.J.B.; Hell, K.; Adjovi, Y.; Fandohan, P.; Koudande, D.O.; Mensah, G.A.; Sanni, A.; Brimer, L. A Review on Aflatoxin Contamination and Its Implications in the Developing World: A Sub-Saharan African Perspective. *Crit. Rev. Food Sci. Nutr.* **2013**, *53*, 349–365. [CrossRef]
26. Kensler, T.W.; Roebuck, B.D.; Wogan, G.N.; Groopman, J.D. Aflatoxin: A 50-Year Odyssey of Mechanistic and Translational Toxicology. *Toxicol. Sci.* **2011**, *120*, S28–S48. [CrossRef]
27. Kumar, P.; Mahato, D.K.; Kamle, M.; Mohanta, T.K.; Kang, S.G. Aflatoxins: A Global Concern for Food Safety, Human Health and Their Management. *Front. Microbiol.* **2017**, *7*, 1–10. [CrossRef] [PubMed]
28. Richard, J.L. Discovery of Aflatoxins and Significant Historical Features. *Toxin Rev.* **2008**, *27*, 171–201. [CrossRef]
29. Rushing, B.R.; Selim, M.I. Aflatoxin B1: A Review on Metabolism, Toxicity, Occurrence in Food, Occupational Exposure, and Detoxification Methods. *Food Chem. Toxicol.* **2019**, *124*, 81–100. [CrossRef] [PubMed]
30. Wild, C.P.; Gong, Y.Y. Mycotoxins and Human Disease: A Largely Ignored Global Health Issue. *Carcinogenesis* **2010**, *31*, 71–82. [CrossRef] [PubMed]
31. Stevens, A.J.; Saunders, C.N.; Spence, J.B.; Newham, A.G. Investigations into" Diseases" of Turkey Poults. *Vet. Rec.* **1960**, *72*, 627–628.
32. Blount, W.P. Disease of Turkey Poults. *Vet. Rec.* **1960**, *72*, 786.
33. Blount, W.P. A New Turkey Disease Problem in England Characterised by Heavy Mortality. *Br. Oil Cake Mills Ltd. Q. Poult. Bull.* **1960**, *27*, 1–3.
34. Van Dorp, D.A.; van Der Zijden, A.S.M.; Beerthuis, R.K.; Sparreboom, S.; Ord, W.O.; De Jong, K.; Keuning, R. Dihydro-Aflatoxin B, a Metabolite of *Aspergillus Flavus*. Remarks on the Structure of Aflatoxin B. *Recl. Trav. Chim. Pays-Bas* **1963**, *82*, 587–592. [CrossRef]
35. Smith, R.H.; McKernan, W. Hepatotoxic Action of Chromatographically Separated Fractions of *Aspergillus Flavus* Extracts. *Nature* **1962**, *195*, 1301–1303. [CrossRef]
36. De Iongh, H.; Beerthuis, R.K.; Vles, R.O.; Barrett, C.B.; Ord, W.O. Investigation of the Factor in Groundnut Meal Responsible for "Turkey X Disease". *Biochim. Biophys. Acta* **1962**, *65*, 548–551. [CrossRef]
37. Allcroft, R.; Carnaghan, R.B.A. Groundnut Toxicity. *Aspergillus Flavus Toxin (Aflatoxin) in Animal Products: Preliminary Communication. Vet. Rec.* **1962**, *74*, 863–864.
38. Asao, T.; Buchi, G.; Abdel-Kader, M.M.; Chang, S.B.; Wick, E.L.; Wogan, G.N. Aflatoxins B and G. *J. Am. Chem. Soc.* **1963**, *85*, 1706–1707. [CrossRef]
39. Austwick, P.K.C.; Ayerst, G. Toxic Products in Groundnuts: Groundnut Microflora and Toxicity. *Chem. Ind.* **1963**, 55–61.
40. Sargkant, K.; Carnaghan, R.B.A.; Allcroft, R. Toxic Products in Groundnuts. Chemistry and Origin. *Chem. Ind.* **1963**, *2*, 50–53.
41. Raper, K.B.; Fennel, D.I. *The Genus Aspergillus*; Williams and Wilkins: Baltimore, MD, USA, 1965.
42. Zhang, K.; Wong, J.W.; Krynitsky, A.J.; Trucksess, M.W. Perspective on Advancing FDA Regulatory Monitoring for Mycotoxins in Foods Using Liquid Chromatography and Mass Spectrometry (Review). *J. AOAC Int.* **2016**, *99*, 890–894. [CrossRef]
43. De Iongh, H.; Vles, R.O.; Van Pelt, J.G. Milk of Mammals Fed an Aflatoxin-Containing Diet. *Nature* **1964**, *202*, 466–467. [CrossRef]

44. Payet, M.; Cros, J.; Quenum, C.; Sankale, M.; Moulanier, M. Deux Observations d'enfants Ayant Consommé de Façon Prolongée Des Farines Souillées Par *Aspergillus Flavus*. *Presse Med.* **1966**, *74*, 649–651.
45. Holzapfel, C.W.; Steyn, P.S.; Purchase, I.F. Isolation and Structure of Aflatoxins M1 and M2. *Tetrahedron Lett.* **1966**, *25*, 2799–2803. [CrossRef]
46. Allcroft, R.; Rogers, H.; Lewis, G.; Nabney, J.; Best, P.E. Metabolism of Aflatoxin in Sheep: Excretion of the 'Milk Toxin'. *Nature* **1966**, *209*, 154–155. [CrossRef] [PubMed]
47. Buechi, G.; Foulkes, D.M.; Kurono, M.; Mitchell, G.F.; Schneider, R.S. Total Synthesis of Racemic Aflatoxin B1. *J. Am. Chem. Soc.* **1967**, *89*, 6745–6753. [CrossRef]
48. Serck-Hanssen, A. Aflatoxin-Induced Fatal Hepatitis? A Case Report from Uganda. *Arch. Environ. Health* **1970**, *20*, 729–731. [CrossRef] [PubMed]
49. IARC Aflatoxins. *IARC Monographs on Evaluation of Carcinogenic Risk of Chemicals to Man*; IARC Press: Lyon, France, 1972; pp. 145–156.
50. Krishnamachari, K.A.; Bhat, R.V.; Nagarajan, V.; Tilak, T.B. Hepatitis Due to Aflatoxicosis. An Outbreak in Western India. *Lancet* **1975**, *305*, 1061–1063. [CrossRef]
51. IARC Aflatoxins. *IARC Monographs on the Evaluation of the Crcinogenic Risk of Chemicals to Man: Some Naturally Occurring Substances*; IARC Press: Lyon, France, 1976; pp. 51–72.
52. FDA—US Food and Drug Administration. CPG Sec. 527.400 Whole Milk, Lowfat Milk, Skim Milk—Aflatoxin M1. Available online: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/cpg-sec-527400-whole-milk-lowfat-milk-skim-milk-aflatoxin-m1 (accessed on 9 February 2020).
53. Essigmann, J.M.; Croy, R.G.; Nadzan, A.M.; Busby, W.F.; Reinhold, V.N.; Büchi, G.; Wogan, G.N. Structural Identification of the Major DNA Adduct Formed by Aflatoxin B1 in Vitro. *Proc. Natl. Acad. Sci. USA* **1977**, *74*, 1870–1874. [CrossRef] [PubMed]
54. FAO. Perspective on Mycotoxins. In *Selected Documents of the Joint FAO/WHO/UNEP, Proceedings of the Conference on Mycotoxins, Nairobi, Kenya, 19–27 September 1977*; FAO Food and Nutrition Paper; FAO: Nairobi, Kenya, 1979; Volume 13, pp. 1–167.
55. Goto, T.; Manabe, M.; Matsuura, S. Application of High-Performance Liquid Chromatography to the Analysis of Aflatoxins in Foods and Feeds. Preparation of Sample and Column. *Agric. Biol. Chem.* **1979**, *43*, 2591–2592. [CrossRef]
56. Bennett, R.A.; Essigmann, J.M.; Wogan, G.N. Excretion of an Aflatoxin-Guanine Adduct in the Urine of Aflatoxin B1-Treated Rats. *Cancer Res.* **1981**, *41*, 650–654.
57. Sorenson, W.G.; Jones, W.; Simpson, J.; Davidson, J.I. Aflatoxin in Respirable Airborne Peanut Dust. *J. Toxicol. Environ. Health* **1984**, *14*, 525–533. [CrossRef]
58. Woychik, N.A.; Hinsdill, R.D.; Chu, F.S. Production and Characterization of Monoclonal Antibodies against Aflatoxin M1. *Appl. Environ. Microbiol.* **1984**, *48*, 1096–1099. [CrossRef] [PubMed]
59. IARC Aflatoxins. *IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans. Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs*; IARC Monographs Supplement 7; International Agency for Research in Cancer: Lyon, France, 1987; Volume 1–42, pp. 83–87. ISBN 92 832 1411 0.
60. Kurtzman, C.P.; Horn, B.W.; Hesseltine, C.W. *Aspergillus Nomius*, a New Aflatoxin-Producing Species Related to *Aspergillus Flavus* and *Aspergillus Tamarii*. *Antonie Van Leeuwenhoek* **1987**, *53*, 147–158. [CrossRef] [PubMed]
61. Sabbioni, G.; Skipper, P.L.; Büchi, G.; Tannenbaum, S.R. Isolation and Characterization of the Major Serum Albumin Adduct Formed by Aflatoxin B1 in Vivo in Rats. *Carcinogenesis* **1987**, *8*, 819–824. [CrossRef]
62. Bosch, F.X.; Muñoz, N. Prospects for Epidemiological Studies on Hepatocellular Cancer as a Model for Assessing Viral and Chemical Interactions. *IARC Sci. Publ.* **1988**, *89*, 427–438.
63. Groopman, J.D.; Hall, A.J.; Whittle, H.; Hudson, G.J.; Wogan, G.N.; Montesano, R.; Wild, C.P. Molecular Dosimetry of Aflatoxin-N7-Guanine in Human Urine Obtained in The Gambia, West Africa. *Cancer Epidemiol. Biomark. Prev.* **1992**, *1*, 221–227.
64. Groopman, J.D.; Zhu, J.Q.; Donahue, P.R.; Pikul, A.; Zhang, L.S.; Chen, J.S.; Wogan, G.N. Molecular Dosimetry of Urinary Aflatoxin-DNA Adducts in People Living in Guangxi Autonomous Region, People's Republic of China. *Cancer Res.* **1992**, *52*, 45–52.
65. Gan, L.S.; Skipper, P.L.; Peng, X.C.; Groopman, J.D.; Chen, J.S.; Wogan, G.N.; Tannenbaum, S.R. Serum Albumin Adducts in the Molecular Epidemiology of Aflatoxin Carcinogenesis: Correlation with Aflatoxin B1 Intake and Urinary Excretion of Aflatoxin M1. *Carcinogenesis* **1988**, *9*, 1323–1325. [CrossRef] [PubMed]
66. Bressac, B.; Kew, M.; Wands, J.; Ozturk, M. Selective G to T Mutations of P53 Gene in Hepatocellular Carcinoma from Southern Africa. *Nature* **1991**, *350*, 429–431. [CrossRef] [PubMed]
67. Yabe, K.; Ando, Y.; Hamasaki, T. A Metabolic Grid among Versiconal Hemiacetal Acetate, Versiconol Acetate, Versiconol and Versiconal during Aflatoxin Biosynthesis. *J. Gen. Microbiol.* **1991**, *137*, 2469–2475. [CrossRef]
68. Ross, R.K.; Yuan, J.M.; Yu, M.C.; Wogan, G.N.; Qian, G.S.; Tu, J.T.; Groopman, J.D.; Gao, Y.T.; Henderson, B.E. Urinary Aflatoxin Biomarkers and Risk of Hepatocellular Carcinoma. *Lancet* **1992**, *339*, 943–946. [CrossRef]
69. IARC International Agency for Research on Cancer. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occuring Substances: Food Items and Costituents, Heterocyclic Aromatic Amines and Mycotoxins*; IARC Press: Lyon, France, 1993; Volume 56, ISBN 92-832-1256-8.
70. Shapira, R.; Paster, N.; Eyal, O.; Menasherov, M.; Mett, A.; Salomon, R. Detection of Aflatoxigenic Molds in Grains by PCR. *Appl. Environ. Microbiol.* **1996**, *62*, 3270–3273. [CrossRef]

71. Lasky, T.; Magder, L. Hepatocellular Carcinoma P53 G > T Transversions at Codon 249: The Fingerprint of Aflatoxin Exposure? *Environ. Health Perspect.* **1997**, *105*, 392–397. [CrossRef]
72. Geiser, D.M.; Pitt, J.I.; Taylor, J.W. Cryptic Speciation and Recombination in the Aflatoxin-Producing Fungus *Aspergillus Flavus*. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 388–393. [CrossRef]
73. Vahl, M.; Jørgensen, K. Determination of Aflatoxins in Food Using LC/MS/MS. *Z. Lebensm. Unters. Forsch.* **1998**, *206*, 243–245. [CrossRef]
74. European Commission. Commission Regulation (EC) No 466/2001 of 8 March 2001 Setting Maximum Levels for Certain Contaminants in Foodstuffs (Text with EEA Relevance). *Off. J. Eur. Comm.* **2001**, 1–13.
75. International Agency for Research on Cancer. *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Traditional Herbal Medicine, Some Mycotoxins, Naphthalene and Strene*; IARC Press: Lyon, France, 2002; Volume 82, ISBN 92-832-1282-7.
76. European Commission. Commission Regulation (EC) No 2174/2003 of 12 December 2003 Amending Regulation (EC) No 466/2001 as Regards Aflatoxins (Text with EEA Relevance). *Off. J. Eur. Union* **2003**, *326*, 12–15.
77. Azziz-Baumgartner, E.; Lindblade, K.; Gieseker, K.; Rogers, H.S.; Kieszak, S.; Njapau, H.; Schleicher, R.; McCoy, L.F.; Misore, A.; DeCock, K.; et al. Case-Control Study of an Acute Aflatoxicosis Outbreak, Kenya, 2004. *Environ. Health Perspect.* **2005**, *113*, 1779–1783. [CrossRef]
78. Yu, J.; Chang, P.-K.; Ehrlich, K.C.; Cary, J.W.; Bhatnagar, D.; Cleveland, T.E.; Payne, G.A.; Linz, J.E.; Woloshuk, C.P.; Bennett, J.W. Clustered Pathway Genes in Aflatoxin Biosynthesis. *Appl. Environ. Microbiol.* **2004**, *70*, 1253–1262. [CrossRef] [PubMed]
79. European Commission. Commision Regulation (EC) No 401/2006 of 23 February 2006 Lying down the Methods of Sampling and Analysis for the Official Control of the Levels of Mycotoxins in Foodstuffs. *Off. J. Eur. Union* **2006**, *70*, 12–34.
80. European Commission. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Off. J. Eur. Union* **2006**, *364*, 5–24.
81. Yard, E.E.; Daniel, J.H.; Lewis, L.S.; Rybak, M.E.; Paliakov, E.M.; Kim, A.A.; Montgomery, J.M.; Bunnell, R.; Abudo, M.U.; Akhwale, W.; et al. Human Aflatoxin Exposure in Kenya, 2007: A Cross-Sectional Study. *Food Addit. Contam. Part A* **2013**, *30*, 1322–1331. [CrossRef] [PubMed]
82. Bandyopadhyay, R.; Atehnkeng, J.; Ortega-Beltran, A.; Akande, A.; Falade, T.D.O.; Cotty, P.J. "Ground-Truthing" Efficacy of Biological Control for Aflatoxin Mitigation in Farmers' Fields in Nigeria: From Field Trials to Commercial Usage, a 10-Year Study. *Front. Microbiol.* **2019**, *10*, 1–18. [CrossRef] [PubMed]
83. Battilani, P.; Rossi, V.; Giorni, P.; Pietri, A.; Gualla, A.; Van der Fels-Klerx, H.J.; Booij, C.J.H.; Moretti, A.; Logrieco, A.; Miglietta, F. Modelling, Predicting and Mapping the Emergence of Aflatoxins in Cereals in the EU Due to Climate Change. *EFSA Support. Publ.* **2012**, *9*, 1–172. [CrossRef]
84. International Agency for Research on Cancer. Aflatoxins. In *Chemical Agents and Related Occupations: A Review of Human Carcinogens*; IARC Press: Lyon, France, 2012; Volume 100F, pp. 225–248. ISBN 978 92 832 1323 9.
85. Duarte, S.C.; Almeida, A.M.; Teixeira, A.S.; Pereira, A.L.; Falcão, A.C.; Pena, A.; Lino, C.M. Aflatoxin M1 in Marketed Milk in Portugal: Assessment of Human and Animal Exposure. *Food Control* **2013**, *30*, 411–417. [CrossRef]
86. Tsakiris, I.N.; Tzatzarakis, M.N.; Alegakis, A.K.; Vlachou, M.I.; Renieri, E.A.; Tsatsakis, A.M. Risk Assessment Scenarios of Children's Exposure to Aflatoxin M1 Residues in Different Milk Types from the Greek Market. *Food Chem. Toxicol.* **2013**, *56*, 261–265. [CrossRef]
87. Santini, A.; Raiola, A.; Ferrantelli, V.; Giangrosso, G.; Macaluso, A.; Bognanno, M.; Galvano, F.; Ritieni, A. Aflatoxin M1 in Raw, UHT Milk and Dairy Products in Sicily (Italy). *Food Addit. Contam. Part B* **2013**, *6*, 181–186. [CrossRef] [PubMed]
88. European Food Safety Authority. Effect on Public Health of a Possible Increase of the Maximum Level for 'Aflatoxin Total' from 4 to 10 Mg/Kg in Peanuts and Processed Products Thereof, Intended for Direct Human Consumption or Use as an Ingredient in Foodstuffs. *EFSA J.* **2018**, *16*, 1–32. [CrossRef]
89. Herrman, T.J.; Hoffman, V.; Muiruri, A.; McCormick, C. Aflatoxin Proficiency Testing and Control in Kenya. *J. Food Prot.* **2019**, *83*, 142–146. [CrossRef] [PubMed]
90. Frisvad, J.C.; Hubka, V.; Ezekiel, C.N.; Hong, S.-B.; Nováková, A.; Chen, A.J.; Arzanlou, M.; Larsen, T.O.; Sklenář, F.; Mahakarnchanakul, W.; et al. Taxonomy of *Aspergillus* Section *Flavi* and Their Production of Aflatoxins, Ochratoxins and Other Mycotoxins. *Stud. Mycol.* **2019**, *93*, 1–63. [CrossRef] [PubMed]
91. European Food Safety Authority. Risk Assessment of Aflatoxins in Food. *EFSA J.* **2020**, *18*, 1–112. [CrossRef]
92. Singh, P.; Orbach, M.J.; Cotty, P.J. *Aspergillus Texensis*: A Novel Aflatoxin Producer with S Morphology from the United States. *Toxins* **2018**, *10*, 513. [CrossRef] [PubMed]
93. Singh, P.; Callicott, K.A.; Orbach, M.J.; Cotty, P.J. Molecular Analysis of S-Morphology Aflatoxin Producers from the United States Reveals Previously Unknown Diversity and Two New Taxa. *Front. Microbiol.* **2020**, *11*, 1–16. [CrossRef] [PubMed]
94. Liu, W.; Wang, L.; Yang, X.; Zeng, H.; Zhang, R.; Pu, C.; Zheng, C.; Tan, Y.; Luo, Y.; Feng, X.; et al. Environmental Microcystin Exposure Increases Liver Injury Risk Induced by Hepatitis B Virus Combined with Aflatoxin: A Cross-Sectional Study in Southwest China. *Environ. Sci. Technol.* **2017**, *51*, 6367–6378. [CrossRef]
95. Guo, H.; Ji, J.; Wang, J.; Sun, X. Co-Contamination and Interaction of Fungal Toxins and Other Environmental Toxins. *Trends Food Sci. Technol.* **2020**, *103*, 162–178. [CrossRef]

96. Renu, K.; Chakraborty, R.; Myakala, H.; Koti, R.; Famurewa, A.C.; Madhyastha, H.; Vellingiri, B.; George, A.; Valsala Gopalakrishnan, A. Molecular Mechanism of Heavy Metals (Lead, Chromium, Arsenic, Mercury, Nickel and Cadmium)—Induced Hepatotoxicity—A Review. *Chemosphere* **2021**, *271*, 129735. [CrossRef]
97. Guan, Y.; Chen, J.; Nepovimova, E.; Long, M.; Wu, W.; Kuca, K. Aflatoxin Detoxification Using Microorganisms and Enzymes. *Toxins* **2021**, *13*, 46. [CrossRef]
98. Sipos, P.; Peles, F.; Brassó, D.L.; Béri, B.; Pusztahelyi, T.; Pócsi, I.; Győri, Z. Physical and Chemical Methods for Reduction in Aflatoxin Content of Feed and Food. *Toxins* **2021**, *13*, 204. [CrossRef]
99. Fan, T.; Xie, Y.; Ma, W. Research Progress on the Protection and Detoxification of Phytochemicals against Aflatoxin B1-Induced Liver Toxicity. *Toxicon* **2021**, *195*, 58–68. [CrossRef]
100. Hamid, A.S.; Tesfamariam, I.G.; Zhang, Y.; Zhang, Z.G. Aflatoxin B1-Induced Hepatocellular Carcinoma in Developing Countries: Geographical Distribution, Mechanism of Action and Prevention (Review). *Oncol. Lett.* **2013**, *5*, 1087–1092. [CrossRef] [PubMed]
101. Williams, J.H.; Phillips, T.D.; Jolly, P.E.; Stiles, J.K.; Jolly, C.M.; Aggarwal, D. Human Aflatoxicosis in Developing Countries: A Review of Toxicology, Exposure, Potential Health Consequences, and Interventions. *Am. J. Clin. Nutr.* **2004**, *80*, 1106–1122. [CrossRef] [PubMed]
102. Singh, U.; Gupta, S.; Gupta, M. A Review on Study on Biological Ill Effects and Health Hazards f Aflatoxins. *Int. J. Adv. Med.* **2021**, *3*, 1–8.
103. Pickova, D.; Ostry, V.; Malir, F. A Recent Overview of Producers and Important Dietary Sources of Aflatoxins. *Toxins* **2021**, *13*, 186. [CrossRef] [PubMed]
104. World Health Organization. Aflatoxins. Available online: https://www.who.int/foodsafety/FSDigest_Aflatoxins_EN.pdf (accessed on 9 February 2020).
105. Ostry, V.; Malir, F.; Toman, J.; Grosse, Y. Mycotoxins as Human Carcinogens—The IARC Monographs Classification. *Mycotoxin Res.* **2017**, *33*, 65–73. [CrossRef]
106. Afshar, P.; Shokrzadeh, M.; Raeisi, S.N.; Ghorbani-HasanSaraei, A.; Nasiraii, L.R. Aflatoxins Biodetoxification Strategies Based on Probiotic Bacteria. *Toxicon* **2020**, *178*, 50–58. [CrossRef]
107. Benkerroum, N. Chronic and Acute Toxicities of Aflatoxins: Mechanisms of Action. *Int. J. Environ. Res. Public Health* **2020**, *17*, 423. [CrossRef]
108. Hua, Z.; Liu, R.; Chen, Y.; Liu, G.; Li, C.; Song, Y.; Cao, Z.; Li, W.; Li, W.; Lu, C.; et al. Contamination of Aflatoxins Induces Severe Hepatotoxicity Through Multiple Mechanisms. *Front. Pharmacol.* **2020**, *11*, 605823. [CrossRef]
109. Pickova, D.; Ostry, V.; Malir, J.; Toman, J.; Malir, F. A Review on Mycotoxins and Microfungi in Spices in the Light of the Last Five Years. *Toxins* **2020**, *12*, 789. [CrossRef] [PubMed]
110. Zhou, H.; Wang, J.; Ma, L.; Chen, L.; Guo, T.; Zhang, Y.; Dai, H.; Yu, Y. Oxidative DNA Damage and Multi-Organ Pathologies in Male Mice Subchronically Treated with Aflatoxin B1. *Ecotoxicol. Environ. Saf.* **2019**, *186*, 1–10. [CrossRef] [PubMed]
111. Li, X.; Lv, Z.; Chen, J.; Nepovimova, E.; Long, M.; Wu, W.; Kuca, K. *Bacillus Amyloliquefaciens* B10 Can Alleviate Liver Apoptosis and Oxidative Stress Induced by Aflatoxin B1. *Food Chem. Toxicol.* **2021**, *151*, 112124. [CrossRef]
112. Owumi, S.; Najophe, E.S.; Farombi, E.O.; Oyelere, A.K. Gallic Acid Protects against Aflatoxin B1-Induced Oxidative and Inflammatory Stress Damage in Rats Kidneys and Liver. *J. Food Biochem.* **2020**, *44*, 13316. [CrossRef]
113. Hassan, A.A.; Abu Hafsa, S.H.; Elghandour, M.M.M.Y.; Kanth Reddy, P.R.; Monroy, J.C.; Salem, A.Z.M. Dietary Supplementation with Sodium Bentonite and Coumarin Alleviates the Toxicity of Aflatoxin B1 in Rabbits. *Toxicon* **2019**, *171*, 35–42. [CrossRef]
114. Śliżewska, K.; Cukrowska, B.; Smulikowska, S.; Cielecka-Kuszyk, J. The Effect of Probiotic Supplementation on Performance and the Histopathological Changes in Liver and Kidneys in Broiler Chickens Fed Diets with Aflatoxin B1. *Toxins* **2019**, *11*, 112. [CrossRef] [PubMed]
115. Park, S.; Lee, J.-Y.; You, S.; Song, G.; Lim, W. Neurotoxic Effects of Aflatoxin B1 on Human Astrocytes in Vitro and on Glial Cell Development in Zebrafish in Vivo. *J. Hazard. Mater.* **2020**, *386*, 121639. [CrossRef]
116. Pang, V.F.; Chiang, C.-F.; Chang, C.-C. The in Vitro Effects of Aflatoxin B1 on Physiological Functions of Swine Alveolar Macrophages. *Vet. Med. Sci.* **2020**, *6*, 919–925. [CrossRef] [PubMed]
117. El-Sayed Mostafa, H.; Ahmed Allithy, A.N.; Abdellatif, N.A.; Anani, M.; Fareed, S.A.; El-Shafei, D.A.; Alaa El-Din, E.A. Amelioration of Pulmonary Aflatoxicosis by Green Tea Extract: An in Vivo Study. *Toxicon* **2021**, *189*, 48–55. [CrossRef] [PubMed]
118. Akinrinde, A.S.; Adebiyi, O.E.; Asekun, A. Amelioration of Aflatoxin B1-Induced Gastrointestinal Injuries by Eucalyptus Oil in Rats. *J. Complement. Integr. Med.* **2019**, *17*, 1–11. [CrossRef]
119. Pu, J.; Yuan, Q.; Yan, H.; Tian, G.; Chen, D.; He, J.; Zheng, P.; Yu, J.; Mao, X.; Huang, Z.; et al. Effects of Chronic Exposure to Low Levels of Dietary Aflatoxin B1 on Growth Performance, Apparent Total Tract Digestibility and Intestinal Health in Pigs. *Animals* **2021**, *11*, 336. [CrossRef] [PubMed]
120. Hernández-Ramírez, J.O.; Nava-Ramírez, M.J.; Merino-Guzmán, R.; Téllez-Isaías, G.; Vázquez-Durán, A.; Méndez-Albores, A. The Effect of Moderate-Dose Aflatoxin B1 and *Salmonella Enteritidis* Infection on Intestinal Permeability in Broiler Chickens. *Mycotoxin Res.* **2020**, *36*, 31–39. [CrossRef] [PubMed]
121. Choi, S.-Y.; Kim, T.H.; Hong, M.-W.; Park, T.S.; Lee, H.; Lee, S.-J. Transcriptomic Alterations Induced by Aflatoxin B1 and Ochratoxin A in LMH Cell Line. *Poult. Sci.* **2020**, *99*, 5265–5274. [CrossRef]
122. Tadee, A.; Mahakunakorn, P.; Porasuphatana, S. Oxidative Stress and Genotoxicity of Co-Exposure to Chlorpyrifos and Aflatoxin B1 in HepG2 Cells. *Toxicol. Ind. Health* **2020**, *36*, 336–345. [CrossRef] [PubMed]

123. Wang, X.; Li, L.; Zhang, G. Quercetin Protects the Buffalo Rat Liver (BRL-3A) Cells from Aflatoxin B1-Induced Cytotoxicity via Activation of Nrf2-ARE Pathway. *World Mycotoxin J.* **2020**, *13*, 299–312. [CrossRef]
124. Wu, K.; Jia, S.; Zhang, J.; Zhang, C.; Wang, S.; Rajput, S.A.; Sun, L.; Qi, D. Transcriptomics and Flow Cytometry Reveals the Cytotoxicity of Aflatoxin B1 and Aflatoxin M1 in Bovine Mammary Epithelial Cells. *Ecotoxicol. Environ. Saf.* **2021**, *209*, 1–9. [CrossRef] [PubMed]
125. Dey, D.K.; Kang, S.C. Aflatoxin B1 Induces Reactive Oxygen Species-Dependent Caspase-Mediated Apoptosis in Normal Human Cells, Inhibits Allium Cepa Root Cell Division, and Triggers Inflammatory Response in Zebrafish Larvae. *Sci. Total Environ.* **2020**, *737*, 139704. [CrossRef]
126. Jiang, Y.; Hansen, P.J.; Xiao, Y.; Amaral, T.F.; Vyas, D.; Adesogan, A.T. Aflatoxin Compromises Development of the Preimplantation Bovine Embryo through Mechanisms Independent of Reactive Oxygen Production. *J. Dairy Sci.* **2019**, *102*, 10506–10513. [CrossRef] [PubMed]
127. Milićević, D.R.; Škrinjar, M.; Baltić, T. Real and Perceived Risks for Mycotoxin Contamination in Foods and Feeds: Challenges for Food Safety Control. *Toxins* **2010**, *2*, 572. [CrossRef]
128. Grenier, B.; Oswald, I. Mycotoxin Co-contamination of Food and Feed: Meta-Analysis of Publications Describing Toxicological Interactions. *World Mycotoxin J.* **2011**, *4*, 285–313. [CrossRef]
129. Streit, E.; Naehrer, K.; Rodrigues, I.; Schatzmayr, G. Mycotoxin Occurrence in Feed and Feed Raw Materials Worldwide -Long Term Analysis with Special Focus on Europe and Asia. *J. Sci. Food Agric.* **2013**, *93*, 2892–2899. [CrossRef]
130. Smith, M.-C.; Madec, S.; Coton, E.; Hymery, N. Natural Co-occurrence of Mycotoxins in Foods and Feeds and Their in Vitro Combined Toxicological Effects. *Toxins* **2016**, *8*, 94. [CrossRef]
131. Huang, S.; Zheng, N.; Fan, C.; Cheng, M.; Wang, S.; Jabar, A.; Wang, J.; Cheng, J. Effects of Aflatoxin B1 Combined with Ochratoxin A and/or Zearalenone on Metabolism, Immune Function, and Antioxidant Status in Lactating Dairy Goats. *Asian Australas. J. Anim. Sci.* **2018**, *31*, 505–513. [CrossRef]
132. Hou, L.; Gan, F.; Zhou, X.; Zhou, Y.; Qian, G.; Liu, Z.; Huang, K. Immunotoxicity of Ochratoxin A and Aflatoxin B1 in Combination Is Associated with the Nuclear Factor Kappa B Signaling Pathway in 3D4/21 cells. *Chemosphere* **2018**, *199*, 718–727. [CrossRef]
133. Ji, J.; Wang, Q.; Wu, H.; Xia, S.; Guo, H.; Blaženović, I.; Zhang, Y.; Sun, X. Insights into Cellular Metabolic Pathways of the Combined Toxicity Responses of Caco-2 Cells Exposed to Deoxynivalenol, Zearalenone and Aflatoxin B1. *Food Chem. Toxicol.* **2019**, *126*, 106–112. [CrossRef] [PubMed]
134. Eze, U.A.; Huntriss, J.; Routledge, M.N.; Gong, Y.Y. Toxicological Effects of Regulated Mycotoxins and Persistent Organochloride Pesticides: In Vitro Cytotoxic Assessment of Single and Defined Mixtures on MA-10 Murine Leydig Cell Line. *Toxicol. In Vitro* **2018**, *48*, 93–103. [CrossRef]
135. Gao, Y.; Bao, X.; Meng, L.; Liu, H.; Wang, J.; Zheng, N. Aflatoxin B1 and Aflatoxin M1 Induce Compromised Intestinal Integrity through Clathrin-Mediated Endocytosis. *Toxins* **2021**, *13*, 184. [CrossRef]
136. Zhao, Q.; Yang, Z.-S.; Cao, S.-J.; Chang, Y.-F.; Cao, Y.-Q.; Li, J.-B.; Yao, Z.-X.; Wen, Y.-P.; Huang, X.-B.; Wu, R.; et al. Acute Oral Toxicity Test and Assessment of Combined Toxicity of Cadmium and Aflatoxin B1 in Kunming Mice. *Food Chem. Toxicol.* **2019**, *131*, 1–7. [CrossRef]
137. Olugbami, J.O.; Damoiseaux, R.; Odunola, O.A.; Gimzewski, J.K. Mitigation of Aflatoxin B1- and Sodium Arsenite-Induced Cytotoxicities in HUC-PC Urinary Bladder Cells by Curcumin and Khaya Senegalensis. *J. Basic Clin. Physiol. Parmacol.* **2020**, *31*. [CrossRef] [PubMed]
138. Liu, W.; Wang, L.; Zheng, C.; Liu, L.; Wang, J.; Li, D.; Tan, Y.; Zhao, X.; He, L.; Shu, W. Microcystin-LR Increases Genotoxicity Induced by Aflatoxin B1 through Oxidative Stress and DNA Base Excision Repair Genes in Human Hepatic Cell Lines. *Environ. Pollut.* **2018**, *233*, 455–463. [CrossRef]
139. Wang, L.; He, L.; Zeng, H.; Fu, W.; Wang, J.; Tan, Y.; Zheng, C.; Qiu, Z.; Luo, J.; Lv, C.; et al. Low-Dose Microcystin-LR Antagonizes Aflatoxin B1 Induced Hepatocarcinogenesis through Decreasing Cytochrome P450 1A2 Expression and Aflatoxin B1-DNA Adduct Generation. *Chemosphere* **2020**, *248*, 126036. [CrossRef]
140. Meneely, J.P.; Hajšlová, J.; Krska, R.; Elliott, C.T. Assessing the Combined Toxicity of the Natural Toxins, Aflatoxin B1, Fumonisin B1 and Microcystin-LR by High Content Analysis. *Food Chem. Toxicol.* **2018**, *121*, 527–540. [CrossRef] [PubMed]
141. Chen, Y.; Liu, Y. Non-Coplanar and Coplanar Polychlorinated Biphenyls Potentiate Genotoxicity of Aflatoxin B1 in a Human Hepatocyte Line by Enhancing CYP1A2 and CYP3A4 Expression. *Environ. Pollut.* **2019**, *246*, 945–954. [CrossRef]
142. Fishbein, A.; Hammock, B.D.; Serhan, C.N.; Panigrahy, D. Carcinogenesis: Failure of Resolution of Inflammation? *Pharmacol. Ther.* **2021**, *218*, 107670. [CrossRef]
143. Peraica, M.; Radić, B.; Lucić, A.; Pavlović, M. Toxic Effects of Mycotoxins in Human. *Bull. World Health Organ.* **1999**, *77*, 754–766. [PubMed]
144. Ismail, A.; Gonçalves, B.L.; de Neeff, D.V.; Ponzilacqua, B.; Coppa, C.F.S.C.; Hintzsche, H.; Sajid, M.; Cruz, A.G.; Corassin, C.H.; Oliveira, C.A.F. Aflatoxin in Foodstuffs: Occurrence and Recent Advances in Decontamination. *Food Res. Int.* **2018**, *113*, 74–85. [CrossRef] [PubMed]
145. Liu, Y.; Wu, F. Global Burden of Aflatoxin-Induced Hepatocellular Carcinoma: A Risk Assessment. *Environ. Health Perspect.* **2010**, *118*, 818–824. [CrossRef] [PubMed]
146. International Agency for Research on Cancer/World Health Organization. Cancer Today. Available online: https://gco.iarc.fr/today/home (accessed on 28 March 2021).

147. Resham, S. Why Hepatocellular Carcinoma (Hcc)'s Management and Control Is Challenging in the Developing Countries? Problems vs. Strategies. *J. Integr. Oncol. S* **2016**, *1*, 2. [CrossRef]
148. McCullough, A.K.; Lloyd, R.S. Mechanisms Underlying Aflatoxin-Associated Mutagenesis—Implications in Carcinogenesis. *DNA Repair* **2019**, *77*, 76–86. [CrossRef]
149. Ferreira, R.G.; Cardoso, M.V.; de Souza Furtado, K.M.; Espíndola, K.M.M.; Amorim, R.P.; Monteiro, M.C. Epigenetic Alterations Caused by Aflatoxin B1: A Public Health Risk in the Induction of Hepatocellular Carcinoma. *Transl. Res.* **2018**, *204*, 51–71. [CrossRef]
150. El-Serag, H.B. Epidemiology of Viral Hepatitis and Hepatocellular Carcinoma. *Gastroenterology* **2012**, *142*, 1264–1273. [CrossRef]
151. Kew, M.C. Hepatocellular Carcinoma in Developing Countries: Prevention, Diagnosis and Treatment. *World J. Hepatol* **2012**, *4*, 99–104. [CrossRef] [PubMed]
152. Habibi, N.; Nassiri-Toosi, M.; Sharafi, H.; Alavian, S.M.; Shams-Ghahfarokhi, M.; Razzaghi-Abyaneh, M. Aflatoxin B $_1$ Exposure and the Risk of Hepatocellular Carcinoma in Iranian Carriers of Viral Hepatitis B and C. *Toxin Rev.* **2019**, *38*, 234–239. [CrossRef]
153. Kew, M.C. Synergistic Interaction between Aflatoxin B1 and Hepatitis B Virus in Hepatocarcinogenesis. *Liver Int.* **2003**, *23*, 405–409. [CrossRef]
154. Bbosa, G.S.; Kitya, D.; Odda, J.; Ogwal-Okeng, J. Aflatoxins Metabolism, Effects on Epigenetic Mechanisms and Their Role in Carcinogenesis. *Health* **2013**, *5*, 14–34. [CrossRef]
155. Groopman, J.D.; Johnson, D.; Kensler, T.W. Aflatoxin and Hepatitis B Virus Biomarkers: A Paradigm for Complex Environmental Exposures and Cancer Risk. *Cancer Biomark.* **2005**, *1*, 5–14. [CrossRef]
156. Monson, M.S.; Coulombe, R.A.; Reed, K.M. Aflatoxicosis: Lessons from Toxicity and Responses to Aflatoxin B1 in Poultry. *Agriculture* **2015**, *5*, 742–777. [CrossRef]
157. Agbetiameh, D.; Ortega-Beltran, A.; Awuah, R.T.; Atehnkeng, J.; Islam, M.-S.; Callicott, K.A.; Cotty, P.J.; Bandyopadhyay, R. Potential of Atoxigenic *Aspergillus Flavus* Vegetative Compatibility Groups Associated with Maize and Groundnut in Ghana as Biocontrol Agents for Aflatoxin Management. *Front. Microbiol.* **2019**, *10*, 02069. [CrossRef]
158. Lewis, M.H.; Carbone, I.; Luis, J.M.; Payne, G.A.; Bowen, K.L.; Hagan, A.K.; Kemerait, R.; Heiniger, R.; Ojiambo, P.S. Biocontrol Strains Differentially Shift the Genetic Structure of Indigenous Soil Populations of *Aspergillus Flavus*. *Front. Microbiol* **2019**, *10*, 1738. [CrossRef]
159. Senghor, L.A.; Ortega-Beltran, A.; Atehnkeng, J.; Callicott, K.A.; Cotty, P.J.; Bandyopadhyay, R. The Atoxigenic Biocontrol Product Aflasafe SN01 Is a Valuable Tool to Mitigate Aflatoxin Contamination of Both Maize and Groundnut Cultivated in Senegal. *Plant Disease* **2020**, *104*, 510–520. [CrossRef]
160. Ben Taheur, F.; Kouidhi, B.; Al Qurashi, Y.M.A.; Ben Salah-Abbès, J.; Chaieb, K. Review: Biotechnology of Mycotoxins Detoxification Using Microorganisms and Enzymes. *Toxicon* **2019**, *160*, 12–22. [CrossRef]
161. Li, P.; Su, R.; Yin, R.; Lai, D.; Wang, M.; Liu, Y.; Zhou, L. Detoxification of Mycotoxins through Biotransformation. *Toxins* **2020**, *12*, 121. [CrossRef]
162. Nešić, K.; Habschied, K.; Mastanjević, K. Possibilities for the Biological Control of Mycotoxins in Food and Feed. *Toxins* **2021**, *13*, 198. [CrossRef] [PubMed]
163. Peles, F.; Sipos, P.; Kovács, S.; Győri, Z.; Pócsi, I.; Pusztahelyi, T. Biological Control and Mitigation of Aflatoxin Contamination in Commodities. *Toxins* **2021**, *13*, 104. [CrossRef] [PubMed]
164. Pinton, P.; Suman, M.; Buck, N.; Dellafiora, L.; De Meester, J.; Stadler, D.; Rito, E. *Practical Guidance to Mitigation of Mycotoxins during Food Processing. Report, Commissioned by the Process.-Related Compounds and Natural Toxins Task Force*; ILSI Europe: Brussel, Belgium, 2019; ISBN 978-90-78637-45-5.
165. Karlovsky, P.; Suman, M.; Berthiller, F.; De Meester, J.; Eisenbrand, G.; Perrin, I.; Oswald, I.P.; Speijers, G.; Chiodini, A.; Recker, T. Impact of Food Processing and Detoxification Treatments on Mycotoxin Contamination. *Mycotox Res.* **2016**, *32*, 179–205. [CrossRef]
166. Bandyopadhyay, R.; Ortega-Beltran, A.; Akande, A.; Mutegi, C.; Atehnkeng, J.; Kaptoge, L.; Senghor, A.L.; Adhikari, B.N.; Cotty, P.J. Biological Control of Aflatoxins in Africa: Current Status and Potential Challenges in the Face of Climate Change. *World Mycotoxin J.* **2016**, *9*, 771–789. [CrossRef]
167. Warnatzsch, E.A.; Reay, D.S.; Camardo Leggieri, M.; Battilani, P. Climate Change Impact on Aflatoxin Contamination Risk in Malawi's Maize Crops. *Front. Sustain. Food Syst.* **2020**, *4*, 591792. [CrossRef]
168. Cotty, P.J. Virulence and Cultural Characteristics of Two *Aspergillus Flavus* Strains Pathogenic on Cotton. *Phytopathology* **1989**, *79*, 808–814. [CrossRef]
169. Adhikari, B.N.; Bandyopadhyay, R.; Cotty, P.J. Degeneration of Aflatoxin Gene Clusters in *Aspergillus Flavus* from Africa and North America. *AMB Express* **2016**, *6*, 62. [CrossRef]
170. Moore, G.G. Practical Considerations Will Ensure the Continued Success of Pre-Harvest Biocontrol Using Non-Aflatoxigenic *Aspergillus Flavus* Strains. *Crit. Rev. Food Sci. Nutr.* **2021**, 1–18. [CrossRef]
171. Mauro, A.; Garcia-Cela, E.; Pietri, A.; Cotty, P.J.; Battilani, P. Biological Control Products for Aflatoxin Prevention in Italy: Commercial Field Evaluation of Atoxigenic *Aspergillus Flavus* Active Ingredients. *Toxins* **2018**, *10*, 30. [CrossRef]
172. Savić, Z.; Dudaš, T.; Loc, M.; Grahovac, M.; Budakov, D.; Jajić, I.; Krstović, S.; Barošević, T.; Krska, R.; Sulyok, M.; et al. Biological Control of Aflatoxin in Maize Grown in Serbia. *Toxins* **2020**, *12*, 162. [CrossRef] [PubMed]

173. Kabak, B.; Dobson, A.; Var, I. Strategies to Prevent Mycotoxin Contamination of Food and Animal Feed: A Review. *Crit. Rev. Food Sci. Nutr.* **2006**, *46*, 593–619. [CrossRef]
174. Chavez, R.A.; Cheng, X.; Stasiewicz, M.J. A Review of the Methodology of Analyzing Aflatoxin and Fumonisin in Single Corn Kernels and the Potential Impacts of These Methods on Food Security. *Foods* **2020**, *9*, 297. [CrossRef] [PubMed]
175. Siwela, A.H.; Siwela, M.; Matindi, G.; Dube, S.; Nziramasanga, N. Decontamination of Aflatoxin-Contaminated Maize by Dehulling. *J. Sci. Food Agric.* **2005**, *85*, 2535–2538. [CrossRef]
176. Aly, S.E. Distribution of Aflatoxins in Product and By-Products during Glucose Production from Contaminated Corn. *Food/Nahrung* **2002**, *46*, 341–344. [CrossRef]
177. Lefyedi, M.L.; Taylor, J.R.N. Effect of Dilute Alkaline Steeping on the Microbial Contamination, Toxicity and Diastatic Power of Sorghum Malt. *J. Inst. Brew.* **2006**, *112*, 108–116. [CrossRef]
178. Raters, M.; Matissek, R. Thermal Stability of Aflatoxin B1 and Ochratoxin A. *Mycotoxin Res.* **2008**, *24*, 130–134. [CrossRef]
179. Herzallah, S.; Alshawabkeh, K.; Fataftah, A.A. Aflatoxin Decontamination of Artificially Contaminated Feeds by Sunlight, γ-Radiation, and Microwave Heating. *J. Appl. Poult. Res.* **2008**, *17*, 515–521. [CrossRef]
180. Ghanem, I.; Orfi, M.; Shamma, M. Effect of Gamma Radiation on the Inactivation of Aflatoxin B1 in Food and Feed Crops. *Braz. J. Microbiol.* **2008**, *39*, 787–791. [CrossRef]
181. Di Stefano, V.; Pitonso, R.; Avellone, G. Effect of Gamma Irradiation on Aflatoxins and Ochratoxin A Reduction in Almond Samples. *J. Food Res.* **2014**, *3*, 113–118. [CrossRef]
182. Stanley, J.; Patras, A.; Pendyala, B.; Vergne, M.J.; Bansode, R.R. Performance of a UV-A LED System for Degradation of Aflatoxins B1 and M1 in Pure Water: Kinetics and Cytotoxicity Study. *Sci. Rep.* **2020**, *10*, 1–12. [CrossRef]
183. Vijayalakshmi, S.; Nadanasabhapathi, S.; Kumar, R.; Sunny Kumar, S. Effect of PH and Pulsed Electric Field Process Parameters on the Aflatoxin Reduction in Model System Using Response Surface Methodology: Effect of PH and PEF on Aflatoxin Reduction. *J. Food Sci. Technol.* **2018**, *55*, 868–878. [CrossRef] [PubMed]
184. Bulut, N.; Atmaca, B.; Evrendilek, G.A.; Uzuner, S. Potential of Pulsed Electric Field to Control *Aspergillus Parasiticus*, Aflatoxin and Mutagenicity Levels: Sesame Seed Quality. *J. Food Saf.* **2020**, *40*, 1–12. [CrossRef]
185. Masimango, N.; Remacle, J.; Ramaut, J.L. The Role of Adsorption in the Elimination of Aflatoxin B1 from Contaminated Media. *Eur. J. Appl. Microbiol. Biotechnol.* **1978**, *6*, 101–105. [CrossRef]
186. Čolović, R.; Puvača, N.; Cheli, F.; Avantaggiato, G.; Greco, D.; Đuragić, O.; Kos, J.; Pinotti, L. Decontamination of Mycotoxin-Contaminated Feedstuffs and Compound Feed. *Toxins* **2019**, *11*, 617. [CrossRef]
187. Food Drug Administration. Title 21—Food and Drugs. Available online: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/cfrsearch.cfm?fr=184.1155 (accessed on 9 May 2020).
188. Doyle, M.P.; Applebaum, R.S.; Brackett, R.E.; Marth, E.H. Physical, Chemical and Biological Degradation of Mycotoxins in Foods and Agricultural Commodities. *J. Food Prot.* **1982**, *45*, 964–971. [CrossRef] [PubMed]
189. Di Natale, F.; Gallo, M.; Nigro, R. Adsorbents Selection for Aflatoxins Removal in Bovine Milks. *J. Food Eng.* **2009**, *95*, 186–191. [CrossRef]
190. Soufiani, G.R.N.; Razmara, M.; Kermanshahi, H.; Barrientos Velázquez, A.L.; Daneshmand, A. Assessment of Aflatoxin B1 Adsorption Efficacy of Natural and Processed Bentonites: In Vitro and in Vivo Assays. *Appl. Clay Sci.* **2016**, *123*, 129–133. [CrossRef]
191. Diaz, D.E.; Hagler, W.M.; Blackwelder, J.T.; Eve, J.A.; Hopkins, B.A.; Anderson, K.L.; Jones, F.T.; Whitlow, L.W. Aflatoxin Binders II: Reduction of Aflatoxin M1 in Milk by Sequestering Agents of Cows Consuming Aflatoxin in Feed. *Mycopathologia* **2004**, *157*, 233–241. [CrossRef]
192. Assaf, J.C.; Nahle, S.; Chokr, A.; Louka, N.; Atoui, A.; El Khoury, A. Assorted Methods for Decontamination of Aflatoxin M1 in Milk Using Microbial Adsorbents. *Toxins* **2019**, *11*, 304. [CrossRef]
193. Aiko, V.; Edamana, P.; Mehta, A. Decomposition and Detoxification of Aflatoxin B1 by Lactic Acid. *J. Sci. Food Agric.* **2016**, *96*, 1959–1966. [CrossRef]
194. Park, D.L.; Lee, L.S.; Price, R.L.; Pohland, A.E. Review of the Decontamination of Aflatoxins by Ammoniation: Current Status and Regulation. *J. Assoc. Off. Anal. Chem.* **1988**, *71*, 685–703. [CrossRef] [PubMed]
195. Intanoo, M.; Kongkeitkajorn, M.B.; Suriyasathaporn, W.; Phasuk, Y.; Bernard, J.K.; Pattarajinda, V. Effect of Supplemental Kluyveromyces Marxianus and Pichia Kudriavzevii on Aflatoxin M1 Excretion in Milk of Lactating Dairy Cows. *Animals* **2020**, *10*, 709. [CrossRef] [PubMed]
196. Tao, F.; Zhao, K.; Zhao, Q.; Xiang, F.; Han, G. A Novel Site-Specific Integration System for Genetic Modification of *Aspergillus flavus*. *G3 Genes Genomes Genet.* **2020**, *10*, 605–611. [CrossRef] [PubMed]
197. Masanga, J.O.; Matheka, J.M.; Omer, R.A.; Ommeh, S.C.; Monda, E.O.; Alakonya, A.E. Downregulation of Transcription Factor AflR in *Aspergillus Flavus* Confers Reduction to Aflatoxin Accumulation in Transgenic Maize with Alteration of Host Plant Architecture. *Plant Cell Rep.* **2015**, *34*, 1379–1387. [CrossRef]
198. Thakare, D.; Zhang, J.; Wing, R.A.; Cotty, P.J.; Schmidt, M.A. Aflatoxin-Free Transgenic Maize Using Host-Induced Gene Silencing. *Sci. Adv.* **2017**, *3*, 1–8. [CrossRef] [PubMed]
199. Sharma, K.K.; Pothana, A.; Prasad, K.; Shah, D.; Kaur, J.; Bhatnagar, D.; Chen, Z.-Y.; Raruang, Y.; Cary, J.W.; Rajasekaran, K.; et al. Peanuts That Keep Aflatoxin at Bay: A Threshold That Matters. *Plant Biotechnol. J.* **2018**, *16*, 1024–1033. [CrossRef]

Příloha 5

Natural occurrence of ochratoxin A in spices marketed in the Czech Republic during 2019–2020

foods

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Natural Occurrence of Ochratoxin A in Spices Marketed in the Czech Republic during 2019–2020

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Abstract: Spices are a popular ingredient in cuisine worldwide but can pose a health risk as they are prone to fungal infestation and mycotoxin contamination. The purpose of this study was to evaluate ochratoxin A (OTA) in 54 single-kind traditional and less traditional spices, each of which was purchased in six samples of different batches (324 samples in total) at the Czech market during 2019–2020. The HPLC-FLD method with pre-treatment by immunoaffinity columns was employed to determine OTA. The limits of detection and quantification were 0.03 ng g^{-1} and 0.10 ng g^{-1}, respectively. A total of 101 (31%) samples of 19 spice kinds were positive at concentrations ranging from 0.11–38.46 ng g^{-1}. Only turmeric was contaminated with an OTA level exceeding the European Union limits. However, most spices have no regulation, thus further extensive monitoring of various mycotoxins in various kinds of spices is necessary. Chilli and black pepper are the most studied spices for OTA contamination, however, many other kinds of spice can also be highly contaminated, but studies on them are less common, rare, or have not yet been performed. The uniqueness of this study lies in the wide range of spice types studied for the presence of OTA on the Czech market.

Keywords: spices; ochratoxin A; immunoaffinity columns; HPLC-FLD

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1. Introduction

There are several definitions for spices that may to some extent overlap with herbs [1–3]. Unlike herbs, which are defined as plants with non-woody tissues, spices are considered a culinary term rather than a botanical category [2]. This study is guided by the simple definition that spices are all parts of a plant that are used to improve meals in their colour, flavour, or even texture. These parts can be leaves, seeds, roots, fruits, bark, buds, or stalks [3].

The importance of spice may vary through countries worldwide [2]. Although generally thought to represent only a small portion of the human diet, they cannot be neglected as they may contribute to the overall intake of mycotoxins from all foodstuffs [4]. Spices are a widespread commodity [2] as they are exported worldwide, mainly from developing countries where they are mostly grown. Approximately 15.9 million tonnes of spices (excluding garlic and onion together exceeding 100 million tonnes) were produced in 2019 [5]. Asian countries were the largest producers of spices (share of production 75.7%; 12.1 million tonnes), followed by African (19.9%; 3.2 million tonnes), American (3.8%; 0.6 million tonnes), European (0.5%; 0.08 million tonnes), and Oceanian (0.1%; 0.012 million tonnes) producing countries in 2019.

Unfortunately, spices are susceptible to fungal infestation and mycotoxin contamination. The local subtropical/tropical climate conditions in most spice-producing countries such as high temperatures in combination with heavy rainfalls pose a suitable environment for mould infestation and thus mycotoxin production in spices. Moreover, following

good agricultural, hygienic, and manufacturing practices is particularly difficult in these countries, which is also likely to contribute to the deterioration of spices by moulds and mycotoxins [6–9]. *Aspergillus carbonarius*, *A. flavus*, *A. ochraceus*, *A. parasiticus*, *A. tamarii*, *A. versicolor*, *Penicillium citrinum*, *P. verrucosum*, and *Fusarium verticillioides* are considered the most common moulds in spices. However, *Alternaria alternata*, *Rhizopus oryzae,* and *R. nigricans* have also been found in some spices such as cumin and coriander [4]. Ochratoxin A (OTA), along with aflatoxins B1, B2, G1, and G2, citrinin, fumonisins B1 and B2, trichothecenes such as deoxynivalenol, nivalenol, T-2 toxin, and HT-2 toxin, zearalenone, altenuene, alternariol, tenuazonic acid, and sterigmatocystin, has been confirmed in spices by many studies [4].

This study focused on OTA (PubChem CID: 442530) [10], which is considered the second most important mycotoxin from the public health point of view [11]. Moreover, it is infamous mainly for its nephrotoxic and less hepatotoxic effects, however, teratogenic, genotoxic, immunotoxic, and neurotoxic effects have also been reported [12]. The International Agency for Research on Cancer classifies OTA into group 2B, which is a possible carcinogen for humans [13,14].

A recent study [4] describing the situation of spice mycotoxin and mould contamination revealed that besides the well-known and most studied spices such as chilli and black pepper, many other types of spices also deserve attention and need to be monitored for various mycotoxins. Table 1 shows a summary of the results of recent studies on the determination of OTA in several types of spices [15–46].

Table 1. Overview of studies dealing with the contamination of spices with OTA from a global perspective.

Country	Spices	*n*+/*n*	*n*+%	Mean (ng g^{-1})	Range Min–Max (ng g^{-1})	LOQ (ng g^{-1})	References
Africa							
Cameroon	Black pepper	2/20	10	1.53	1.15–1.91	1.00	[15]
	Cloves	0/40	0	-	-	1.00	
	White pepper	8/20	40	3.30	1.81–4.89	1.00	
Ivory Coast	Black pepper	0/30	0	-	-	0.20	[16]
	Chilli	25/30	83	68.97 ↑	0.04–907.57	0.20	
	Ginger	15/30	50	0.22	0.04–0.56	0.20	
	Black pepper	7/12	58	4.56	0.27–13.95	0.16	[17]
	Chilli	4/12	33	1.50	0.23–4.45	0.16	
	Dawadawa	2/12	17	1.40	1.26–1.55	0.16	
	Ginger	3/12	25	0.22	0.17–0.31	0.16	
Nigeria	Ginger	57/120	48	3.75	0.17–12.02	0.30	[18]
South Africa	Chilli	2/18	11	16.00	7.00–25.00	4.20	[19]
	Fruit chutney spices	1/4	25	6.00 *	6.00 *	4.20	
	Onion	0/8	0	-	-	4.20	
	Paprika	1/7	14	11.00 *	11.00 *	4.20	
	Vegetable spice	0/1	0	-	-	4.20	
America							
Brazil	Black pepper	0/15	0	-	-	N/S	[20]
	Chilli	0/15	0	-	-	N/S	
	Cinnamon	0/13	0	-	-	N/S	
	Cloves	0/12	0	-	-	N/S	
	Fennel	0/15	0	-	-	N/S	
	Oregano	0/12	0	-	-	N/S	
	Rosemary	0/15	0	-	-	N/S	
	White pepper	0/15	0	-	-	N/S	

Table 1. *Cont.*

Country	Spices	*n*+/*n*	*n*+%	Mean (ng g^{-1})	Range Min–Max (ng g^{-1})	LOQ (ng g^{-1})	References
Asia							
China	Aniseed	5/80	6	0.73	N/S	0.50	[21]
	Chilli	15/80	19	6.77	N/S	0.50	
	Cinnamon	4/80	5	1.10	N/S	0.50	
	Cumin	5/29	17	1.46	N/S	0.50	
	Curry powder	5/11	46	2.44	N/S	0.50	
	Fennel	0/40	0	-	-	0.50	
	Pepper	0/80	0	-	-	0.50	
	Prickly ash	8/80	10	3.17	N/S	0.50	
	Liquorice	2/31	6	2.00	0.06–3.93		[22]
India	Chilli	40/55	73	97.10 ↑	N/S	N/S	[23]
	Black pepper	33/42	79	154.10 ↑	N/S	N/S	
	Caraway	12/25	48	63.20 ↗	N/S	N/S	
	Coriander	9/30	30	47.60 ↗	N/S	N/S	
	Cumin	0/28	0	-	-	N/S	
	Fennel	14/25	56	98.10 ↗	N/S	N/S	
	Fenugreek	18/35	51	83.20 ↗	N/S	N/S	
	Ginger	20/36	56	82.80 ↑	N/S	N/S	
	Turmeric	20/35	57	125.90 ↑	N/S	N/S	
	Black pepper	31/55	56	155.00	N/S	N/S	[24]
	Cardamom	11/32	34	68.00	N/S	N/S	
	Fennel	8/35	23	10.00	N/S	N/S	
	Mace	18/30	60	128.00	N/S	N/S	
	Turmeric	21/42	50	162.00	N/S	N/S	
Indonesia	Chilli	3/6	50	44.87 ↑	23.70–84.60	1.77	[25]
Iran	Black pepper	10/23	43	3.31	0.70–7.64	0.06	[26]
	Cinnamon	8/23	35	5.46	0.45–16.10	0.06	
	Chilli	4/23	17	5.66	0.56–18.64	0.06	
	Turmeric	7/23	30	2.77	0.60–8.49	0.06	
	Black pepper	20/20	100	49.29	15.91–197.64	1.23	[27]
	Cinnamon	2/20	10	18.5	0.70–139.44	1.23	
	Chilli	0/20	0	-	-	1.23	
	Turmeric	0/20	0	-	-	1.23	
Korea	Chilli	6/56	11	2.38	4.51 M	0.31	[28]
Lebanon	Allspice	0/3	0	-	-	1.50	[29]
	Anise	1/3	33	2.60 *	2.60 *	1.50	
	Basil	0/2	0	-	-	1.50	
	Bay leaf	0/2	0	-	-	1.50	
	Black pepper	1/4	25	2.30 *	2.30 *	1.50	
	Caraway	0/2	0	-	-	1.50	
	Cardamom	0/4	0	-	-	1.50	
	Chilli	2/7	29	7.70	N/S	1.50	
	Cinnamon	0/3	0	-	-	1.50	
	Cloves	0/2	0	-	-	1.50	
	Coriander	0/2	0	-	-	1.50	
	Cumin	1/5	20	3.50 *	3.50 *	1.50	
	Fennel	0/2	0	-	-	1.50	
	Fenugreek	0/4	0	-	-	1.50	
	Garlic	1/2	50	5.10 *	5.10 *	1.50	
	Ginger	0/3	0	-	-	1.50	
	Marjoram	1/2	50	0.75 *	0.75 *	1.50	
	Mint	0/3	0	-	-	1.50	
	Nutmeg	1/2	50	33.90 * ↑	33.90 *	1.50	

Table 1. *Cont.*

Country	Spices	*n*+/*n*	*n*+%	Mean (ng g^{-1})	Range Min–Max (ng g^{-1})	LOQ (ng g^{-1})	References
Lebanon	Onion	0/4	0	-	-	1.50	
	Oregano	0/3	0	-	-	1.50	
	Paprika	2/3	67	11.40	N/S	1.50	
	Parsley	0/1	0	-	-	1.50	
	Rosemary	1/2	50	0.75 *	0.75 *	1.50	
	Saffron	0/1	0	-	-	1.50	
	Sage	1/3	33	4.20 *	4.20 *	1.50	
	Sumac	0/2	0	-	-	1.50	
	Thyme	0/3	0	-	-	1.50	
	Turmeric	1/2	50	2.40 *	2.40 *	1.50	
	White pepper	0/2	0	-	-	1.50	
	Allspice	N/S	ND	-	-	1.50	[30]
	Anise	N/S	D	2.6	N/S	1.50	
	Black pepper	N/S	D	2.30	N/S	1.50	
	Cardamom	N/S	ND	-	-	1.50	
	Caraway	N/S	ND	-	-	1.50	
	Cinnamon	N/S	ND	-	-	1.50	
	Cloves	N/S	ND	-	-	1.50	
	Coriander	N/S	ND	-	-	1.50	
	Cumin	N/S	D	3.50	N/S	1.50	
	Fennel	N/S	ND	-	-	1.50	
	Garlic powder	N/S	ND	-	-	1.50	
	Ginger	N/S	ND	-	-	1.50	
	Nutmeg	N/S	D	34.00 ↑	N/S	1.50	
	Onion powder	N/S	ND	-	-	1.50	
	Paprika	N/S	D	11.40	N/S	1.50	
	Red chilli	N/S	D	7.70	N/S	1.50	
	Turmeric	N/S	D	2.40	N/S	1.50	
	White pepper	N/S	ND	-	-	1.50	
Malaysia	Coriander	1/1	100	0.91 *	0.91 *	0.33	[31]
	Cumin	1/2	50	20.40 * ↗	20.40 *	0.33	
	Curry	8/8	100	2.36	0.14–9.59	0.33	
	Chilli	1/2	50	0.62 *	0.62 *	0.33	
	Fennel	1/2	50	1.26 *	1.26 *	0.33	
	Black pepper	0/1	0	-	-	0.33	
	Turmeric	2/2	100	1.89	0.20–3.58	0.33	
	White pepper	0/1	0	-	-	0.33	
	Chilli	0/10	0	-	-	0.30	[32]
	Chilli	65/80	81	7.15	0.20–101.20	0.06	[33]
Pakistan	Chilli crushed, restaurant	14/28	50	19.80	48.70 M	0.18	[34]
	Chilli powdered, restaurant	12/29	41	22.90 ↑	58.10 M	0.18	
	Chilli crushed, open market	11/29	38	16.90	54.30 M	0.18	
	Chilli powdered, open market	13/34	38	21.40 ↑	64.50 M	0.18	
	Chilli	99/242	41	N/S	120.90 M	0.30	[35]
Saudi Arabia	Cardamom	38/80	48	60.14 ↗	30.00–78.00	3.33	[36]
Sri Lanka	Chilli flakes	13/26	50	4.90	15.00 M	N/S	[37]
	Chilli pods	2/18	11	N/S	5.30 M	N/S	
	Red chilli powder	20/42	48	16.00	282.00 M	N/S	
	Black pepper	N/S	D	N/S	79.00 M ↑	N/S	[38]
	Chilli	35/86	41	N/S	282.00 M	N/S	

Table 1. *Cont.*

Country	Spices	*n*+/*n*	*n*+%	Mean (ng g^{-1})	Range Min–Max (ng g^{-1})	LOQ (ng g^{-1})	References
Turkey	Black pepper	4/23	17	0.34	3.48 ^{M}	0.19	[39]
	Cumin	1/19	5	0.63 *	0.63 *	0.19	
	Red chilli flakes	18/24	75	12.34	53.04 ^{M}	0.19	
	Red chilli powder	12/22	55	13.46	98.20 ^{M}	0.19	
Europe							
Czech Republic	Black pepper	11/12	92	0.83	2.82 ^{M}	0.20	[40]
	Caraway	2/12	17	0.19	0.71 ^{M}	0.20	
	Chilli pepper dried	11/12	92	6.70	32.70 ^{M}	0.20	
	Coriander seed	4/12	33	0.46	1.96 ^{M}	0.20	
	Fiery paprika powder	12/12	100	19.00	5.5–91.80	0.20	
	Ginger root dried	7/12	58	2.04	12.70 ^{M}	0.20	
	Liquorice	12/12	100	15.80	3.8–36.70	0.20	
	Nutmeg	12/12	100	8.70	0.3–60.70	0.20	
	Sweet paprika powder	12/12	100	16.00	1.1–38.40	0.20	
Hungary	Black pepper	0/6	0	-	-	0.60	[41]
	Chilli	1/5	20	2.1 *	2.1 *	0.60	
	Red pepper, ground	32/70	46	N/S	0.4–66.2	0.60	
	White pepper	0/5	0	-	-	0.60	
Italy	Paprika	17/31	55	39.64 ↑	0.11–177.40	0.22	[42]
	Chilli	15/25	60	N/S	2.16–16.35	2.13	[43]
	Pepper	4/30	13.3	N/S	1.61–15.85	2.61	
Latvia	Basil	0/50	0	-	-	2.40	[44]
	Black pepper	0/50	0	-	-	1.50	
	Nutmeg	N/S	D	N/S	14.00 *	1.50	
	Oregano	0/50	0	-	-	2.40	
	Thyme	0/50	0	-	-	2.40	
Poland	Allspice	1/5	20	0.20 *	0.20 *	0.30	[45]
	Basil	1/3	33	0.05 *	0.05 *	0.30	
	Black pepper, grain	4/4	100	23.57 ↑	N/S	0.30	
	Black pepper, ground	4/5	80	9.46	N/S	0.30	
	Cayenne pepper	5/8	63	45.64 ↑	N/S	0.30	
	Cinnamon	3/4	75	2.14	N/S	0.30	
	Cloves	1/2	50	0.48 *	0.48 *	0.30	
	Curry	5/5	100	19.01 ↗	N/S	0.30	
	Garlic	2/3	67	0.11	N/S	0.30	
	Marjoram	4/5	80	7.13	N/S	0.30	
	Nutmeg	2/5	40	2.73	N/S	0.30	
	Oregano, whole	1/2	50	9.38 *	9.38 *	0.30	
	Oregano, crushed	2/4	50	22.12 ↗	N/S	0.30	
	Rosemary	1/2	50	5.07 *	5.07 *	0.30	
	Tarragon	1/1	100	6.98 *	6.98 *	0.30	
	Thyme	3/3	100	15.59 ↗	N/S	0.30	
	Turmeric	1/1	100	11.72 *	11.72 *	0.30	
	White pepper	6/7	86	29.41 ↑	N/S	0.30	
Spain	Chilli	35/35	100	N/S	0.62–44.60	0.10	[46]
	Paprika	63/64	98	N/S	281.00 ^{M}	0.10	

Note: n: number of samples; n+: number of positive samples: n+%: per cent of positive samples; *: the only measured concentration; ^{M}: the maximum concentration (the whole range is not known); -: no data; N/S: not specified; D: detected (the quantity of positive samples is not known); ND: not detected; ↑: the average OTA concentrations in regulated spices exceeding the relevant European Union limits EC No. 1881/2006 as in force [47]; ↗: the average OTA concentrations exceeding the limit of 15 ng g^{-1}, which is currently proposed for 'all spices' by the European Commission [48].

However, several single-kind spices have never (or not recently) been tested for OTA. Therefore, this study aims to determine OTA in a wider range of spice types to obtain an overview of the current state of the OTA contamination of spices sold on the Czech market. As far as the authors know, globally, this is the first study dealing with OTA in many kinds of spices that are based on a single plant species.

2. Materials and Methods

2.1. Sample Collecting

Fifty-four single-kind of traditional and less traditional spices (six samples of different batches per kind of spice, 324 samples in total, each in the amount of 30–100 g) were collected in the years 2019–2020. Of this number, 300 samples (92.6%) were imported and 24 samples (7.4%) were of Czech provenance. The characterisation of all single-kind spice samples was performed (see Table 2). All samples were stored in consumer packaging or polypropylene bags at laboratory temperature (21 ± 0.5 °C) in a dry place in the dark until sample preparation before analysis. Most samples originated from Asian and European countries (see Figure 1).

Table 2. The characterisation of spice samples.

No.	Spices	Latin Name	Form	Country of Origin
1	Allspice	*Pigmenta officinalis* Lindl.	milled	Mexico
2	Anise	*Pimpinella anisum* L.	whole	Egypt
3	Asafoetida *	*Ferula assa-foetida* L.	milled	India
4	Basil	*Ocimum basilicum* L.	scrubbed	Egypt
5	Bay leaf	*Laurus nobilis* L.	milled	Turkey
6	Black cumin *	*Nigella sativa* L.	whole	India
7	Black pepper	*Piper nigrum* L.	milled	Spain
8	Calamint *	*Saturea hortensis* L.	scrubbed	Germany
9	Caraway	*Carum carvi* L.	milled	Czech Republic
10	Cardamom	*Elateria cardamomum* L.	milled	Guatemala
11	Cayenne pepper	*Capsicum frutescens* L.	milled	Indonesia
12	Celery root *	*Apium graveolens* L.	whole	Czech Republic
13	Chervil *	*Anthriscus cerefolium* (L.) Hoffm.	scrubbed	Germany
14	Chilli crushed with seeds	*Capsicum frutescens L.*	crushed	Thailand
15	Chilli milled	*Capsicum frutescens* L.	milled	India
16	Chives *	*Allium schoenoprasum* L.	chopped	China
17	Cinnamon	*Cinnamomum burmannii* (Nees & Th. Nees) Nees ex Blume	milled	Indonesia
18	Citronella *	*Cymbopogon citratus* (DC- ex Nees) Stapf	cut	Albania
19	Clove	*Eugenia caryophyllata* L.	milled	Madagascar
20	Coriander	*Coriandrum sativum* L.	milled	Czech Republic
21	Cumin	*Cuminum cyminum* L.	milled	India
22	Dried dill tip *	*Anetum graveolens* L.	chopped	Czech Republic
23	Fennel	*Foeniculum vulgare* Mill.	whole	Egypt
24	Fenugreek	*Trigonella foenum-graecum* L.	milled	India
25	Galangal root *	*Alpinia glanga* (L.) Wild.	milled	China
26	Garlic	*Allium sativum* L.	granulated	China
27	Ginger	*Zingiber officinale* Roscoe	milled	Peru
28	Green pepper *	*Piper nigrum* L.	milled	India
29	Juniper *	*Juniperus communis* L.	milled	Pakistan
30	Lemon peel *	*Citrus limon* (L.) Burm. f.	milled	Spain
31	Liquorice root	*Glycyrhiza glabra* L.	crushed	China
32	Lovage *	*Levisticum officinale* W.D.J. Koch	cut	Poland
33	Mace	*Myristica fragrans* Houtt.	milled	Indonesia
34	Marjoram	*Majorana hortensis* L.	scrubbed	Egypt
35	Mint	*Mentha piperita* L.	milled	Egypt
36	Nutmeg	*Myristica fragrans* Houtt.	milled	Indonesia
37	Orange peel *	*Citrus aurantium* L.	milled	Spain
38	Oregano	*Origanum vulgare* L.	cut	Turkey

Table 2. *Cont.*

No.	Spices	Latin Name	Form	Country of Origin
39	Parsley	*Petroselinum sativum* Hoffm.	chopped	Poland
40	Pink pepper *	*Schinus terebinthifolius* Raddi	whole	Brazil
41	Rosemary	*Rosmarinus officinalis* L.	cut	Morocco
42	Saffron	*Crocus sativus* L.	whole	Spain
43	Sage	*Salvia officinalis* L.	scrubbed	Germany
44	Sichuan pepper *	*Zanthoxylum piperitum* (L.) DC.	whole	China
45	Star anise *	*Illicium verum* Hook. f.	milled	India
46	Sumac	*Rhus coriaria* L.	milled	Turkey
47	Sweet paprika	*Capsicum annuum* L.	milled	Hungary
48	Tarragon cut	*Artemisia dracunculus* L.	cut	Poland
49	Thyme	*Thymus vulgaris* L.	whole	Poland
50	Turmeric	*Curcuma longa* L.	milled	India
51	Vanilla *	*Vanilla planifolia* Jacks. Ex Andrews	milled	Tahiti
52	White pepper	*Piper nigrum* L.	milled	Vietnam
53	White mustard *	*Sinapis alba* L.	milled	Ukraine
54	Wild garlic *	*Allium usrinum* L.	cut	Bulgaria

Notes: *: spices in which OTA has never (or not recently) been studied according to the available literature (see Table 1).

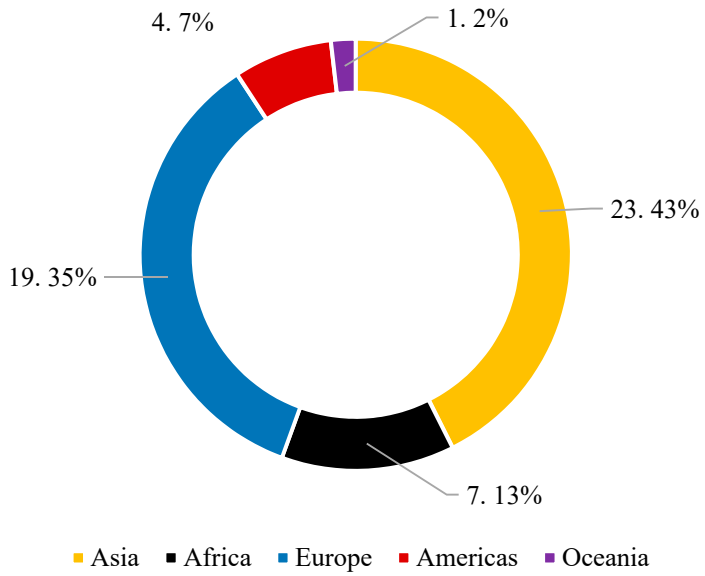

Figure 1. The origin of spice samples.

2.2. Sample Preparation—OTA Purification by Immunoaffinity Chromatography

All spice samples were properly homogenised using a laboratory homogenizer. The separation step was performed using a modified method according to Zimmerli and Dick [49] using immunoaffinity chromatography to increase both the selectivity and sensitivity of the method. The immunoaffinity chromatography uses immunoaffinity columns (IACs) operating specifically on the principle of antigen–antibody. The method consists of binding the antigen (OTA) by special anti-OTA antibodies anchored in the column. After the application of the washing solution, the other potentially interfering substances are removed from the column. The bound OTA is then released with acidified methanol from the antigen–antibody complex [49].

2.3. Chemicals and Apparatus

Methanol (CH_3OH), acetonitrile (C_2H_3N), and chloroform ($CHCl_3$) (all of HiPerSolv CHROMANORM gradient grade), formic acid 85% (HCOOH) pro-analysis (p.a.), orthophosphoric acid 85% (H_3PO_4) (HiPerSolv CHROMANORM), glacial acetic acid (CH_3COOH), di-sodium hydrogen phosphate anhydrous (Na_2HPO_4), sodium hydrogen

carbonate ($NaHCO_3$), sodium chloride (NaCl), and sodium hydroxide (NaOH) (all of AnalaR NORMAPUR) were purchased from VWR (Stříbrná Skalice, Czech Republic). All chemicals were stored at laboratory temperature 21 ± 0.5 °C. Analytical standard of OTA (*Petromyces albertensis*, ≥98%, HPLC) was purchased from VWR (Stříbrná Skalice, Czech Republic) and produced by Sigma-Aldrich spol. s r.o. (Prague, Czech Republic). The analytical standard was stored in a laboratory freezer at −20 °C. Immunoaffinity columns (IACs) OCHRAPREP® were purchased from Jemo Trading spol. s r.o., Profood (Bratislava, Slovakia) and produced by R-Biopharm (Darmstadt, Germany). Nylon syringe filters (13 mm, 0.22 μm) produced by Labstore (Inverness, Highland, UK) and were purchased from HPST s.r.o. (Prague, Czech Republic).

All solutions were prepared in ultrapure water using a Milli-Q system (Millipore, Milford, MA, USA) (hereinafter referred to as 'water'). The resistivity of ultrapure water was >18.2 MΩ.cm at 25 °C

A IKA A 10 basic homogeniser manufactured by IKA—WERKE GMBH & CO. KG (Staufen, Germany) was purchased from Fisher Scientific, spol. s r.o. (Pardubice, Czech Republic); an analytical balance KERN EW1500-2 manufactured by KERN & SOHN GmbH (Balingen, Germany) was purchased from Fisher Scientific, spol. s r.o. (Pardubice, Czech Republic); a Reax Multi shaker manufactured by Heidolph Instruments GmbH & Co. KG (Schwabach, Germany) was purchased from Fisher Scientific, spol. s r.o. (Pardubice, Czech Republic); and a laboratory centrifuge MPW 351e manufactured by MPW MED. Instruments (Warsaw, Poland) was purchased from Unimed Praha, spol. s r.o. (Prague, Czech Republic).

HPLC-FLD, Agilent 1260 Infinity II coupled to 1260 Infinity II Fluorescence Detector manufactured by Agilent (Santa Clara, CA, USA) was purchased from HPST s.r.o. (Prague, Czech Republic).

2.4. Solution Preparation

2.4.1. 3% Solution of Sodium Hydrogen Carbonate ($NaHCO_3$)

A total of 30 g of $NaHCO_3$ was quantitatively transferred to a 1000 mL volumetric flask (hereinafter referred to as 'flask') and dissolved in a small amount of water. After dissolving the batch, the flask was made up with water.

2.4.2. Phosphate Saline Buffer Containing 15% Methanol (PBS-15% Methanol)

PBS consists of two solutions: solution A (0.02 mol L^{-1} Na_2HPO_4 at pH 7.4) and solution B (0.29 mol L^{-1} NaCl). Solution A: A total of 1.42 g of $NaHPO_4$ was quantitatively transferred to a 500 mL flask and dissolved in a small amount of water. After dissolving the batch, the flask was made up with water. The pH at 7.4 was adjusted with 85% H_3PO_4. Solution B: A total of 8.47 g of NaCl was quantitatively transferred to a 500 mL flask and dissolved in a small amount of water. After dissolving the batch, the flask was made up with water. PBS was obtained by mixing both prepared solutions A and B in a ratio of 1:1. PBS is stable for one year. PBS-15% methanol was obtained by mixing 850 mL of PBS and 150 mL of methanol.

2.4.3. 3% Buffer Solution of Ortho-Phosphoric Acid (H_3PO_4) and Sodium Chloride (NaCl) at pH 1.6

A total of 116.9 g of NaCl was quantitatively transferred to a 1000 mL flask and dissolved in a small amount of water. After dissolving the batch, a total of 33.7 mL of H_3PO_4 was pipetted into the flask. The flask was made up with water. The pH at 1.6 was adjusted with NaOH. The solution is stable for six months.

2.4.4. Elution Solution of Methanol (CH_3OH) Acidified by Glacial Acetic Acid (CH_3COOH)

A total of 2 mL of CH_3COOH was pipetted into a 100 mL flask. The flask was made up with CH_3OH.

All of these solutions were kept at 5 ± 0.5 °C. Before direct use, they were tempered at a laboratory temperature of 21 ± 0.5 °C.

2.4.5. OTA Working Standard Solution at a Concentration of 40 μg L^{-1} (25 mL)

A total of 100 μL of OTA stock solution at concentration of 1000 μg L^{-1} was pipetted into a 25 mL flask. The flask was made up with CH_3OH.

Stock and working standard solutions were kept at −20 ± 0.5 °C.

2.4.6. Calibration OTA Standards

A total of six calibration OTA standards (0.10, 0.25, 0.50, 1.00, 2.00, and 4.0 ng mL^{-1}) were prepared with a linear response on each day of the measurement from the working solution (40 μg L^{-1}) by its dilution in the mobile phase (MP) in a ratio reaching the target concentration. The determination coefficient was 0.9999. A blank sample consisting of the mobile phase was also prepared fresh daily.

2.4.7. Mobile Phase (MP)

The MP consisted of two solutions: solution A (acetonitrile:acetic acid, 99:1) and solvent B (water:acetic acid, 99:1). Solvents were used in ratio 40:60; A:B.

2.5. Workflow

2.5.1. OTA Extraction

A total of 2 g of the sample was weighed into a polypropylene centrifuge tube (hereinafter referred to as the tube), 10 mL of buffer was added and left to shake using Vortex (1 min). The extraction step with 4 × 5 mL of chloroform was performed using Vortex (3 min) and a centrifuge (15 min; 3305× *g*; at laboratory temperature 21 ± 0.5 °C). The lower chloroform phase was collected into a glass vial and left to evaporate under nitrogen at 45 °C to dryness. The residue was dissolved in 5 mL of chloroform using Vortex (5 min). The dissolved residuum was transferred to a new tube. Extraction with 2 × 5 mL of 3% solution of sodium bicarbonate was performed using Vortex (3 min) and a centrifuge (5 min; 2000× *g*, at laboratory temperature 21 ± 0.5 °C) to achieve a compact thin layer between two phases. The upper aqueous bicarbonate phase was collected into a new tube in which 1 mL of chloroform and 0.5 mL of 85% formic acid had been prepared. The re-extraction of aqueous bicarbonate with 2 × 2 mL of chloroform was performed using the Vortex (3 min) and centrifuge (5 min; 2000× *g*, at laboratory temperature 21 ± 0.5 °C) to achieve a compact thin layer between two phases. The lower chloroform phase at the bottom of the tube was collected into a glass vial and left to evaporate to dryness under a nitrogen stream at 45 °C.

2.5.2. OTA Separation

The residue was dissolved in 20 mL of PBS–methanol 15% using Vortex (5 min). A laboratory ultrasonic bath was used (10 min) to enhance the dissolution of the residue.

The IACs were brought to the laboratory temperature (at 21 ± 0.5 °C for approximately $\frac{1}{2}$–1 h) and the buffer was released. A total of 20 mL of PBS–methanol 15% was transferred to the reservoir above the IAC and left to pass through IAC (at one drop per second; 2 mL min^{-1}). The IACs were purified with 20 mL of water (at one drop per s) followed by brief air sieving (1–2 s). The elution of potential OTA was performed with 1.5 mL of methanol:acetic acid (98:2) into a small glass vial (at one drop per second) and followed by strong air sieving (30 s). The 1.5 mL eluate was evaporated under a nitrogen stream at 45 °C to dryness and kept in the laboratory fridge at 4 °C until analysis with HPLC-FLD. Before analysis, samples were dissolved in 0.5 mL of MP using an ultrasonic bath (5 min; 37 kHz; at laboratory temperature 21 ± 0.5 °C) and passed through a nylon syringe filter (13 mm, 0.22 μm) into a vial for HPLC.

2.5.3. Analysis of Ochratoxin A in Spices by HPLC-FLD

HPLC-FLD was employed for the determination of OTA. The column (Kinetex C18, 2.6 µm, 100 Å, 50 × 21 mm) coupled with a security GuardTM column (Phenomenex C18, 4 × 2.0 mm) was purchased from Chromservis s.r.o. (Prague, Czech Republic) and were used and kept at 30 °C. The MP was set at a flow rate of 0.2 mL min^{-1}. The injection volume was 8.0 µL. Fluorescence detection was performed at an excitation wavelength of 333 nm and an emission wavelength of 465 nm, PMT gain 18, attenuation 100 LU. Chromatography software Agilent OpenLab software was used to collect the chromatographic data. The method was validated. The limit of detection (LOD) was 0.03 ng g^{-1} and the limit of quantification (LOQ) was 0.1 ng g^{-1}. The recovery of the method was verified using samples spiked with OTA. No reference material for the determination of OTA in spices was available during the period of this research. Therefore, the recovery was performed via spiked spice samples at OTA concentration levels of 0.5 and 2.0 ng g^{-1}. OTA levels of 0.5 ng g^{-1} and 2.0 ng g^{-1} were added to the matrix before the extraction step, both concentrations in triplicate. The same concentrations were added after the separation step on immunoaffinity columns to the eluate, both concentrations in triplicate again. A total of 12 spiked samples were analysed for OTA. The recovery was determined for both concentration levels based on matrix effect—the ratio of the mean concentrations of samples with spiked matrix and samples with spiked eluate. The mean recovery was 74.2%, which fulfils the requirements of Regulation (EC) No. 401/2006 [50]. The repeatability standard deviation (RSD) was 0.76%. The mean measurement uncertainty was 4.03% including all kind of spices. OTA retention time was 5.4 min. The calibration curve consisted of six levels of concentrations (0.10, 0.25, 0.50, 1.00, 2.00, and 4.00 ng mL^{-1}). All samples with a concentration outside the calibration curve were diluted or concentrated to reach value within the calibration curve. The chromatographs of OTA standard solution (4.00 ng mL^{-1}) and one of the samples (33-mace) are shown in Figure 2.

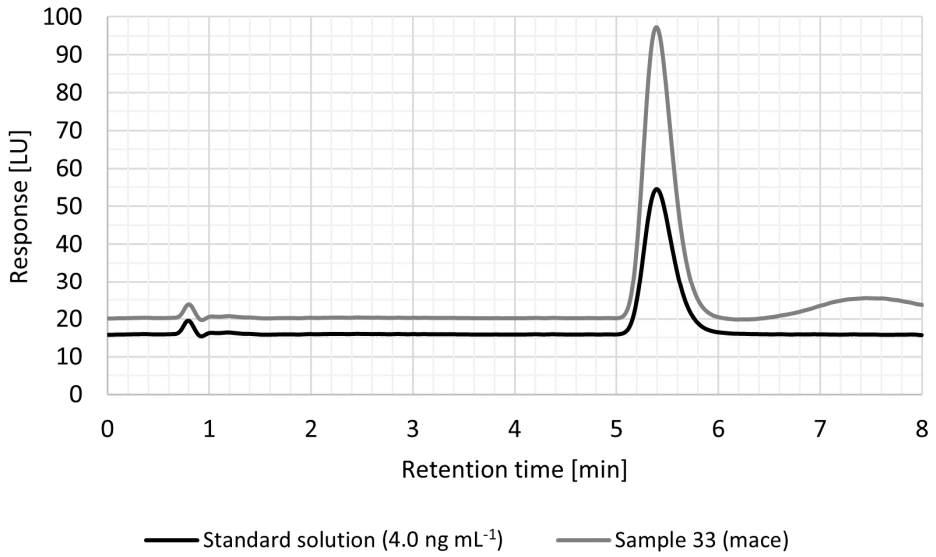

Figure 2. HPLC-FLD chromatogram showing peaks of OTA in spice sample 33 (mace) and standard solution (4.0 ng mL^{-1}) at a retention time of 5.4 min.

3. Results

A total of 101 (31%) spice samples of 19 spice kinds were positive (exceeding LOQ of 0.1 ng g^{-1}) for OTA (see Table 3). The concentrations of positive samples were in the range of 0.11 ng g^{-1} (for pink pepper) to 38–46 ng g^{-1} (for turmeric).

Table 3. The concentrations of OTA in a total of 20 positive single-kind spices available on the Czech market.

No.	Kind of Spice	Incidence *n+/n*	Mean ± SD (ng g^{-1})	Median (ng g^{-1})	95th Perc. (ng g^{-1})	Range Min–Max (ng g^{-1})
50	Turmeric	6/6	19.82 ± 11.93	17.04	36.01	5.13–38.46
31	Liquorice root	6/6	11.94 ± 3.27	12.10	16.18	7.57–17.42
15	Chilli milled	6/6	7.50 ± 1.34	7.78	8.96	5.28–9.27
33	Mace	6/6	5.27 ± 0.83	5.25	6.22	3.94–6.33
27	Ginger	6/6	3.40 ± 0.48	3.46	3.91	2.18–3.93
11	Cayenne pepper	6/6	2.59 ± 0.61	2.71	3.21	1.67–3.27
47	Sweet paprika	6/6	2.26 ± 0.60	1.99	3.06	1.73–3.12
14	Chilli crushed with seeds	6/6	1.43 ± 0.48	1.41	1.98	0.82–2.03
51	Vanilla	6/6	1.42 ± 0.33	1.49	1.72	0.82–1.74
37	Orange peel	6/6	1.04 ± 0.30	1.04	1.41	0.63–1.47
21	Cumin	5/6	0.46 ± 0.27	0.55	0.70	<LOQ–0.72
36	Nutmeg	5/6	0.43 ± 0.30	0.49	0.78	<LOQ–0.84
53	White mustard	5/6	0.38 ± 0.30	0.32	0.76	<LOQ–0.79
52	White pepper	5/6	0.36 ± 0.23	0.37	0.61	<LOQ–0.62
7	Black pepper	5/6	0.31 ± 0.20	0.37	0.52	<LOQ–0.53
19	Clove	5/6	0.29 ± 0.18	0.33	0.48	<LOQ–0.50
30	Lemon peel	5/6	0.18 ± 0.12	0.18	0.32	<LOQ–0.36
46	Sumac	5/6	0.14 ± 0.08	0.14	0.24	<LOQ–0.26
40	Pink pepper	1/6	0.11 *	0.11 *	-	<LOQ–0.11

Note: n: number of samples; n+ = positive samples > LOQ = 0.10 ng g^{-1}; SD = standard deviation; 95th perc = 95% percentile; *: the only one positive sample; left censored data: samples that contained OTA levels below LOQ were assigned a value 0 ng g^{-1} for statistical processing (<LOQ = 0 ng g^{-1})—the lower bound approach (LB) [51].

4. Discussion

4.1. Comparison of OTA Results in Spices with Other Relevant Studies in the World

To our knowledge, the set of spices analysed in this study has not been comprehensively analysed for OTA in other research papers, which makes it difficult to compare the whole dataset with the existing studies. Moreover, some types of spices included in this study such as asafoetida, black cumin, calamint, celery root, chervil, chives, citronella, dried dill tip, galangal root, green pepper, juniper, lemon peel, lovage, orange peel, pink pepper, Sichuan pepper, star anise, vanilla, white mustard, and wild garlic have never, or not recently, been analysed for the presence of OTA in other studies. The benefit of this study is certainly a positive OTA finding in some of these previously unanalysed spices such as lemon peel, orange peel, pink pepper, vanilla, and white mustard.

Therefore, we focused on evaluating our above-detection limit results in relation to the studies listed in Table 1. The comparability was possible with spices such as turmeric, liquorice, chilli, mace, ginger, cayenne pepper, sweet paprika, cumin, nutmeg, white pepper, black pepper, clove, and sumac.

To evaluate the general occurrence of OTA in given spices, we used categories from our previous study by Picková et al. [4]. These categories are based on the percentage of a total number of positive findings out of a total number of samples tested in a given spice based on recent relevant studies since 2015. These categories are: 'no occurrence' (0%), 'rare occurrence' (up to 5%), 'low occurrence' (up to 25%), 'moderate occurrence' (up to 50%), 'high occurrence' (up to 75%), and 'very high occurrence' (more than 75%).

OTA in turmeric was found to be of a 'moderate occurrence'. Across all studies presented in Table 1, the average OTA concentration in turmeric was in the range of 1.89–162.00 ng g^{-1} [23,24,26,29–31,45] or was not detected at all [27]. In our study, the average concentration of 17.04 ng g^{-1} in turmeric fell within the range found in the literature. The greatest similarity of results could be observed with the average OTA concentration of 11.72 ng g^{-1} in the Polish study [45]. In our study, it was the only one spice kind with OTA concentration exceeding the EU limit of 15 ng g^{-1} [47]. Given the origin of our samples of turmeric in India, undoubtedly the largest producer of spices in the world [5], the average concentration of 17.04 ng g^{-1} does not seem to be as severe as the average concentration of 162.00 ng g^{-1} found in the Indian study [24].

OTA in liquorice was found to be of a 'moderate occurrence' [9]. Across all studies mentioned in Table 1, the average OTA concentration in liquorice was found in the range

of 2.00–15.80 ng g^{-1} in only two studies [22,40]. In our study, liquorice with the average OTA concentration of 11.94 ng g^{-1} was within the range found in the literature and was nearly in line with the OTA concentration of 15.80 ng g^{-1} in the Czech study [40]. The EU limit of 20 ng g^{-1} for OTA was not exceeded in any of the liquorice samples [47].

OTA in chilli was found to be of a 'moderate occurrence' [4]. Across all of the studies mentioned in Table 1, the average OTA concentration was found in the range of 0.62–97.10 ng g^{-1} [16,17,19,21,23,25,26,29–31,33,37,39,40], but it was not detected in another study [32]. In our study, both average OTA concentrations of 7.50 ng g^{-1} in milled chilli and 1.43 ng g^{-1} in crushed chilli with seeds were within the range found in the literature. The average OTA concentration for milled chilli was very similar to the average OTA concentrations of 6.77 ng g^{-1} in Chinese [21], 7.70 ng g^{-1} in Lebanese [29], 7.15 ng g^{-1} in Malaysian [33], and 6.7 ng g^{-1} in Czech studies [40], while for crushed chilli with seeds to the OTA concentrations of 1.50 ng g^{-1} in the Ivory Coast study [17]. The EU limit of 20 ng g^{-1} for OTA was not exceeded in any of the chilli samples [47].

OTA in mace was found to be of a 'high occurrence' [4]. It is necessary to note that this statement was based on only one Indian study in which the average OTA concentration of 128 ng g^{-1} was found in mace [24]. In contrast, the average OTA concentration of 5.27 ng g^{-1} in mace was much lower in this study.

OTA in ginger was found to be of a 'moderate occurrence' [4]. Across all studies mentioned in Table 1, the average OTA concentration was found in the range of 0.22–82.80 ng g^{-1} [16–18,23], but it was not detected at all in other studies [29,30]. In our study, the average OTA concentration of 3.40 ng g^{-1} in ginger was in the range found in the literature. The greatest similarity was observed with the average OTA concentration of 3.75 ng g^{-1} found in the Nigerian study [18]. The EU limit of 15 ng g^{-1} for OTA was not exceeded in any of the ginger samples [47].

The average OTA concentration of 2.59 ng g^{-1} in cayenne pepper in our study was not in line with the average OTA concentration of 45.64 ng g^{-1} in the Polish study, which was the only study available for the comparison. The EU limit of 15 ng g^{-1} for OTA was not exceeded in any of the cayenne pepper samples [47].

OTA in paprika was found to be of a 'high occurrence' [4]. Across all studies mentioned in Table 1, the average OTA concentration was found in the range of 11.00–39.64 ng g^{-1} [19,29,30,40,42,46], but was not detected in another study [27]. In our study, the average OTA concentration of 2.26 ng g^{-1} in sweet paprika was not in the range found in the literature, as it was lower than the average OTA concentration of 11.00 ng g^{-1} in the Lebanese study [29]. The EU limit of 15 ng g^{-1} for OTA was not exceeded in any of the sweet paprika samples [47].

OTA in cumin was found to be of a 'low occurrence' [4]. Across all studies mentioned in Table 1, the average OTA concentration was found in the range of 0.63–20.4 ng g^{-1} [21,29,31,39], but it was not detected at all in another study [23]. In our study, the average OTA concentration of 0.46 ng g^{-1} in cumin was not within the range found in the literature as it was lower than the average OTA concentration of 0.63 ng g^{-1} in the Turkish study [39].

OTA in nutmeg was found to be of a 'very high occurrence' [4]. Across all studies mentioned in Table 1, the average OTA concentration was found in the range of 2.73–34.00 ng g^{-1} [29,30,40,44,45]. In our study, the average OTA concentration of 0.43 ng g^{-1} in nutmeg was not within the range found in the literature as it was lower than the average OTA concentration of 2.73 ng g^{-1} in the Polish study [45]. The EU limit of 15 ng g^{-1} for OTA was not exceeded in any of the nutmeg samples [47].

OTA in white pepper was found to be of a 'low occurrence' [4]. Across all studies mentioned in Table 1, the average OTA was found in the range of 3.30–29.41 ng g^{-1} in only two studies [15,45], but it was not detected at all in other studies [20,29–31,41]. In our study, the average OTA concentration of 0.36 ng g^{-1} in white pepper was not within the range found in the literature as it was lower than the average OTA concentration of 3.30 ng g^{-1} in the Cameroonian study [39]. The EU limit of 15 ng g^{-1} for OTA was not exceeded in any of the white pepper samples [47].

OTA in black pepper was found to be of a 'moderate occurrence' [4]. Across all studies mentioned in Table 2, the average OTA concentration was in the range of 0.34–155.00 ng g^{-1} [15,17,23–25,27,29–31,39,40,45] or was not detected at all [16,20,31,41,44]. In our study, the average OTA concentration of 0.31 ng g^{-1} in black pepper was not within the range found in the literature as it was found to be very similar, but slightly lower, than the OTA concentration of 0.0.34 ng g^{-1} in the Turkish study [39]. The EU limit of 15 ng g^{-1} for OTA was not exceeded in any of the black pepper samples [47].

OTA in cloves was found to be of a 'none occurrence' [4], however, the study by Pickova et al. [4] dealt with only the most recent publications concerning spices since 2015 [15,20,29,30]. There has been one case of a positive finding with the average OTA concentration of 0.48 ng g^{-1} in cloves in an older Poland study [45] with which our result is in agreement as the average OTA concentration of 0.29 ng g^{-1} was in cloves.

OTA in sumac was found to be of a 'none occurrence' [4]. This statement was based on only one study in Lebanon [29]. In contrast, our study provided a positive finding of OTA in sumac with an average concentration of 0.14 ng g^{-1}, which can be considered a benefit of the study.

OTA was not found in the other spice kinds included in this study. Our under-detection limit results were in line with the statement of 'none occurrence' in cases of allspice [29,30], basil [29,44], bay leaf [29], mint [29], oregano [20,29,44], parsley [29], saffron [29], and thyme [29,44]. However, there is one older Polish study that contradicts this statement and thus our results, as it presented positive results for the presence of OTA in allspice, basil, oregano, and thyme [45].

4.2. Comparison of OTA Results in Spices with the Maximal Limits of the EU Legislation

Commission Regulation (EC) No. 1881/2006 [47] is one of the most extensive and stringent regulations setting maximum limits for certain contaminants including mycotoxins in foodstuffs, as amended, and is suitable for comparing the results obtained, especially because of its complexity with regard to spices. Moreover, all samples were purchased in the Czech Republic, which is one of the 27 Member States of the EU, therefore only the EU limits were considered. Results showed that only one sample (50-turmeric) was contaminated with OTA at a concentration exceeding the maximal limit set by the European Union. A comparison of OTA concentrations that have been found so far in regulated spices with the maximal limits of the EU legislation is presented in Table 4.

Table 4. The concentrations of OTA in positive single-kind spices available on the Czech market and comparison with the European Union legislation.

Number of Sample	Kind of Spice	OTA Concentration [1] (ng g^{-1})	EU Limits [2] (ng g^{-1})
50	Turmeric	19.82 [3]	15
31	Liquorice root	11.94	20/80 [4]
15	Chilli milled	7.50	20
27	Ginger	3.40	15
11	Cayenne pepper	2.59	20
47	Sweet paprika	2.26	15
14	Chilli crushed with seeds	1.43	20
36	Nutmeg	0.43	15
52	White pepper	0.36	15
7	Black pepper	0.31	15
40	Pink pepper	0.11	15

[1] Positive samples are all samples with concentrations exceeding the limit of quantification of 0.13 ng g^{-1}; [2] EU limits refer to the maximum levels of OTA in spices under the European Union—Regulation No. 1881/2006 as in force [47]; [3] OTA concentration exceeding the maximum permitted limit set by the European Union legislation; [4] The maximum limit of OTA of 20 ng g^{-1} is valid for '*Liquorice root, an ingredient for herbal infusion*'. The maximum limit of OTA of 80 ng g^{-1} is valid for '*Liquorice extract for use in food in particular beverages and confectionary*' provided that it is pure and an undiluted extract is obtained whereby 1 kg of extract is obtained from 3 to 4 kg of liquorice root [47].

4.3. Proposal for New Maximum Limits for OTA in the EU and FAO/WHO Codex Alimentarius

The issue of OTA was also recently discussed in the meeting from 14 to 15 July 2021 at the Working Group for Agricultural Contaminants of the Directorate-General for Health and Food Safety, the European Commission. Amendments to the draft maximum levels for OTA in food for which there are currently no limits and the draft maximum limits for spices for which there are currently limits are currently under discussion and consideration (see Table 5) [48].

Table 5. The draft proposal of the maximum limits of OTA in spices.

Food	Proposal of Maximum Limits (ng g^{-1})
All spices including dried spices except *Capsicum* spp.	15
Capsicum spp. (dried fruits, whole or ground, including chilli, ground chilli, cayenne pepper and red pepper—paprika)	20
Mixtures of spices	15

Processed according to [48].

The issue of OTA was also recently discussed in the report of the 14th Session of the Codex Committee on Contaminants in Foods by the Codex Alimentarius Commission (virtual) 3–7 and 13 May 2021. They discussed the maximum limits for OTA in nutmeg, dried chilli and paprika, ginger, pepper, and turmeric for comments and consideration by the Session of the Codex Committee on Contaminants in Foods in the year 2022. Maximum limits of 15–20 ng g^{-1} for OTA in spices should be established [52].

4.4. The Occurrence of OTA in Spices on Data by RASFF (2016–2021)

Rapid Alert System for Food and Feed (RASFF) is a key tool ensuring food safety in the context of the EU and enables one to orientate oneself in the issue of OTA occurrence in various foods including spices. Notifications reporting the presence of OTA in spices are also valuable information for completing the idea of the current state of distributed spices. Based on the RASFF database, a total of 58 OTA notifications have been related to spices since 2016 (see Figure 3). The most prevalent notifications concerned OTA in chilli (33%), sweet paprika powder (21%), and nutmeg (17%). Most OTA notifications originated in India (17%, mostly chilli) and Indonesia (16%, mostly nutmeg).

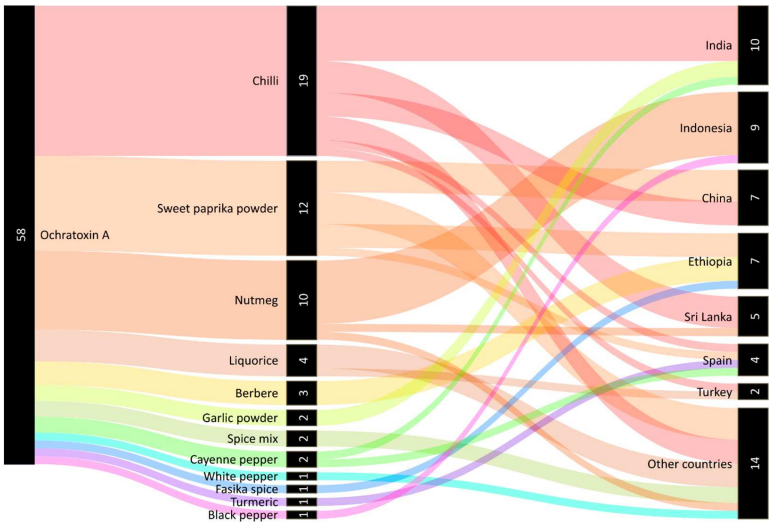

Figure 3. Notifications of ochratoxin A in spices by the Rapid Alert System for Food and Feed (RASFF) since 2016 (current to the 20 July 2021). Notes: The category 'Other countries' includes all countries of origin with only one notification for spices: Azerbaijan, Bangladesh, France, Germany, Hungary, Italy, Lebanon, Peru, Portugal, Thailand, Ukraine, the United Kingdom, and Vietnam, or notification of unknown origin. Processed according to the RASFF database [53] using The Sankey Diagram Generator online tool and vector graphics editor Inkscape 0.92.

5. Conclusions

Human dietary exposure to OTA from foodstuffs is very common. Despite various effective methods for OTA mitigation and the reduction of possible health risks of OTA in foodstuffs, OTA is still a persistent problem. Although spices are not among the main sources of daily OTA intake in humans, they may contribute significantly to the co-exposure with major OTA sources such as cereals, wine, pork meat, and coffee. This may result in an additive effect and thus an increase in OTA toxicity. The significance of this study lies in the analysis of a large number of types of spices for OTA, focusing only on single-species spices, not mixtures of spices. In this study, the analysis of 54 single-kind species showed a total of 19 (35%) OTA-positive spice kinds, meaning that at least one sample of a given spice kind exceeded LOQ by its concentration. Among these OTA-positive spice kinds were turmeric, liquorice root, chilli milled, mace, ginger, cayenne pepper, sweet paprika, chilli crushed with seeds, vanilla, orange peel, cumin, nutmeg, white mustard, white pepper, black pepper, clove, lemon peel, sumac, and pink pepper. This study therefore demonstrates that the Czech population is exposed to OTA through various contaminated single-kind spices available on the Czech market.

As can be seen, the spice kinds with OTA-positive findings included regulated spice kinds but also those for which regulation has not yet been set, namely mace, vanilla, orange peel, cumin, white mustard, cloves, lemon peel, sumac, and basil. Fortunately, promising discussions are already taking place in the European Commission, in which, among other things, a limit for 'all spices' has been proposed at 15 ng g^{-1}, but has not been adopted yet. Taking into consideration this proposed limit for all hitherto unregulated spices, none of the analysed spice samples exceeded this value in this study.

In terms of public health protection, where food safety is an important preventive component, it is necessary to regulate various mycotoxin contents in various spices. Hence, our future research will focus not only on OTA monitoring, but also on the other mycotoxins in spices, as it will be important to verify the mycotoxin intake from this commodity.

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References

1. Chomchalow, N. Spice Production in Asia—An Overview. *AU J. Technol.* **2001**, *5*, 1–14.
2. Sherman, P.W.; Billing, J. Darwinian Gastronomy: Why We Use Spices: Spices Taste Good Because They Are Good for Us. *BioScience* **1999**, *49*, 453–463. [CrossRef]
3. Uhl, S.R. *Handbook of Spices, Seasonings, and Flavorings*, 2nd ed.; CRC Press: Boca Raton, FL, USA, 2006; ISBN 978-1-4200-0436-6.
4. Pickova, D.; Ostry, V.; Malir, J.; Toman, J.; Malir, F. A Review on Mycotoxins and Microfungi in Spices in the Light of the Last Five Years. *Toxins* **2020**, *12*, 789. [CrossRef]
5. FAOSTAT. Food and Agriculture Organization of the United Nations. Available online: http://www.fao.org/faostat/en/#data/QC/visualize (accessed on 25 February 2020).
6. Botana, L.M.; Sainz, M.J. (Eds.) *Climate Change and Mycotoxins*; Walter de Gruyter GmbH: Berlin, Germany, 2015; ISBN 978-3-11-033305-3.

7. Marroquín-Cardona, A.G.; Johnson, N.M.; Phillips, T.D.; Hayes, A.W. Mycotoxins in a Changing Global Environment—A Review. *Food Chem. Toxicol.* **2014**, *69*, 220–230. [CrossRef] [PubMed]
8. Oguntoyinbo, F.A. Safety Challenges Associated with Traditional Foods of West Africa. *Food Rev. Int.* **2014**, *30*, 338–358. [CrossRef]
9. Yogendrarajah, P.; Van Poucke, C.; De Meulenaer, B.; De Saeger, S. Development and Validation of a QuEChERS Based Liquid Chromatography Tandem Mass Spectrometry Method for the Determination of Multiple Mycotoxins in Spices. *J. Chromatogr. A* **2013**, *1297*, 1–11. [CrossRef]
10. PubChem. Available online: https://pubchem.ncbi.nlm.nih.gov/ (accessed on 7 June 2021).
11. Bhat, R.; Rai, R.V.; Karim, A.A. Mycotoxins in Food and Feed: Present Status and Future Concerns. *Compr. Rev. Food Sci. Food Saf.* **2010**, *9*, 57–81. [CrossRef] [PubMed]
12. EFSA. European Food Safety Authority Risk Assessment of Ochratoxin A in Food. *EFSA J.* **2020**, *18*, e06113. [CrossRef]
13. IARC. *International Agency for Research on Cancer. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occuring Substances: Food Items and Costituents, Heterocyclic Aromatic Amines and Mycotoxins*; IARC Press: Lyon, France, 1993; Volume 56, ISBN 92-832-1256-8.
14. Ostry, V.; Malir, F.; Toman, J.; Grosse, Y. Mycotoxins as Human Carcinogens—The IARC Monographs Classification. *Mycotoxin Res.* **2017**, *33*, 65–73. [CrossRef]
15. Nguegwouo, E.; Sone, L.E.; Tchuenchieu, A.; Tene, H.M.; Mounchigam, E.; Njayou, N.F.; Nama, G.M. Ochratoxin A in Black Pepper, White Pepper and Clove Sold in Yaoundé (Cameroon) Markets: Contamination Levels and Consumers' Practices Increasing Health Risk. *Int. J. Food Contam.* **2018**, *5*, 1. [CrossRef]
16. Manda, P.; Adanou, K.M.; Ardjouma, D.; Adepo, A.J.B.; Dano, D.S. Occurrence of Ochratoxin A in Spices Commercialized in Abidjan (Côte d'Ivoire). *Mycotoxin Res.* **2016**, *32*, 137–143. [CrossRef] [PubMed]
17. Fofana-Diomande, A.; Kuaou, K.; Narcisse, A.; Sory, T.; Dembele, A. Study of the Contamination of Some Spices from Côte d'Ivoire by Mycotoxins (AFB1 and OTA). *J. Chem. Biol. Phys. Sci.* **2019**, *9*, 389–399. [CrossRef]
18. Lippolis, V.; Irurhe, B.; Porricelli, A.; Cortese, M.; Schena, R.; Imafidon, T.; Oluwadun, A.; Pascale, M. Natural Co-Occurrence of Aflatoxins and Ochratoxin A in Ginger (Zingiber Officinale) from Nigeria. *Food Control* **2016**, *73*, 1061–1067. [CrossRef]
19. Motloung, L.; De Saeger, S.; De Boevre, M.; Detavernier, C.; Audenaert, K.; Adebo, O.A.; Njobeh, P.B. Study on Mycotoxin Contamination in South African Food Spices. *World Mycotoxin J.* **2018**, *11*, 401–409. [CrossRef]
20. Garcia, M.V.; Mallmann, C.A.; Copetti, M.V. Aflatoxigenic and Ochratoxigenic Fungi and Their Mycotoxins in Spices Marketed in Brazil. *Food Res. Int.* **2018**, *106*, 136–140. [CrossRef] [PubMed]
21. Zhao, X.; Yuan, Y.; Zhang, X.; Yue, T. Identification of Ochratoxin A in Chinese Spices Using HPLC Fluorescent Detectors with Immunoaffinity Column Cleanup. *Food Control* **2014**, *46*, 332–337. [CrossRef]
22. Huang, Y.; Tang, G.; Zhang, T.; Fillet, M.; Crommen, J.; Jiang, Z. Supercritical Fluid Chromatography in Traditional Chinese Medicine Analysis. *J. Pharm. Biomed. Anal.* **2018**, *147*, 65–80. [CrossRef] [PubMed]
23. Jeswal, P.; Kumar, D. Mycobiota and Natural Incidence of Aflatoxins, Ochratoxin A, and Citrinin in Indian Spices Confirmed by LC-MS/MS. *Int. J. Microbiol.* **2015**, *2015*, 242486. [CrossRef] [PubMed]
24. Jeswal, P.; Kumar, D. Natural Occurrence of Toxigenic Mycoflora and Ochratoxin A & Aflatoxins in Commonly Used Spices from Bihar State (India). *J. Environ. Sci. Toxicol. Food Technol.* **2015**, *9*, 50–55. [CrossRef]
25. Wikandari, R.; Mayningsih, I.C.; Sari, M.D.P.; Purwandari, F.A.; Setyaningsih, W.; Rahayu, E.S.; Taherzadeh, M.J. Assessment of Microbiological Quality and Mycotoxin in Dried Chili by Morphological Identification, Molecular Detection, and Chromatography Analysis. *Int. J. Environ. Res. Public Health* **2020**, *17*, 1847. [CrossRef]
26. Jalili, M. Natural Occurrence of Ochratoxin A Contamination in Commercial Spices in Tehran. *Nutr. Food Sci. Res.* **2016**, *3*, 25–30. [CrossRef]
27. Zareshahrabadi, Z.; Bahmyari, R.; Nouraei, H.; Khodadadi, H.; Mehryar, P.; Asadian, F.; Zomorodian, K. Detection of Aflatoxin and Ochratoxin A in Spices by High-Performance Liquid Chromatography. *J. Food Qual.* **2020**, *2020*, 8858889. [CrossRef]
28. Kim, S.; Lee, S.; Nam, T.-G.; Seo, D.; Yoo, M. Comparison of a Newly Developed Liquid Chromatography with Tandem Mass Spectrometry Method and Enzyme-Linked Immunosorbent Assay for Detection of Multiple Mycotoxins in Red Pepper Powder. *J. Food Prot.* **2017**, *80*, 1347–1354. [CrossRef] [PubMed]
29. El Darra, N.; Gambacorta, L.; Solfrizzo, M. Multimycotoxins Occurrence in Spices and Herbs Commercialized in Lebanon. *Food Control.* **2019**, *95*, 63–70. [CrossRef]
30. Al Ayoubi, M.; Solfrizzo, M.; Gambacorta, L.; Watson, I.; El Darra, N. Risk of Exposure to Aflatoxin B1, Ochratoxin A, and Fumonisin B1 from Spices Used Routinely in Lebanese Cooking. *Food Chem. Toxicol.* **2021**, *147*, 111895. [CrossRef] [PubMed]
31. Ali, N.; Hashim, N.H.; Shuib, N.S. Natural Occurrence of Aflatoxins and Ochratoxin A in Processed Spices Marketed in Malaysia. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* **2015**, *32*, 518–532. [CrossRef]
32. Alsharif, A.M.A.; Choo, Y.-M.; Tan, G.-H. Detection of Five Mycotoxins in Different Food Matrices in the Malaysian Market by Using Validated Liquid Chromatography Electrospray Ionization Triple Quadrupole Mass Spectrometry. *Toxins* **2019**, *11*, 196. [CrossRef]
33. Jalili, M.; Jinap, S. Natural Occurrence of Aflatoxins and Ochratoxin A in Commercial Dried Chili. *Food Control* **2012**, *24*, 160–164. [CrossRef]
34. Iqbal, S.; Rafique Asi, M.; Zuber, M.; Akhtar, J.; Saif, M. Natural Occurrence of Aflatoxins and Ochratoxin A in Commercial Chilli and Chilli Sauce Samples. *Food Control* **2013**, *30*, 621–625. [CrossRef]

35. Iqbal, S.Z.; Asi, M.R.; Mehmood, Z.; Mumtaz, A.; Malik, N. Survey of Aflatoxins and Ochratoxin A in Retail Market Chilies and Chili Sauce Samples. *Food Control* **2017**, *81*, 218–223. [CrossRef]
36. Gherbawy, Y.A.; Shebany, Y.M. Mycobiota, Total Aflatoxins and Ochratoxin A of Cardamom Pods. *Food Sci. Technol. Res.* **2018**, *24*, 87–96. [CrossRef]
37. Yogendrarajah, P.; Jacxsens, L.; De Saeger, S.; De Meulenaer, B. Co-Occurrence of Multiple Mycotoxins in Dry Chilli (*Capsicum Annum*, L.) Samples from the Markets of Sri Lanka and Belgium. *Food Control* **2014**, *46*, 26–34. [CrossRef]
38. Jacxsens, L.; Pratheeb, Y.; Meulenaer, B. Risk Assessment of Mycotoxins and Predictive Mycology in Sri Lankan Spices: Chilli and Pepper. *Procedia Food Sci.* **2016**, *6*, 326–330. [CrossRef]
39. Ozbey, F.; Kabak, B. Natural Co-Occurrence of Aflatoxins and Ochratoxin A in Spices. *Food Control* **2012**, *28*, 354–361. [CrossRef]
40. Ostry, V.; Malir, F.; Dofkova, M.; Skarkova, J.; Pfohl-Leszkowicz, A.; Ruprich, J. Ochratoxin A Dietary Exposure of Ten Population Groups in the Czech Republic: Comparison with Data over the World. *Toxins* **2015**, *7*, 3608–3635. [CrossRef] [PubMed]
41. Fazekas, B.; Tar, A.; Kovács, M. Aflatoxin and Ochratoxin a Content of Spices in Hungary. *Food Addit. Contam.* **2005**, *22*, 856–863. [CrossRef] [PubMed]
42. Gambacorta, L.; Magistà, D.; Perrone, G.; Murgolo, S.; Logrieco, A.F.; Solfrizzo, M. Co-Occurrence of Toxigenic Moulds, Aflatoxins, Ochratoxin A, Fusarium and Alternaria Mycotoxins in Fresh Sweet Peppers (*Capsicum Annuum*) and Their Processed Products. *World Mycotoxin J.* **2018**, *11*, 159–174. [CrossRef]
43. Prelle, A.; Spadaro, D.; Garibaldi, A.; Gullino, M.L. Co-Occurrence of Aflatoxins and Ochratoxin A in Spices Commercialized in Italy. *Food Control* **2014**, *39*, 192–197. [CrossRef]
44. Reinholds, I.; Pugajeva, I.; Bavrins, K.; Kuckovska, G.; Bartkevics, V. Mycotoxins, Pesticides and Toxic Metals in Commercial Spices and Herbs. *Food Addit. Contam. Part B* **2016**, *10*, 5–14. [CrossRef] [PubMed]
45. Waskiewicz, A.; Beszterda, M.; Bocianowski, J.; Golinski, P. Natural Occurrence of Fumonisins and Ochratoxin A in Some Herbs and Spices Commercialized in Poland Analyzed by UPLC–MS/MS Method. *Food Microbiol.* **2013**, *36*, 426–431. [CrossRef]
46. Santos, L.; Marín, S.; Sanchis, V.; Ramos, A.J. Co-Occurrence of Aflatoxins, Ochratoxin A and Zearalenone in *Capsicum* Powder Samples Available on the Spanish Market. *Food Chem.* **2010**, *122*, 826–830. [CrossRef]
47. European Commission. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 Setting Maximum Levels for Certain Contaminants in Foodstuffs. *Off. J. Eur. Union* **2006**, *L364*, 5–24.
48. MoA. The Ministry of Agriculture of the Czech Republic: Current Discussed Topics in the Field of Contaminants in Food—July 2021. Available online: https://www.bezpecnostpotravin.cz/aktualni-diskutovana-temata-v-oblasti-kontaminantu-v-potravinach-cervenec-2021.aspx (accessed on 15 May 2021).
49. Zimmerli, B.; Dick, R. Determination of Ochratoxin A at the Ppt Level in Human Blood, Serum, Milk and Some Foodstuffs by High-Performance Liquid Chromatography with Enhanced Fluorescence Detection and Immunoaffinity Column Cleanup: Methodology and Swiss Data. *J. Chromatogr. B Biomed. Sci. Appl.* **1995**, *666*, 85–99. [CrossRef]
50. European Commission. Commision Regulation (EC) No 401/2006 of 23 February 2006 Lying down the Methods of Sampling and Analysis for the Official Control of the Levels of Mycotoxins in Foodstuffs. *Off. J. Eur. Union* **2006**, *L70*, 1–42.
51. EFSA, European Food Safety Authority. Management of Left-Censored Data in Dietary Exposure Assessment of Chemical Substances. *EFSA J.* **2010**, *8*, 1–96. [CrossRef]
52. FAO/WHO. Joint FAO/WHO Food Standards Programme. In Proceedings of the Codex Alimentarius Commission 44th Session, Geneva, Switzerland, 8–13 November 2021; Report of the 14th Session of the Codex Committee on Contaminants in Foods (Virtual) 3–7 and 13 May 2021. Available online: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/fr/?lnk=1&url=https%253A%252F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-735-14%252FREPORT%252FFinalReport%252FREP21_CFe.pdf (accessed on 20 July 2021).
53. RASFF. Rapid Alert System for Food and Feed Portal Database. Available online: https://webgate.ec.europa.eu/rasff-window/portal/ (accessed on 20 July 2021).

Příloha 6

Investigation of ochratoxin A in blood sausages in the Czech Republic: Comparison with data over Europe

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Investigation of ochratoxin a in blood sausages in the Czech Republic: Comparison with data over Europe

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ABSTRACT

Blood sausages consisting of groats, pork, porcine offal, fat, blood, and spices are very popular in the Czech Republic. All these ingredients are potential sources of dietary exposure to ochratoxin A (OTA). OTA has a strong affinity to serum proteins in porcine blood. Thus, the contamination of blood sausages with OTA can be expected. This study aims to evaluate OTA in 200 samples of porcine blood sausages purchased at the Czech market during 2020–2021. The analytical method high-performance liquid chromatography coupled with fluorescence detection with pre-treatment using immunoaffinity columns was employed to determine OTA. The limit of detection was 0.03 ng/g and the limit of quantification 0.10 ng/g. Recovery was 71.6 %. All samples were positive at contents ranging from 0.15 to 5.68 ng/g with a mean of 1.47 ng/g, and a median of 1.26 ng/g. A total of 66% of these samples contained OTA content exceeding the maximum limit of 1 ng/g set in Italy. This study demonstrates that the Czech population is exposed to OTA from blood sausages. The proposed preliminary action limit for OTA in blood sausages should be set at 1 ng/g. No regulatory limits for OTA in blood sausages have been established yet in the European Union legislation. To protect human health, further monitoring of OTA in these products is necessary.

1. Introduction

Blood sausages (also somewhere known as blood pudding or black pudding) have been made for thousands of years (Edwards, 1988). During that time, many recipes have been developed and vary across countries all around the world (see Table S1 in the supplementary materials) (Anjos et al., 2019; AtlasMedia Ltd., 2022; Belleggia et al., 2020; Fellendorf et al., 2016; Gašperlin et al., 2014; Kim et al., 2021; Santos et al., 2003; Sinclair, 2005). As the name implies, animal blood, namely from pig, sheep, cow, lamb, and goose, is the key ingredient in all recipes for blood sausages. Other parts of the animal such as meat, fat, and offal are also used. Other ingredients are fillers such as oatmeal, breadcrumbs, barley, buckwheat, other grains, and regional spices (Meats and Sausages, 2022). A general blood sausage-making process includes six steps: 1) raw material selection, 2) preliminary preparation of raw materials such as weighting, size reduction, premixing, precooking, and curing, 3) mixing, 4) stuffing, 5) cooking, and 6) chilling (Ramos et al., 2013).

Our study focused on blood sausages manufactured in the Czech Republic. Porcine blood, meat from pig head or belly, fat, and offal are preferred in them as the animal ingredients. The usual fillings are grains such as barley groats or, in some cases, white buns (AtlasMedia Ltd., 2022). Black pepper, cumin, marjoram, allspice, fried onions and occasionally garlic are the most used spices (Jandásek, 2014). Blood sausages are typically encapsulated in pork intestine casing (AtlasMedia Ltd., 2022). They are usually consumed heated either by frying or baking at 150–180 °C for 30–40 min, rarely cold, and most often served with sauerkraut and boiled potatoes, but bread is also common.

Meat and meat products are commonly contaminated with the pillar mycotoxin ochratoxin A (OTA) (PubChem CID 442530) (Pleadin et al., 2021; PubChem, 2021) that is produced by several species of microscopic filamentous fungi of the *Aspergillus* and *Penicillium* genera (Malir et al., 2016; Ostry et al., 2013; Vlachou et al., 2022). OTA is classified as possibly carcinogenic in humans, group 2B (IARC, 1993). OTA is

Abbreviations: ACN, Acetonitrile; EU, European Union; HPLC-FD, high-performance liquid chromatography coupled with fluorescence detection; IACs, immunoaffinity columns; MeOH, methanol, OTA, ochratoxin A; TDS, Total Diet Study.

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considered a cumulative toxin with relatively rapid absorption and slow elimination (EFSA, 2020). In the human bloodstream, 99% of OTA binds to plasma proteins, mainly to albumin, and only a small OTA fraction occurs in the free form (Malir et al., 2013). The organs of rapid kinetics are kidneys, liver, testes, intestine and of slow elimination are muscles and adipose tissue (EFSA, 2020). OTA is nephrotoxic, hepatotoxic, genotoxic, teratogenic, embryotoxic, neurotoxic, and immunotoxic (Malir et al., 2016; Zhao & Ambrose, 2017).

In terms of OTA contamination, pork products pose the highest risk among animal products (Galtier et al., 1981) and have historically been considered a significant source of dietary exposure to OTA for humans (Pleadin et al., 2021). The overall estimated contribution of animal products to human OTA exposure generally did not exceed 3%; however, in certain regions where the consumption of traditional meat products like blood puddings was popular, it can reach up to 10 % (EFSA, 2004; Mitchell et al., 2017; Walker, 2002). The most important current contributors to the chronic dietary exposure to OTA were 'Preserved meat (ham, pork)','Cheese' and 'Grains and grain-based products' (EFSA, 2020). The digestive tract of monogastric species such as pigs is less effective in breaking down OTA than that of polygastric species due to the absence of rumen in which OTA is microbially degraded to less toxic ochratoxin α (Battacone et al., 2010; EFSA, 2004; Liu et al., 2022; Pleadin et al., 2021). The feed composition also plays an important role because pig feed contains more cereals that are risky from the point of view of OTA contamination (Battacone et al., 2010).

After the exposure of pigs to OTA-contaminated feed, blood sausages are the most contaminated porcine final products (Persi et al., 2014; Pleadin et al., 2014). This can be attributed not only to the use of less valuable parts of the pig such as blood, non-muscular parts and offal, but also to the addition of other possibly OTA-contaminated materials such as cereals and cereal products, and, to a lesser extent, spices (Meucci et al., 2019; Ostry et al., 2013; Vlachou et al., 2022).

The aims of our study were: 1) evaluation of the OTA contamination of 200 blood sausages purchased at the Czech market using pre-purification on immunoaffinity columns (IACs) followed by high-performance liquid chromatography coupled with fluorescence detection (HPLC-FD) and 2) comparison of the results with those from foreign studies dealing with the natural occurrence of OTA in blood sausages.

2. Material and methods

2.1. Sample collection and classification

A total of 200 blood sausage samples were collected in retail shops in 18 different cities throughout the whole area of the Eastern Bohemia during years 2020–2021. These cities are Chlumec nad Cidlinou, Chotovice, Dvur Kralove, Horice, Hradec Kralove, Jaromer, Jicin, Kosmonosy, Mestec Kralove, Mlada Boleslav, Mnichovo Hradiste, Nova Paka, Novy Bydzov, Pardubice, Prague, Smirice, Sobotka, and Trebechovice pod Orebem.

Samples, all originated in the Czech Republic, were purchased in a quantity of 71–518 g based on the packaging. They included blood sausages purchased at the counter as well as packaged in a protective atmosphere. All samples were homogenized and stored in disposable transparent polypropylene containers for food storage at −20 ± 0.5 °C until sample preparation before processing.

The collection of blood sausage samples for the OTA analysis was carried out within the national food sampling rules and was based on the methodological design known as the "Total Diet Study" that is suitable for the surveillance of chronic dietary exposure and respects consumer behavior of people in the Czech Republic (types of shops, season of the purchase, brands, etc.) (EFSA, 2011). All steps are described and documented in standard operating procedure to ensure the accuracy and precision of food sampling.

Collected blood sausages were classified according to EFSA standardized food classification and description system FoodEx2 (EFSA, 2015). Analytical measurements were carried out with individual sampled items and culinary treatment was not applied. Detailed information about sampled blood sausage is presented in Table S2 in supplementary materials.

2.2. Chemicals and apparatus

Acetonitrile (ACN), methanol (MeOH) (all of HiPerSolv CHROMANORM gradient grade), hydrochloric acid, glacial acetic acid, disodium hydrogen phosphate anhydrous, sodium dihydrogen phosphate dihydrate, sodium chloride (all of AnalaR NORMAPUR grade) were purchased from VWR (Stribrná Skalice, Czech Republic). Laboratory cleaning agent RBS® 35 was purchased from P-LAB a.s. (Prague, Czech Republic). All solutions (see section 2.3.) were prepared in ultrapure water prepared using a Milli-Q system (Millipore, Milford, MA, USA), hereinafter referred to as 'water'. The resistivity of water was > 18.2 MΩ.cm at 25 °C.

OTA standard (*Petromyces albertensis*, ≥98%, HPLC) was from Sigma-Aldrich (Prague, Czech Republic). The analytical standard was stored in a laboratory freezer at − 20 ± 0.5 °C.

Filter papers Whatman No. 4 or KA-2M were obtained from Merck (Prague, Czech Republic) and papirna Pernstejn (Pernstejn, Czech Republic), respectively. Nylon syringe filters (13 mm, 0.22 μm) originated from Labstore (Inverness, Highland, United Kingdom). Immunoaffinity columns (IACs) OCHRAPREP® were obtained from R-Biopharm (Darmstadt, Germany) and vacuum filtration kit MORTON frita S3 from KAVALIERGLASS (Prague, Czech Republic).

Two homogenizers, A - Ultra-Turrax T 50 digital and B - Ultra-Turrax T 10 basic, were manufactured by IKA®-Werke GmbH & Co. KG (Staufen, Germany). The Reax Multi shaker was from Heidolph Instruments GmbH & Co. KG (Schwabach, Germany). The chromatographic HPLC system Agilent 1260 Infinity II was coupled to 1260 Infinity II Fluorescence Detector, both manufactured by Agilent (Santa Clara, CA, USA). Analytical column (Kinetex C18, 2.6 μm, 100 Å, 50x21 mm) was used with security Guard^{TM} column (Phenomenex C18, 4x2.0 mm), both from Phenomenex (Prague, Czech Republic).

2.3. Standards and solutions preparation

2.3.1. Phosphate buffered saline (PBS) at pH 7.4

A total of 0.62 g of sodium dihydrogen phosphate dihydrate, 2.271 g of di-sodium hydrogen phosphate anhydrous, and 9 g of sodium chloride were all quantitatively transferred into a 1,000 mL flask. The flask was made up with water. The pH of the solution was adjusted at 7.4 with hydrochloric acid.

2.3.2. Elution solution of methanol acidified by glacial acetic acid

A total of 2 mL of glacial acetic acid was pipetted into a 100 mL flask. The flask was made up with MeOH.

All of the solutions 2.3.1–2.3.3 were kept at 5 ± 0.5 °C. Before direct use, they were tempered at a laboratory temperature of 21 ± 0.5 °C.

2.3.3. OTA stock solution in methanol at a concentration of 10,000 μg/L

A total of 1,000 μg of solid OTA standard was dissolved in 5 mL of MeOH in the original standard vial. The dissolved standard solution was quantitatively transferred into a 100 mL flask. The flask was made up with MeOH. The stock solution was kept at −20 ± 0.5 °C. The stock solution is stable for 1 year.

2.3.4. OTA working standard solution at a concentration of 40 μg/L

A total of 100 μL of OTA stock solution at a concentration of 10,000 μg/L was pipetted into a 25 mL flask. The flask was made up with MeOH. The working solution was kept at −20 ± 0.5 °C.

2.3.5. Calibration OTA standards

Six-point calibration was performed using six calibration standard

solutions that were prepared from the working solution by its dilution in the mobile phase (*vide infra*) in a ratio reaching the target concentrations of 0.1, 0.25, 0.5, 1.0, 2.0, and 4.0 ng/mL. The determination coefficient was 0.9999. A blank sample consisting of the mobile phase and all calibration solution were prepared fresh daily. The calibration curve is shown in Figure S1 in supplementary materials.

2.3.6. HPLC mobile phase

The HPLC mobile phase comprised a 40:60 mixture of 1% acetic acid and ACN and 1% aqueous acetic acid.

All glassware contaminated with OTA was decontaminated according to systematic instruction for decontamination of OTA reporting an immersion of glassware in 50 °C bath containing 2% RBS 35 for at least 30 min. This way of decontamination is declared to be effective in decontaminating 99.99 % OTA content (Ostry et al., 2021).

2.4. Workflow

2.4.1. Homogenization of samples

A total of 200 blood sausage samples in weight range 71–518 g were homogenized using homogenizer A. The homogenizer and all components used for homogenization were washed and rinsed with MeOH between each sample to avoid sample contamination.

2.4.2. Sample preparation—OTA purification by immunoaffinity chromatography

A total of 5 g of homogenized sample was weighed and transferred into a centrifuge tube was mixed with 20 mL 60% aqueous ACN solution and homogenized again using homogenizer B for 2 min before left to shake for 5 min using a shaker. The sample was filtered using filter paper cartridge and a vacuum filtration kit. The filtrate (4 mL) was diluted with 44 mL PBS buffer (relevant to 1 g of sample) and transferred to the reservoir above the immunoaffinity column IAC. All solutions were allowed to drop at a flow rate of 1 drop/s through IAC that had been previously brought to the laboratory temperature of 21 ± 0.5 °C. The IACs were purified twice with 10 mL of PBS buffer, then briefly sieved with air for 1–2 s after each OTA adsorption. The elution of OTA was achieved with 1 mL elution solution consisting of acidified MeOH into a small glass vial at a rate of 1 drop/s. The eluates were returned to the IACs and left to drip four more times. Finally, another 0.5 mL of fresh elution solution was added and passed through IACs followed by strong air sieving for 30 s. The eluate with a volume of 1.5 mL was evaporated under nitrogen gas at 45 °C to dryness and kept in the laboratory refrigerator at 5 ± 0.5 °C until analysis. The dry samples were dissolved in 0.5 mL of mobile phase using an sonication bath and passed through a nylon syringe filter into a vial for HPLC analysis.

2.5. HPLC-FD conditions

Both security GuardTM (Phenomenex C18, 4x2.0 mm) and HPLC (Kinetex C18, 2.6 μm, 100 Å, 50x21 mm) columns were kept at 30 °C. The mobile phase flow rate was 0.2 mL/min and the injection volume was 8.0 μL. Fluorescence detection was carried out at an excitation wavelength of 333 nm and an emission wavelength of 465 nm, PMT gain 18, and attenuation 100 LU. Chromatography software Agilent OpenLab software was used to collect the chromatographic data.

2.6. Method validation

The limit of detection (LOD) was 0.03 ng/g and the limit of quantification (LOQ) was 0.10 ng/g, based on the formulas according to the IUPAC: LOD $= \frac{3 \bullet \sigma}{b}$ and LOQ $= \frac{10 \bullet \sigma}{b}$, where "σ" means the standard deviation of ten peak areas of the lowest calibration point of 0.1 ng/g and "b" means the slope of the calibration curve. No suitable certified reference material for the determination of OTA was available during the period of this research. No interlaboratory comparative examinations Proficiency testing (e.g. FERA, UK) were done. The recovery of the method was verified using samples spiked with OTA at concentration levels of 0.5, 1.0, and 2.0 ng/g that were added: a) to the matrix before the extraction step, b) after the separation step on immunoaffinity columns to the eluate. Each concentration level was measured in triplicate: therefore, a total of 18 spiked samples were analyzed to determine the mean recovery. The sample without OTA addition was marked as the "blank sample" and the measured OTA concentration was subtracted from the concentrations of samples with OTA addition. The mean recovery was 71.6% that fulfils the requirements of Regulation (EC) No. 401/2006 (Commission, 2006). The repeatability RSD was 2.05%. The mean measurement uncertainty was 4.8%. OTA retention time was 5.2 min. The calibration curve consisted of six concentration levels of 0.10, 0.25, 0.50, 1.00, 2.00, and 4.00 ng/g. All samples with a concentration outside the calibration curve were diluted to reach the value within the calibration curve.

3. Theory

3.1. Natural occurrence of ochratoxin a in blood sausages and its ingredients

The OTA contamination of pork products can occur either directly as a result of ochratoxigenic microfungi *Penicillium nordicum, P. verrucosum, Aspergillus westerdijkiae,* and *A. ochraceus* growing on these products, or indirectly as a result of animal feeding with OTA contaminated feed leading to the carryover (Bernáldez et al., 2018; Ferrara et al., 2016; Ostry et al., 2013; Parussolo et al., 2019; Rodríguez et al., 2012). OTA then occurs in the pig tissues and then in the final products as demonstrated with several studies. A few of them have shown that more toxin can persist in the kidneys, lungs, and liver (Persi et al., 2014; Pleadin et al., 2014, 2016). However, the blood is generally considered the most contaminated material (Altafini et al., 2017; Aoudia et al., 2009; Bertuzzi et al., 2013; Curtui et al., 2001; Duarte et al., 2012; EFSA, 2004; Lusky et al., 1993; Rossi et al., 2006). The smallest quantities of toxin are usually found in the muscles and fat (Altafini et al., 2017; Bertuzzi et al., 2013; Curtui et al., 2001; EFSA, 2004; Gallo et al., 2020; Krogh et al., 1976, 1979; Lusky et al., 1993; Malagutti et al., 2005; Persi et al., 2014; Pleadin et al., 2014, 2016; Raja et al., 2008; Rossi et al., 2006). In the bloodstream, OTA binds to serum proteins, especially albumin, which significantly affects its biological half-life (EFSA, 2004; Galtier et al., 1981). In pigs, the OTA biological half-life of 72–120 h is the longest known among common farm animals (Galtier et al., 1981; Marin et al., 2009; Sreemannarayana et al., 1988). Therefore, the addition of porcine blood can significantly increase the OTA content in the final products (Lusky et al., 1993; Persi et al., 2014). This appears to be the reason why blood sausages proved to be the most contaminated final product that originated from OTA-fed pigs (Persi et al., 2014; Pleadin et al., 2014). All other used porcine tissues contribute to this contamination. Table S3 in the supplementary materials provides an overview of several studies dealing with natural OTA contamination of porcine tissues.

The non-animal ingredients of blood sausages are also potentially dangerous in terms of OTA contamination. Cereals (barley groats) and cereal products (white buns), which are used in Czech blood sausages as a filling, are repeatedly found to be contaminated with OTA (González-Osnaya et al., 2007; Hassan et al., 2019; Ibáñez-Vea et al., 2012; Pleadin et al., 2018; Polisenska et al., 2010; Tam et al., 2011; Zaied et al., 2009; Zinedine et al., 2007). Finally, the added spices can also contribute to the OTA content in the blood sausages (Meucci et al., 2019) since OTA has been detected in spices in the past (Pickova et al., 2020, 2021).

Considering the OTA contamination of animal and plant ingredients of blood sausages reported in the literature, the contamination can also be expected in the final products. Unfortunately, studies dealing with the natural occurrence of OTA in blood sausages are scarce as shown in Table 1 with only a few studies from Belgium (Tangni et al., 2021), the

Table 1
List of studies dealing with the natural occurrence of ochratoxin A in blood sausages.

Country[1]	n+/n	n+(%)	Mean (ng/g)	Range (ng/g)	LOD[2] (ng/g)	LOQ[3] (ng/g)	Recovery (%)	Method[5]	Reference
GER	44/57	77	0.16	<LOD-3.16	0.01	N/S[4]	67–88	IAC, HPLC-FD	(Gareis & Scheuer, 1999, 2000)
CZE	0/12	0	–	–	0.1	0.3	85	IAC, HPLC-FD	(Ostry et al., 2011)
BEL	0/20	0	–	–	N/S	0.4	80–85	LC-MS/MS	(Tangni et al., 2021)

HPLC-FD, high-performance liquid chromatography coupled with fluorescence detection; LC-MS/MS, liquid chromatography with tandem mass spectrometry.
[1] GER, Germany; CZE, Czech Republic; BEL, Belgium.
[2] LOD, limit of detection.
[3] LOQ, limit of quantification.
[4] N/S, not specified.
[5] IAC, immunoaffinity column clean-up step.

Czech Republic (Ostry et al., 2011), and Germany (Gareis & Scheuer, 1999, 2000).

3.2. Regulation limits regarding OTA in pork and derived products

Although OTA has been reported in various animal-derived products in most countries of the world, the regulations of OTA presence in these products have not been established yet. The European Union (EU) regulation 1881/2006 setting maximum limits for certain contaminants, as amended, includes maximum limits for OTA in various foodstuffs but does not concern any meat, meat products, edible offal, or other foods of animal origin (EC, 2006a). A total of 27 member states including the Czech Republic, are bound by the EU legislation. Several countries summarized in Table 2 set themselves maximum limits on commodities not specified by the EU to protect consumers from OTA contaminated animal products, (Duarte et al., 2011; FAO, 2004).

To minimize the risk of the OTA occurrence in animal products, it is also necessary to pay more attention to the monitoring of feed quality that is considered a key step in preventing the carryover effect (Asefa et al., 2011; Pleadin et al., 2015; Tangni et al., 2021). The EU Commission recommendation 2006/576/EC on the presence of certain mycotoxins, including OTA, in products intended for animal feeding, sets a guidance value of 50 ng/g for complementary and complete feeding stuff for pigs. The use of cereals and cereal products contaminated up to the guidance value of 250 ng/g is permitted provided the contamination of the total daily dose does not exceed the established limit for complete feeding stuffs (EC, 2006b).

3.3. Dietary exposure assessment of OTA in blood sausages

Data from the nation-wide dietary survey (SISP04) were used for calculations of the dietary exposure (Ruprich et al., 2004). This survey was conducted by repeated 24 h recall on an age and gender representative sample of the Czech population. The Czech food consumption data (SISP04) were used as reported to the EFSA Comprehensive European Food Consumption Database. The Comprehensive Food Consumption Database is the source of information on food consumption across the EU containing detailed data for a number of EU countries (EFSA 2018).

Chronic food consumption for consumer only (average consumption of 2 surveyed days) of blood sausages is presented in Table S4 in supplementary materials. The chronic dietary exposure distribution of OTA in blood sausages in ng/kg bw/day is calculated by multiplying the arithmetic mean of analytically determined concentrations with individual chronic food intake in population groups of interest.

$$DE = (C \times V)$$

DE is the dietary exposure for an individual (ng/kg bw/day), C is the arithmetic mean of analytically determined concentrations of OTA (ng/kg), and V is the individual chronic food consumption (kg/kg bw/day) (Ruprich et al., 2004; Ostry et al., 2020).

Table 2
Individual national maximum levels of ochratoxin A in foodstuffs of animal origin set by European Union member countries.

Country	Foodstuffs	Maximum limit (ng/g)
Denmark	Porcine kidney[1]	10
	Porcine kidney[2]	25
Estonia	Porcine liver	10
Italy	Porcine meat and derived products	1
Slovakia	Meat	5

[1] Viscera condemned, visibly damaged kidneys are analysed chemically.
[2] Whole carcass condemned, visibly damaged kidneys are analysed chemically. Processed according to (FAO, 2004; Ministero della Sanita, 1999).

3.4. Risk characterization of OTA in blood sausages

Direct and indirect genotoxic and non-genotoxic modes of action might each contribute to tumour formation. Since recent studies have raised uncertainty regarding the mode of action for kidney carcinogenicity, it is inappropriate to establish a health-based guidance value (HBGV) and a margin of exposure (MOE) approach was applied (EFSA, 2012). For the characterization of non-neoplastic effects, a benchmark dose lower confidence limit for a 10% response ($BMDL_{10}$) of 4.73 μg/kg body weight (bw) per day was calculated from kidney lesions observed in pigs (EFSA, 2020).

$$MOE = BMDL_{10}/\text{exposure dose}$$

MOE of 200 or higher is considered of low concern from a public health point of view with respect to the non-carcinogenic effect. For characterization of neoplastic effects, a $BMDL_{10}$ of 14.5 μg/kg bw per day was calculated from kidney tumours seen in rats. MOE of 10,000 or higher is considered of low concern from a public health point of view with respect to the carcinogenic effect while MOE less than 10,000 represents a high public health concern (EFSA, 2020).

4. Results and discussion

4.1. Ochratoxin a occurrence in blood sausage

All 200 porcine blood sausage samples were positive, exceeding LOQ of 0.1 ng/g for OTA. The entire data set is given in the Table S5 in supplementary materials. The mean OTA concentration was 1.47 ng/g. The summary descriptive statistical data processing of OTA natural occurrence in blood sausage samples is given in Table 3.

Only very few studies deal with the natural occurrence of OTA in blood sausages and affect rather low number of samples typically counting 12–57 (Gareis & Scheuer, 1999, 2000; Ostry et al., 2011;

Table 3
Statistical data on ochratoxin A in blood sausages.

n	n+ %	Mean (ng/g)	Median (ng/g)	90th percentile (ng/g)	Range (ng/g)
200	100	1.47	1.26	2.77	0.15–5.68

Tangni et al., 2021). Therefore, our study is unique in a large number of 200 samples. All of them were positive on OTA occurrence with the concentration levels exceeding LOQ.

In contrast, the Belgian study using LC-MS/MS method without the previous IAC clean-up step has reported no occurrence of OTA in blood sausages (Tangni et al., 2021). Comparable results have been reported in the Czech study using the HPLC-FD method coupled with the IAC clean-up step (Ostry et al., 2011). This could be due to the relatively high LOQs of 0.3 and 0.4 ng/g that were determined in the Czech and Belgium studies, respectively. Our methodology similar to the latter enabled the LOQ of 0.1 ng/g. The differences were due to different sensitivity of the methods, equipment, and the composition of the sample depending on used ingredients. Therefore, our results can only be partially compared with the German study that used the HPLC-FD method coupled with the IAC clean-up step (Gareis & Scheuer, 1999, 2000). All parameters such as OTA positivity of 100% in our study vs. 77 % in the German study, mean concentration of 1.47 vs. 0.16 ng/g, and maximum concentration of 5.68 vs. 3.16 ng/g were higher in our study compared to the German one. Thus, our study provides new worrying information about the dietary exposure to OTA from blood sausages and related products.

As mentioned above, no regulation of the occurrence of OTA in foodstuffs of animal origin exists for the EU. Unfortunately, the European Commission's latest discussion from December 2021 does not even mention that any limits regarding these products should come into force any time soon (MoA, 2021). Therefore, our results were compared with the Italian OTA limit of 1 ng/g in porcine meat and derived products (FAO, 2004; Ministero della Sanita, 1999), as only this limit is relevant for comparison. Our study indicates that the Italian limit was exceeded in 132 (66 %) samples.

It is generally known that heat treatment used during production does not significantly reduce content of mycotoxins in meat and meat products (Pleadin et al., 2021). However, it has been demonstrated that the OTA content in blood sausages can be reduced by 9.1, 13.8, 70.9, 76.6, and 85.9% via cooking at 100 °C for 30 min, frying at 170 °C for 30 min, and baking at 190, 200, and 220 °C for 60 min, respectively (Pleadin et al., 2014). Considering our results and the above-mentioned heat treatment, frying, which is the typical heat treatment of blood sausages before consumption, would occur insufficient in reducing the OTA content. It appears possible to achieve an average OTA concentration in our samples lower than the Italian limit only by long-term baking. However, it should understood that the baking temperatures and times shown above do not correspond to the typical heat treatment and food preparation of the blood sausages before consumption.

4.2. Estimation of dietary exposure dose and risk characterization of OTA in blood sausages

Calculation of dietary exposure dose and MOE for OTA for whole population (consumers only, 95th percentile) is presented in Table 4. The 95th percentile chronic exposure of OTA through blood sausages was determined in the whole population (consumer only) 0.004 μg/kg bw/day.

MOE of more than 200 (non-neoplastic effects) indicates a low health concern with the exception of MOEs for high consumers in the whole population. MOE < 10,000 is considered of high concern from a public health point of view with respect to the neoplastic (carcinogenic) effect. This would indicate a possible health concern if genotoxicity is direct. Uncertainty in this assessment is high and the risk can be overestimated.

The proposed preliminary action limit for OTA in blood sausages could be set at 1 μg/kg.

5. Conclusion

OTA is a common contaminant of animal products, but in general, animal products, unlike cereals and cereal products, are not a major public health hazard in terms of human dietary exposure to OTA.

Table 4
Calculation of dietary exposure dose and margin of exposure (MOE) for ochratoxin A for whole population (consumers only, 95th percentile).

Monitored parameter	Units	Result
Ochratoxin A in blood sausage (the arithmetic mean)	μg/kg	1.47
Expected consumption of blood sausage in the whole population p95 (consumer only)	g/kg bw/day	2.58
Exposure dose of ochratoxin A for whole population p95 (consumer only)	μg/kg bw/day	0.004
$BMDL_{10}$ (non-neoplastic effects)	μg/kg bw/day	4.73
$BMDL_{10}$ (neoplastic effects)	μg/kg bw/day	14.5
MOE (non-neoplastic effects)	Limit: ≥ 200	1183
MOE (neoplastic effects)	Limit: ≥ 10,000	3625

However, in certain European countries, animal products mainly pork and products with porcine blood pose a significant dietary exposure to OTA. Our study confirmed that all 200 blood sausages purchased on the Czech market were contaminated with OTA above the LOQ. Moreover, a total of 66% of these sausages contained an OTA content exceeding the maximum limit set in Italy, which is the only country to regulate OTA in products of this type. Given the results achieved and the risk assessment evaluation, it is clear that it is desirable to introduce in the EU at least the same, or perhaps even stricter limit for OTA in products from animal tissues. Unfortunately, the literature concerning the natural occurrence of OTA in blood sausages is limited and more monitoring studies are needed to address this issue. Therefore, further monitoring of OTA in these products is necessary to protect human health, e.g. Total Diet Study (TDS). A TDS most accurately represents the levels of the mycotoxins in the edible portion of food at the point of consumption, and takes into account loss during processing, food preparation, and storage. This might be useful as the starting point toward setting future priorities for more detailed collections of data.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

Alarousy, R., & Hakim, A. (2015). Incidence of Fungal Infections and Mycotoxicosis in Pork Meat and Pork By-Products in Egyptian Markets. *World Academy of Science, Engineering and Technology, 9*, 1184–1187.

Altafini, A., Armorini, S., Zaghini, A., Sardi, L., & Roncada, P. (2017). Tissue distribution of ochratoxin A in pigs after administration of two-levels contaminated diets. *World Mycotoxin Journal, 10*(3), 263–272. https://doi.org/10.3920/WMJ2016.2152

Anjos, O., Fernandes, R., Cardoso, S. M., Delgado, T., Farinha, N., Paula, V., et al. (2019). Bee pollen as a natural antioxidant source to prevent lipid oxidation in black pudding. *LWT, 111*, 869–875. https://doi.org/10.1016/j.lwt.2019.05.105

Aoudia, N., Callu, P., Grosjean, F., & Larondelle, Y. (2009). Effectiveness of mycotoxin sequestration activity of micronized wheat fibres on distribution of ochratoxin A in plasma, liver and kidney of piglets fed a naturally contaminated diet. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 47*(7), 1485–1489. https://doi.org/10.1016/j.fct.2009.03.033

Asefa, D. T., Kure, C. F., Gjerde, R. O., Langsrud, S., Omer, M. K., Nesbakken, T., et al. (2011). A HACCP plan for mycotoxigenic hazards associated with dry-cured meat production processes. *Food Control, 22*(6), 831–837. https://doi.org/10.1016/j.foodcont.2010.09.014

AtlasMedia Ltd. (2022). *Blood Sausages.* Tasteatlas. https://www.tasteatlas.com/blood-sausages.

Battacone, G., Nudda, A., & Pulina, G. (2010). Effects of ochratoxin A on livestock production. *Toxins, 2*(7), 1796–1824. https://doi.org/10.3390/toxins2071796

Bauer, J., & Gareis, M. (1987). Ochratoxin A in the food chain. *Zentralblatt fur Veterinarmedizin Reihe B. Journal of veterinary medicine. Series B, 34*(8), 613–627. https://doi.org/10.1111/j.1439-0450.1987.tb00442.x

Belleggia, L., Ferrocino, I., Reale, A., Boscaino, F., Di Renzo, T., Corvaglia, M. R., et al. (2020). Portuguese cacholeira blood sausage: A first taste of its microbiota and volatile organic compounds. *Food Research International, 136*, Article 109567. https://doi.org/10.1016/j.foodres.2020.109567

Bernáldez, V., Rodríguez, A., Delgado, J., Sánchez-Montero, L., & Córdoba, J. J. (2018). Gene Expression Analysis as a Method to Predict OTA Accumulation in Dry-Cured Meat Products. *Food Analytical Methods, 11*(9), 2463–2471. https://doi.org/10.1007/s12161-018-1231-0

Bertuzzi, T., Gualla, A., Morlacchini, M., & Pietri, A. (2013). Direct and indirect contamination with ochratoxin A of ripened pork products. *Food Control, 34*(1), 79–83. https://doi.org/10.1016/j.foodcont.2013.04.011

Cao, X., Li, X., Li, J., Niu, Y., Shi, L., Fang, Z., et al. (2018). Quantitative determination of carcinogenic mycotoxins in human and animal biological matrices and animal-derived foods using multi-mycotoxin and analyte-specific high performance liquid chromatography-tandem mass spectrometric methods. *Journal of Chromatography B, 1073*, 191–200. https://doi.org/10.1016/j.jchromb.2017.10.006

Ceci, E., Bozzo, G., Bonerba, E., Di Pinto, A., & Tantillo, M. G. (2007). Ochratoxin A detection by HPLC in target tissues of swine and cytological and histological analysis. *Food Chemistry, 105*(1), 364–368. https://doi.org/10.1016/j.foodchem.2006.12.019

Chen, D., Cao, X., Tao, Y., Wu, Q., Pan, Y., Huang, L., et al. (2012). Development of a sensitive and robust liquid chromatography coupled with tandem mass spectrometry and a pressurized liquid extraction for the determination of aflatoxins and ochratoxin A in animal derived foods. *Journal of Chromatography A, 1253*, 110–119. https://doi.org/10.1016/j.chroma.2012.06.095

Commission, E.-E. (2006). Commission Regulation (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Off J Eur Union, 70*, 12–34.

Curtui, V. G., & Gareis, M. (2001). A simple HPLC method for the determination of the mycotoxins ochratoxin A and B in blood serum of swine. *Food Additives and Contaminants, 18*(7), 635–643. https://doi.org/10.1080/02652030118636

Curtui, V. G., Gareis, M., Usleber, E., & Märtlbauer, E. (2001). Survey of Romanian slaughtered pigs for the occurrence of mycotoxins ochratoxins A and B, and zearalenone. *Food Additives and Contaminants, 18*(8), 730–738. https://doi.org/10.1080/02652030116824

Dervilly-Pinel, G., Guérin, T., Minvielle, B., Travel, A., Normand, J., Bourin, M., et al. (2017). Micropollutants and chemical residues in organic and conventional meat. *Food Chemistry, 232*, 218–228. https://doi.org/10.1016/j.foodchem.2017.04.013

Dragacci, S., Grosso, F., Bire, R., Fremy, J. M., & Coulon, S. (1999). A French monitoring programme for determining ochratoxin A occurrence in pig kidneys. *Natural Toxins, 7*(4), 167–173. https://doi.org/10.1002/(SICI)1522-7189(199907/08)7:4<167::AID-NT55>3.0.CO;2-Q

Duarte, S. C., Lino, C. M., & Pena, A. (2012). Food safety implications of ochratoxin A in animal-derived food products. *The Veterinary Journal, 192*(3), 286–292. https://doi.org/10.1016/j.tvjl.2011.11.002

Duarte, S. C., Pena, A., & Lino, C. M. (2011). Human ochratoxin A biomarkers-From exposure to effect. *Critical Reviews in Toxicology, 41*(3), 187–2012. https://doi.org/10.3109/10408444.2010.529103

EC. (2006a). - European Commission. Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union, L 364*, 5–24.

EC. (2006b). Commision recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. *Official Journal of the Europen Union, 229*(L229), 7–9.

Edwards, J. (1988). The Roman Cookery of Apicius. *Century.*

EFSA. (2004). Opinion of the Scientific Panel on Contaminants in Food Chain on a request from the Commission related to ochratoxin A (OTA) as undesirable substance in animal feed. Request No EFSA-Q-2003-039. *EFSA Journal, 2*(10), 1–36. https://doi.org/10.2903/j.efsa.2004.101.

Efsa. (2011). Towards a harmonised Total Diet Study approach: A guidance document. *EFSA Journal, 9*(11), 2450. https://doi.org/10.2903/j.efsa.2011.2450

Efsa. (2012). Statement on the applicability of the Margin of Exposure approach for the safety assessment of impurities which are both genotoxic and carcinogenic in substances added to food/feed. *EFSA Journal, 10*(3), 2578. https://doi.org/10.2903/j.efsa.2012.2578

EFSA. (2015). *The food classification and description system FoodEx 2 (revision 2) | EFSA*. European Food Safety Authority. https://www.efsa.europa.eu/en/supporting/pub/en-804.

EFSA. (2018). The EFSA Comprehensive European Food Consumption Database. Last update: 26 April 2018. http://www.efsa.europa.eu/en/datexfoodcdb/datexfooddb.htm.

Efsa. (2020). Risk assessment of ochratoxin A in food. *EFSA Journal, 18*(5), 1–150. https://doi.org/10.2903/j.efsa.2020.6113

FAO. (2004). - *Food and Agriculture Organization. Worldwide regulations for mycotoxins in food and feed in 2003*. Food and Agriculture Organization of the United Nations.

Ferrara, M., Magistà, D., Lippolis, V., Cervellieri, S., Susca, A., & Perrone, G. (2016). Effect of Penicillium nordicum contamination rates on ochratoxin A accumulation in dry-cured salami. *Food Control, 67*. https://doi.org/10.1016/j.foodcont.2016.03.010

Fellendorf, S., OSullivan, M., & Kerry, J. (2016). Effect of different salt and fat levels on the physicochemical properties and sensory quality of black pudding. *Food Science & Nutrition, 5*(2), 237–284. https://doi.org/10.1002/fsn3.390.

Gallo, M., Ferrara, L., Calogero, A., Montesano, D., & Naviglio, D. (2020). Relationships between food and diseases: What to know to ensure food safety. *Food Research International, 137*, Article 109414.

Galtier, P., Alvinerie, M., & Charpenteau, J. L. (1981). The pharmacokinetic profiles of ochratoxin A in pigs, rabbits and chickens. *Food and Cosmetics Toxicology, 19*, 735–738. https://doi.org/10.1016/0015-6264(81)90528-9

Gareis, M., & Scheuer, R. (1999). Prevention of Mycotoxin Contamination of Meat and Meat Products. *JSM Mycotoxins, 1999*(Suppl2), 101–108. https://doi.org/10.2520/myco1975.1999.Suppl2_101

Gareis, M., & Scheuer, R. (2000). Ochratoxin A in meat and meat products. *Archiv Für Lebensmittelhygiene, 51*(4/5), 102–104.

Gašperlin, L., Skvarča, M., Žlender, B., Lušnic, M., & Polak, T. (2014). Quality Assessment of Slovenian *Krvavica*, A Traditional Blood Sausage: Sensory Evaluation: QUALITY ASSESSMENT OF SLOVENIAN *KRVAVICA*. *Journal of Food Processing and Preservation, 38*(1), 97–105. https://doi.org/10.1111/j.1745-4549.2012.00750.x

Giacomo, L., Michele, V., Guido, F., Danilo, M., Luigi, I., & Valentina, M. (2016). Determination of ochratoxin A in pig tissues using enzymatic digestion coupled with high-performance liquid chromatography with a fluorescence detector. *MethodsX, 3*, 171–177. https://doi.org/10.1016/j.mex.2016.03.006

González-Osnaya, L., Soriano, J. M., Moltó, J. C., & Mañes, J. (2007). Dietary intake of ochratoxin A from conventional and organic bread. *International Journal of Food Microbiology, 118*(1), 87–91. https://doi.org/10.1016/j.ijfoodmicro.2007.05.011

Guillamont, E. M., Lino, C. M., Baeta, M. L., Pena, A. S., Silveira, M. I. N., & Vinuesa, J. M. (2005). A comparative study of extraction apparatus in HPLC analysis of ochratoxin A in muscle. *Analytical and Bioanalytical Chemistry, 383*(4), 570–575. https://doi.org/10.1007/s00216-005-0051-4

Hassan, A. A., Abu Hafsa, S. H., Elghandour, M. M. M. Y., Kanth Reddy, P. R., Monroy, J. C., & Salem, A. Z. M. (2019). Dietary Supplementation with sodium bentonite and coumarin alleviates the toxicity of aflatoxin B1 in rabbits. *Toxicon, 171*, 35–42. https://doi.org/10.1016/j.toxicon.2019.09.014

Hort, V., Nicolas, M., Minvielle, B., Maleix, C., Desbourdes, C., Hommet, F., et al. (2018). Ochratoxin A determination in swine muscle and liver from French conventional or organic farming production systems. *Journal of Chromatography B, 1092*, 131–137. https://doi.org/10.1016/j.jchromb.2018.05.040

Hou, Y., Zhou, J., Li, Y., Xie, J., Zhou, L., & Lv, F. (2015). Determination of Ochratoxin A in Pig Kidneys by Immunoaffinity Cleanup and Ultra-High Performance Liquid Chromatography. *Journal of AOAC INTERNATIONAL, 98*(6), 1566–1570. https://doi.org/10.5740/jaoacint.15-170

IARC. (1993). - International Agency for Research on Cancer. IARC Monographs on the evaluation of carcinogenic risks to humans: Some naturally occuring substances: Food items and costituents, heterocyclic aromatic amines and mycotoxins (Vol. 56). IARC Press. http://monographs.iarc.fr/ENG/Monographs/vol56/mono56.pdf.

Ibáñez-Vea, M., González-Peñas, E., Lizarraga, E., & López de Cerain, A. (2012). Co-occurrence of aflatoxins, ochratoxin A and zearalenone in barley from a northern region of Spain. *Food Chemistry, 132*(1), 35–42. https://doi.org/10.1016/j.foodchem.2011.10.023

Jandásek, J. (2014). Seasonings in the production of cooked meat products. *Maso, 2014*(1), 137–145.

Jørgensen, K. (1998). Survey of pork, poultry, coffee, beer and pulses for ochratoxin A. *Food Additives & Contaminants, 15*(5), 550–554. https://doi.org/10.1080/02652039809374680

Jørgensen, K., & Petersen, A. (2002). Content of ochratoxin A in paired kidney and meat samples from healthy Danish slaughter pigs. *Food Additives and Contaminants, 19*(6), 562–567. https://doi.org/10.1080/02652030110113807

Kim, Y., Jang, H., Lim, S., & Hong, S. (2021). Effect of Starch Noodle (Dangmyeon) and Pork Intestines on the Rehydration Stability of Korean Blood Sausage (Sundae). *Food Science of Animal Resources, 41*(1), 153–163. https://doi.org/10.5851/kosfa.2020.e87

Kotowski, K., Grabarkiewicz-Szczesna, J., Waskiewicz, A., Kostecki, M., & Golinski, P. (2000). Ochratoxin A in porcine blood and in consumed feed samples. *Mycotoxin Research, 16*(2), 66–72. https://doi.org/10.1007/BF02946106

Krogh, P., Elling, F., Friis, C., Hald, B., Larsen, A. E., Lillehøj, E. B., et al. (1979). Porcine nephropathy induced by long-term ingestion of ochratoxin A. *Veterinary Pathology, 16*(4), 466–475.

Krogh, P., Elling, F., Hald, B., Larsen, A. E., Lillehoj, E. B., Madsen, A., et al. (1976). Time-dependent disappearance of ochratoxin A residues in tissues of bacon pigs. *Toxicology, 6*(2), 235–242.

Langseth, W., Nymoen, U., & Bergsjø, B. (1993). Ochratoxin A in plasma of Norwegian swine determined by an HPLC column-switching method. *Natural Toxins, 1*(4), 216–221. https://doi.org/10.1002/nt.2620010403

Liu, W.-C., Pushparaj, K., Meyyazhagan, A., Arumugam, V. A., Pappuswamy, M., Bhotla, H. K., et al. (2022). Ochratoxin A as an alarming health threat for livestock and human: A review on molecular interactions, mechanism of toxicity, detection, detoxification, and dietary prophylaxis. *Toxicon, 213*, 59–75. https://doi.org/10.1016/j.toxicon.2022.04.012

Luan, C., Wang, L., Chen, F., Wang, S., Zhao, L., & Shao, L. (2016). Determination of Ochratoxin A in Pig Muscle Using Dispersive Liquid-liquid Microextraction Combined with High-Performance Liquid Chromatography. *Food Analytical Methods, 9*(6), 1490–1494. https://doi.org/10.1007/s12161-015-0330-4

Lusky, K., Tesch, D., & Gobel, R. (1993). Influence of the mycotoxin ochratoxin-A on animal health and formation of residues in pigs and different types of sausages derived from these animals. *Archiv Fur Lebensmittelhygiene, 44*(6), 131–134.

Malagutti, L., Zannotti, M., Scampini, A., & Sciaraffia, F. (2005). Effects of Ochratoxin A on heavy pig production. *Animal Research, 54*(3), 179–184.

Malir, F., Ostry, V., & Novotna, E. (2013). Toxicity of the mycotoxin ochratoxin A in the light of recent data. *Toxin Reviews, 32*(2), 19–33. https://doi.org/10.3109/15569543.2013.782504

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Malir, J., & Toman, J. (2016). Ochratoxin A: 50 years of research. *Toxins, 8*(7), 191. https://doi.org/10.3390/toxins8070191

Marin, D. E., Tăranu, I., Tabuc, C., & Burgehelea, M. (2009). Ochratoxin: Nature, origin, metabolism and toxic effects in pigs. *Archiva Zootechnica, 12*(1), 5–17.

Marquardt, R. R., Frohlich, A. A., Sreemannarayana, O., Abramson, D., & Bernatsky, A. (1988). Ochratoxin A in blood from slaughter pigs in western Canada. *Canadian Journal of Veterinary Research, 52*(2), 186–190.

Matrella, R., Monaci, L., Milillo, M. A., Palmisano, F., & Tantillo, M. G. (2006). Ochratoxin A determination in paired kidneys and muscle samples from swines slaughtered in southern Italy. *Food Control, 17*(2), 114–117. https://doi.org/10.1016/j.foodcont.2004.08.008

Meats and Sausages. (2022). *Blood sausages*. Meats and Sausages. https://www.meatsandsausages.com/sausage-types/blood-sausage.

Meucci, V., Pistoia, A., Bertini, S., Menozzi, A., & Intorre, L. (2019). Natural occurrence of ochratoxin A in confined reared and grazing pigs derived products. *Large Animal Review, 25*(3), 95–99.

Milićević, D., Jurić, V., Stefanović, S., Jovanović, M., & Janković, S. (2008). Survey of Slaughtered Pigs for Occurrence of Ochratoxin A and Porcine Nephropathy in Serbia. *International Journal of Molecular Sciences, 9*(11), 2169–2183. https://doi.org/10.3390/ijms9112169

Ministero della Sanita. (1999). Circolare 9 giugno 1999, n.10. Direttive in materia di controllo ufficiale sui prodotti alimentari: Valori massimi ammissibili di micotossine nelle derrate alimentari di origine nazionale, comunitaria e Paesi terzi. (GU Serie Generale n.135 del 11-06-1999). Cazzeta Ufficiale Della Republic Italiana. http://www.micotossine.it/public/pag_92.pdf.

Mitchell, N. J., Chen, C., Palumbo, J. D., Bianchini, A., Cappozzo, J., Stratton, J., et al. (2017). A risk assessment of dietary Ochratoxin a in the United States. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 100*, 265–273. https://doi.org/10.1016/j.fct.2016.12.037

MoA. (2021). The Ministry of Agriculture of the Czech Republic: Current Discussed Topics in the Field of Contaminants in Food—December 2021. Informacni Centrum Bezpecnosti Potravin. https://www.bezpecnostpotravin.cz/aktualni-diskutovana-temata-v-oblasti-kontaminantu-v-potravinach-prosinec-2021.aspx.

Monaci, L., Tantillo, G., & Palmisano, F. (2004). Determination of ochratoxin A in pig tissues by liquid?liquid extraction and clean-up and high-performance liquid chromatography. *Analytical and Bioanalytical Chemistry, 378*(7), 1777–1782. https://doi.org/10.1007/s00216-004-2497-1

Ominski, K. H., Frohlich, A. A., Marquardt, R. R., Crow, G. H., & Abramson, D. (1996). The incidence and distribution of ochratoxin A in western Canadian swine. *Food Additives & Contaminants, 13*(2), 185–198. https://doi.org/10.1080/02652039609374397

Ostry, V. Skarkova, J., Malir, F. et al. (2011). *Ochratoxin A - health risk assessment for selected population groups in the Czech Republic* (Final report of the IGA project of the Ministry of Health of the Czech Republic č. NT/12051 – 3/2011; pp. 1–31). National Institute of Public Health in Prague, Center for Health , Nutrition and Food in Brno.

Ostry, V., Malir, F., & Ruprich, J. (2013). Producers and important dietary sources of ochratoxin A and citrinin. *Toxins, 5*(9), 1574–1586. https://doi.org/10.3390/toxins5091574

Ostry, V., Dofkova, M., Blahova, J., Malir, F., Kavrik, R., Rehurkova, I., et al. (2020). Dietary exposure assessment of sum deoxynivalenol forms, sum T-2/HT-2 toxins and zearalenone from cereal-based foods and beer. *Food and Chemical Toxicology, 139* (111280), 1–10. https://doi.org/10.1016/j.fct.2020.111280

Ostry, V., Smoldas, J., Rehakova, J., & Ruprich, J. (2021). Systematic instructions for decontamination of selected mycotoxins in laboratories. *Acta Hygienica Epidemiologica et Microbiologica, 5*, 1–45. https://doi.org/10.21101/ahem.a1011

Parussolo, G., Oliveira, M. S., Garcia, M. V., Bernardi, A. O., Lemos, J. G., Stefanello, A., et al. (2019). Ochratoxin A production by Aspergillus westerdijkiae in Italian-type salami. *Food Microbiology, 83*, 134–140. https://doi.org/10.1016/j.fm.2019.05.007

Persi, N., Pleadin, J., Kovacevic, D., Scortichini, G., & Milone, S. (2014). Ochratoxin A in raw materials and cooked meat products made from OTA-treated pigs. *Meat Science, 96*(1), 203–210. https://doi.org/10.1016/j.meatsci.2013.07.005

Pickova, D., Ostry, V., Malir, J., Toman, J., & Malir, F. (2020). A Review on Mycotoxins and Microfungi in Spices in the Light of the Last Five Years. *Toxins, 12*(12), 1–33. https://doi.org/10.3390/toxins12120789

Pickova, D., Toman, J., Ostry, V., & Malir, F. (2021). Natural Occurrence of Ochratoxin A in Spices Marketed in the Czech Republic during 2019–2020. *Foods, 10*(12), 2984. https://doi.org/10.3390/foods10122984

Pleadin, J., Kudumija, N., Kovačević, D., Scortichini, G., Milone, S., & Kmetič, I. (2016). Comparison of ochratoxin A levels in edible pig tissues and in biological fluids after exposure to a contaminated diet. *Mycotoxin Research, 32*(3), 145–151. https://doi.org/10.1007/s12550-016-0249-7

Pleadin, J., Lešić, T., Milićević, D., Markov, K., Šarkanj, B., Vahčić, N., et al. (2021). Pathways of mycotoxin occurrence in meat products: A review. *Processes, 9*(12), 2122. https://doi.org/10.3390/pr9122122

Pleadin, J., Perši, N., Kovačević, D., Vulić, A., Frece, J., & Markov, K. (2014). Ochratoxin A reduction in meat sausages using processing methods practiced in households. *Food Additives & Contaminants: Part B, 7*(4), 239–246. https://doi.org/10.1080/19393210.2014.900119

Pleadin, J., Staver, M. M., Vahčić, N., Kovačević, D., Milone, S., Saftić, L., et al. (2015). Survey of aflatoxin B 1 and ochratoxin A occurrence in traditional meat products coming from Croatian households and markets. *Food Control, 52*, 71–77. https://doi.org/10.1016/j.foodcont.2014.12.027

Pleadin, J., Zadravec, M., Lešić, T., Vahčić, N., Frece, J., Mitak, M., et al. (2018). Co-occurrence of ochratoxin A and citrinin in unprocessed cereals established during a three-year investigation period. *Food Additives & Contaminants: Part B, 11*(1), 20–25. https://doi.org/10.1080/19393210.2017.1389994

Polisenska, I., Pfohl-Leszkowicz, A., Hadjeba, K., Dohnal, V., Jirsa, O., Denesova, O., et al. (2010). Occurrence of ochratoxin A and citrinin in Czech cereals and comparison of two HPLC methods for ochratoxin A detection. *Food Additives & Contaminants: Part A, 27*(11), 1545–1557. https://doi.org/10.1080/19440049.2010.485580

Polovinski Horvatovic, M., Radovic, I., Glamocic, D., Jajic, I., Krstovic, S., Mirkov, M., et al. (2019). The occurrence of ochratoxin A in kidneys of healthy pigs from Vojvodina province, Serbia. *IOP Conference Series: Earth and Environmental Science, 333*(1), Article 012095. https://doi.org/10.1088/1755-1315/333/1/012095

Pozzo, L., Cavallarin, L., Nucera, D., Antoniazzi, S., & Schiavone, A. (2010). A survey of ochratoxin A contamination in feeds and sera from organic and standard swine farms in northwest Italy. *Journal of the Science of Food and Agriculture, 90*(9), 1467–1472. https://doi.org/10.1002/jsfa.3965

PubChem. (2021). PubChem. https://pubchem.ncbi.nlm.nih.gov/.

Raja, A. V., Saikumar, G., Sharma, R., & Dwivedi, P. (2008). Ochratoxicosis in swine: Clinical and pathological changes following prolonged exposure to ochratoxin A. *The Indian Journal of Animal Sciences, 78*(9), 922–928.

Ramos, D. D., Villalobos-Delgado, L. H., Cabeza, E. A., Caro, I., Fernández-Diez, A., & Mateo, J. (2013). Mineral composition of blood sausages–a two-case study. In *Muzzalupo, I. Food Industry* (pp. 93–107). In Tech.

Rodríguez, A., Rodríguez, M., Martín, A., Delgado, J., & Córdoba, J. (2012). Presence of ochratoxin A on the surface of dry-cured Iberian ham after initial fungal growth in the drying stage. *Meat Science, 92*(4), 728–734. https://doi.org/10.1016/j.meatsci.2012.06.029

Rossi, A., Sardi, L., Zaghini, A., & Rizzi, L. (2006). Mycotoxin contaminated diets in pig: In vivo and at slaughtering effects. *Rivista Di Suinicoltura (Italy), 47*(10), 131–134.

Ruprich, J., Dofkova, M., Rehurkova, I., Slamenikova, E., & Resova, D. (2004). Státní Zdravotní Ústav. Individuální Spotřeba Potravin v ČR - Národní Studie SISP04. http://www.szu.cz/tema/bezpecnost-potravin/individualni-spotreba-potravin-v-cr.

Santos, E. M., González-Fernández, C., Jaime, I., & Rovira, J. (2003). Physicochemical and sensory characterisation of Morcilla de Burgos, a traditional Spanish blood sausage. *Meat Science, 65*(2), 893–898. https://doi.org/10.1016/S0309-1740(02)00296-6

Sinclair, C. (2005). *Dictionary of Food International Food and Cooking Terms from A to Z* (2nd ed.). A & C Black Publishers Ltd.

Sreemannarayana, O., Frohlich, A. A., Vitti, T. G., Marquardt, R. R., & Abramson, D. (1988). Studies of the tolerance and disposition of ochratoxin A in young calves. *Journal of Animal Science, 66*(7), 1703–1711. https://doi.org/10.2527/jas1988.6671703x

Tam, J., Pantazopoulos, P., Scott, P. M., Moisey, J., Dabeka, R. W., & Richard, I. D. K. (2011). Application of isotope dilution mass spectrometry: Determination of ochratoxin A in the Canadian Total Diet Study. *Food Additives & Contaminants. Part A, Chemistry, Analysis, Control, Exposure & Risk Assessment, 28*(6), 754–761. https://doi.org/10.1080/19440049.2010.504750

Tangni, E. K., Masquelier, J., & Van Hoeck, E. (2021). Determination of ochratoxin A in edible pork offal: Intra-laboratory validation study and estimation of the daily intake via kidney consumption in Belgium. *Mycotoxin Research, 37*(1), 79–87. https://doi.org/10.1007/s12550-020-00415-7

Vlachou, M., Pexara, A., Solomakos, N., & Govaris, A. (2022). Ochratoxin A in slaughtered pigs and pork products. *Toxins, 14*(2), 67. https://doi.org/10.3390/toxins14020067

Walker, R. (2002). Risk Assessment of Ochratoxin: Current Views of the European Scientific Committee on Food, the Jecfa and the Codex Committee on Food Additives and Contaminants. In J. W. DeVries, M. W. Trucksess, & L. S. Jackson (Eds.), *Tricksess et al. Mycotoxins and Food Safety* (Vol. 504, pp. 249–255). Springer US. https://doi.org/10.1007/978-1-4615-0629-4_26.

Zaied, C., Abid, S., Zorgui, L., Bouaziz, C., Chouchane, S., Jomaa, M., et al. (2009). Natural occurrence of ochratoxin A in Tunisian cereals. *Food Control, 20*(3), 218–222. https://doi.org/10.1016/j.foodcont.2008.05.002

Zhao, Y., & Ambrose, R. P. K. (2017). Structural characteristics of sorghum kernel: Effects of temperature. *International Journal of Food Properties, 20*(11), 2630–2638. https://doi.org/10.1080/10942912.2016.1247099

Zhao, Z., Liu, N., Yang, L., Deng, Y., Wang, J., Song, S., et al. (2015). Multi-mycotoxin analysis of animal feed and animal-derived food using LC–MS/MS system with timed and highly selective reaction monitoring. *Analytical and Bioanalytical Chemistry, 407*(24), 7359–7368. https://doi.org/10.1007/s00216-015-8898-5

Zinedine, A., Juan, C., Idrissi, L., & Mañes, J. (2007). Occurrence of ochratoxin A in bread consumed in Morocco. *Microchemical Journal, 87*(2), 154–158. https://doi.org/10.1016/j.microc.2007.07.004

Supplementary materials

Table S1. International comparison of the composition of blood sausages

Country	Meal local name	Main ingredients	Side dish/serving
Bulgaria	Bahur	Blood of sheep, beef, or pork, animal trimmings, rice, onions, bay leaves, cumin, allspice, pork intestinal casing	Consumed cooked in warm form or cold form after their drying in a dry and cool place.
Croatia, Krpina-Zagorje County	Krvavica	Porcine blood, skin, offal, rice, buckwheat, barley, corn flour, animal intestinal casing	Consumed cooked and served with boiled potatoes, sauerkraut, and sautéed onions.
Czech Republic	Jelito	Porcine blood, ground pork meat from head and belly, offal, marjoram and cumin, grains, plain white buns, animal intestine casing	Consumed fried or baked, rarely cold, and most often served with sauerkraut and boiled potatoes, but bread is also common.
England	Black pudding	Porcine blood, oatmeal, and fat, animal intestine casing	Consumed fried/grilled/boiled, sliced or crumbled. It can be served with vinegar (in Manchester) or as a part of English breakfast (in other parts of the country).
Estonia	Verivorst	Blood, barley, onions, allspice, marjoram, animal intestine casing	Consumed roasted alongside potatoes and pork, butter, sour cream, sauerkraut, and lingonberry compote.
Finland, Tampere	Mustamakkara	Porcine blood, pork meat and fat, crushed rye and flour, animal intestine casing	Consumed with lingonberry jam.
France	Boudin noir aux pommes	Blood sausage with cooked apples, animal intestine casing	Consumed fried and served with chopped cooked apples.
Germany	Blutwurst	Porcine blood, pork fat and meat, marjoram, allspice, thyme, oats, bread, bacon, offal, animal intestine casing	Consumed boiled or fried and served warm alongside mashed potatoes or sauerkraut.
Germany	Zungenwurst	Porcine blood, fat, pieces of cooked tongue, ground pepper and other strong spices, animal intestine casing; Note: It is usually made of animal parts with no fillers or cereals.	Cured and then consumed without prior cooking.

Country	Meal local name	Main ingredients	Side dish/serving
Germany	Rotwurst	Well-seasoned blood sausage containing large chunks of meat and more pieces of fat and flour	Consumed thickly sliced cold or fried
Germany	Thüringer blutwurst	Pig blood, parboiled pork belly, pork rind, liver, offal, spices – marjoram, caraway, cloves, intestine.	Consumed cold or served warm with mashed potatoes and applesauce.
Ireland	Drisheen	Black pudding with milk, salt, blood of pig, cow, or sheep, fat, breadcrumb or oatmeal, animal intestine casing	Usually consumed warm and sliced. Together with herbs, it can also be filled into animal intestines and consumed as blood sausage.
Ireland, Timoleague	Timoleague Brown Pudding	Porcine blood, pork trimmings, onion, seasonings, spices, cereals, oatmeal soaked in blood overnight, animal intestinal casings	Boiled and, after cooling, sliced and consume.
Ireland, Sneem	Sneem black pudding	Blood pudding, beef suet, onions, oat, flake, spice, fresh blood from local pigs, lambs, or cows	Consumed baked/grilled/fried and served cut into 10mm thick slices.
Luxembourg	Träipen	Pork blood, ground pork head meat, lungs, kidneys, tongues or other pork offal, cabbage, onions, stale bread, salt, pepper, savoury, animal intestine casing	Consumed fried or baked and served with apple sauce.
Netherland	Bloedworst	Blood, raisins, oat bran, and pork fat	Consumed either fried or cold with a little mustard.
Poland	Kaszanka	Animal blood, pork offal, barley or buckwheat, spices and fresh herbs, animal intestine casing	Consumed grilled/fried/cooked and served alongside potatoes, sauerkraut, and caramelized onions.
Poland, Silesian Voivodeship	Żymlok	pork blood, ground pork offal, bread rolls soaked in broth, onions, marjoram, nutmeg, pepper, bay leaves, allspice, juniper berries, animal intestine casing	Can be consumed hot or chilled with bread and caramelized onions.
Poland, Silesian Voivodeship	Krupnioki śląskie	Porcine blood, smoked pork, buckwheat, salt, pepper, allspice, onions, garlic, marjoram, animal intestine casing	Consumed hot alongside bread, mustard, pickled vegetable, and a glass of Polish beer.
Portugal	Cacholeira	Pork, liver and other offals, fat, blood, spices – garlic, cumin, sweet paprika, pepper, animal casing. Additional ingredients such as wine are also possible.	Consumed as an appetizer. Or is used to enrich many typical Alentejan dishes.

Country	Meal local name	Main ingredients	Side dish/serving
Portugal	Morcela de assar	Porcine blood, pork fat, bread, onion, coriander, sugar, olive oil, and salt.	Consumed it is, roasted, fried or boiled.
Romania	Sângerete	Pork blood, ground pork meat, fat, boiled rice, garlic, thyme, allspice, coriander, nutmeg, animal intestine casing	Consumed cooked.
Scotland, Lewis and Harris	Stornoway Black Pudding	Blood of sheep, cow, or pig, beef suet, oatmeal, water, onions, salt, pepper, animal intestinal casings; Note. No other seasonings are allowed.	Consumed cooked.
Slovenia	Krvavica	Blood, offal - lungs, tongue, heart, and kidney, pork or beef, animal fat, lard or tallow, pork skin, cracklings, white bread, rice, barley, millet, buckwheat or corn, flour spices – black pepper, marjoram, allspice, cinnamon, cloves, cumin, thyme, nutmeg, and peppermint, roasted onions, rice and millet, animal casing	Consumed baked.
South Korea	Sundae sausage	Porcine blood, meat, rice, glass noodles, vegetable, barley, bean sprouts, kimchi, perilla leaves, soybean paste, scallions, animal intestine casing; Note: Squid or fish can be also included in some regional varieties.	Can be steamed and consumed on its own. It can be incorporated into various meals such as hearty sundaeguk soup.
Spain, Alicante	Botifarra de ceba	Animal blood, fat, onion, salt, pepper, pimentón, oregano, cloves, animal intestinal casing	Can be consumed fresh or fried/grilled/boiled.
Spain, Granada	Morcilla de Granada	Animal blood, pork belly, jowl, salt, oregano, paprika, animal casing	Consumed as a snack with asparagus and bread.
Spain, Province of Burgos	Morcilla de Burgos	Animal blood, animal fat – mainly lard and tallow, chopped and sautéined onion in butter, rice, spices – mainly black pepper and paprika, and cumin., animal intestinal casing; Note: No meat is included.	Can be consumed as it is, but it is mostly consumed after being sautéed or fried.
Sweden	Blodpudding, blodkorv	Porcine blood, flour, beer or svagdricka, butter, seasonings	Consumed cooked, sliced and fried with butter until crispy surface and served along with crispy bacon, lingonberry jam, pork fat, raisins,

Country	Meal local name	Main ingredients	Side dish/serving
			spices, potato cakes and Swedish snaps.
The United Kingdom and Ireland	Black pudding	Porcine blood and lean pork, groats, pork fat, chopped onion, salt, spices, herbs, mancu, oatmeals, filled into beef runners or wide hog casings.	Consumed sliced and usually fried and served along with bacon, breakfast sausages, eggs, beans and bread.
United States of America, Lousiana	Boudin rouge	Porcine blood, boiled ground pork meat, rice, strained pork stock, onions, dried seasonings, pork intestinal casings	Consumed boiled or steamed and served alongside mashed potatoes.

Summarized from Anjos et al., 2019; AtlasMedia Ltd., 2022; Belleggia et al., 2020; Fellendorf et al., 2016; Gašperlin et al., 2014; Kim et al., 2021; Santos et al., 2003; Sinclair, 2005.

Table S2. Sampled food and classification

Food sample	FoodEx2 name	FoodEx2 Code
Blood sausage	Blood-type sausage	A025S

Table S3 Natural OTA contamination of porcine ingredients used in blood sausages and related products

Country	Sample type	n+/n[1]	n+ (%)	Mean (ng/g)	Range (ng/g)	LOD[2] (ng/g)	LOQ[3] (ng/g)	Reference
Europe								
Belgium	Kidney	41/110	37	0.22	1.91[M]	0.06	0.2	(Tangni et al., 2021)
	Liver	0/20	0	-	-	0.06	0.2	
Czech Republic	Kidney	1/12	8	0.04[LB] 0.13[MB] 0.22[UB]	<LOQ-0.46*	0.1	0.3	(Ostry et al., 2011)
	Liver	0/12	0	-	-	0.1	0.3	
	Meat	1/12	8	0.02[LB] 0.11[MB] 0.20[UB]	<LOQ-0.20*	0.1	0.3	
Denmark	Meat conventional	64/76	84	0.11[LB(LOD)]	1.3[M]	0.02-0.03	N/S	(Jørgensen, 1998)
	Meat ecological	4/7	57	0.05[LB(LOD)]	0.12[M]	0.2-0.3	N/S	
	Kidney	230/300	77	0.5[LB(LOD)]	0-15	0.02	0.06	(Jørgensen & Petersen, 2002)
	Meat	67/300	22	0.12 [LB]	0-2.9	0.03	0.09	
France	Kidney	3/300	1	N/S	0.48-1.4	0.17-0.20	0-34-0.50	(Dragacci et al., 1999)
	Meat	4/96	4	N/S	0.2-1.15	0.03	0.10	(Dervilly-Pinel et al., 2017)
	Liver	25/70	36	0.15[LB] 0.18[UB]	<LOQ-3.65	0.03	0.10	(Hort et al., 2018)
Germany	Blood serum	93/191	49	5.8[+]	0.1-67.3	0.1	N/S	(Bauer & Gareis, 1987)
	Kidney	27/61	44	0.43	<LOD-9.33	0.01	N/S	(Gareis & Scheuer, 1999, 2000)
	Liver	10/59	17	0.07	<LOD-2.72	0.01	N/S	
	Meat	10/58	17	0.02	<LOD-0.14	0.01	N/S	
Italy	Kidney	42/54	78	N/S	0.26-3.05	0.14	0.52	(Monaci et al., 2004)
	Kidney	54/54	100	0.29	0.9[M]	0.01	N/S	(Matrella et al., 2006)
	Meat	42/54	78	0.02	N/S	0.01	N/S	
	Intestine	0/5	0	-	-	0.1	0.3	(Ceci et al., 2007)
	Kidney	N/S/5	N/S	25.6	23.9-27.5	0.1	0.3	
	Liver	N/S/5	N/S	4.4	3.2-5.3	0.1	0.3	
	Meat	0/5	0	-	-	0.1	0.3	
	Spleen	N/S/5	N/S	0.4	0.3-0.5	0.1	0.3	
	Stomach	0/5	0	-	-	0.1	0.3	
	Blood serum	285/285	100	N/S	0.03-6.24	0.03	0.1	(Pozzo et al., 2010)
	Kidney	5/5	100	0.37	0.17-0.91	0.001	0.002	(Giacomo et al., 2016)
	Liver	5/5	100	0.35	0.07-0.59	0.001	0.002	
	Meat	5/5	100	0.13	0.09-0.2	0.001	0.002	
Norway	Plasma	178/216	82	0.5[+]	12.5[M]	0.1	N/S	(Langseth et al., 1993)
Poland	Blood serum	26/45	58	N/S	0.3-69.5	2.0	N/S	(Kotowski et al., 2000)

Country	Sample type	n+/n[1]	n+ (%)	Mean (ng/g)	Range (ng/g)	LOD[2] (ng/g)	LOQ[3] (ng/g)	Reference
Portugal	Meat	1/13	8	0.01	0.12*	0.01	0.04	(Guillamont et al., 2005)
Romania	Blood serum	49/52	94	N/S	0.1-13.4	0.1	N/S	(Curtui & Gareis, 2001)
	Blood serum	51/52	98	2.43+	0.05-13.4	0.1	N/S	(Curtui et al., 2001)
	Kidney	41/52	79	0.54+	3.18M	0.01	N/S	
	Liver	39/52	75	0.16+	0.61M	0.01	N/S	
	Meat	9/52	17	0.15+	0.53M	0.01	N/S	
Serbia	Blood	28/90	31	3.7LB	0.22-220.8	0.1	N/S	(Milićević et al., 2008)
	Kidney	30/90	33	1.26LB	0.17-52.5	0.01	N/S	
	Liver	24/90	27	0.63LB	0.22-14.5	0.01	N/S	
	Kidney	14/95	15	1.36+	0.1-3.97	N/S	0.1	(Polovinski Horvatovic et al., 2019)
Americas								
Canada	Blood serum	910/1200	76	N//S	229M	N//S	N//S	(Marquardt et al., 1988)
	Blood serum	N/S/1588	36	5.1 14.1+	0.3-211	0.3	N/S	(Ominski et al., 1996)
Asia								
China	Fat	0/3	0	-	-	N/S	N/S	(Chen et al., 2012)
	Kidney	0/3	0	-	-	N/S	N/S	
	Liver	1/3	33	1.46*	1.46*	N/S	N/S	
	Meat	1/3	33	1.25*	1.25*	N/S	N/S	
	Kidney	35/40	88	N/S	0.102-0.323	0.03	0.1	(Hou et al., 2015)
	Liver	0/10	0	-	-	0.05	0.1	(Zhao et al., 2015)
	Meat	0/10	0	-	-	0.05	0.1	
	Meat	3/8	38	N/S	1.2-3.0	0.21	0.70	(Luan et al., 2016)
	Liver	0/5	0	-	-	0.07	0.25	(Cao et al., 2018)
	Muscle	1/5	20	0.88*	0.88*	0.07	0.25	
Africa								
Egypt	Kidney	10/10	100	N/S	7,51M	0.01	N/S	(Alarousy & Hakim, 2015)
	Liver	10/10	100	N/S	3,78M	0.01	N/S	
	Meat	10/10	100	N/S	6,19M	0.01	N/S	

*Notes: +, the mean value is based only on positive samples, LB, lower bound - concentrations below LOQ are regarded as zero, MB, middle bound - concentrations below LOD are regarded as ½ LOQ, UB, upper bound - concentrations below LOD are regarded as LOD, LB(LOD), concentrations below LOD are regarded as zero; M, the only maximum concentration is available; *, the only one positive sample*

Table S4 Expected consumption of blood sausages for the consumer only (g/kg bw/day)

Population group	Avg[1]	SD[2]	p50[3]	p95[4]	p99[5]
Children 4–6 years	0	0	0	0	0
Men 18–59 years	1.0091	0.661	0.917	2.000	2.154
Women 18–59 years	0.641	0	0	0	0
Men 60+	0	0	0	0	0
Women 60+	1.523	1.081	1.051	2.843	3.088
The whole population	1.150	0.771	0.976	2.576	3.035

[1] Avg, average; [2] SD, standard deviation; [3] p50, 50th percentile; [4] p50, 95th percentile; [5] p99, 99th percentile

Table S5 Ochratoxin A concentrations in blood sausage samples

no.	OTA (ng/g)	no.	OTA (ng/g)	no.	OTA (ng/g)	no.	OTA (ng/g)	no.	OTA (ng/g)
1	0.21	41	1.51	81	0.56	121	1.23	161	0.52
2	0.30	42	1.28	82	1.42	122	2.81	162	1.82
3	0.15	43	1.43	83	0.97	123	1.91	163	0.80
4	1.00	44	1.10	84	0.95	124	3.69	164	0.79
5	0.16	45	0.86	85	2.04	125	5.62	165	0.92
6	0.62	46	1.41	86	2.40	126	5.68	166	0.68
7	0.90	47	1.00	87	0.50	127	3.51	167	1.17
8	0.74	48	0.57	88	0.86	128	2.61	168	1.08
9	0.35	49	0.79	89	1.31	129	2.08	169	2.10
10	0.80	50	1.12	90	0.81	130	1.92	170	2.29
11	0.38	51	0.72	91	0.79	131	3.03	171	2.78
12	0.35	52	0.84	92	1.51	132	2.53	172	0.48
13	0.67	53	0.92	93	0.61	133	2.37	173	1.52
14	1.29	54	1.45	94	1.53	134	2.87	174	1.48
15	1.02	55	1.45	95	1.72	135	1.63	175	1.16
16	2.04	56	1.90	96	2.96	136	1.27	176	1.02
17	1.42	57	0.95	97	3.25	137	2.03	177	1.71
18	1.84	58	1.73	98	0.91	138	1.13	178	1.07
19	3.70	59	0.95	99	0.73	139	4.17	179	1.17
20	1.66	60	1.01	100	1.08	140	3.03	180	0.90
21	1.19	61	0.96	101	0.31	141	2.30	181	2.03
22	0.63	62	1.77	102	0.89	142	3.39	182	1.36
23	1.07	63	1.04	103	1.30	143	1.32	183	1.55
24	1.78	64	1.55	104	2.00	144	1.97	184	1.31
25	0.18	65	0.78	105	0.91	145	1.15	185	1.74
26	0.73	66	1.76	106	1.59	146	1.24	186	1.49
27	0.84	67	1.03	107	1.51	147	0.88	187	1.94
28	0.52	68	0.48	108	0.75	148	1.03	188	1.85
29	0.68	69	2.43	109	1.08	149	0.78	189	2.60
30	0.35	70	2.76	110	0.92	150	3.09	190	1.20
31	0.80	71	2.20	111	1.88	151	1.59	191	1.02
32	0.73	72	2.93	112	2.39	152	1.15	192	1.56

no.	OTA (ng/g)	no.	OTA (ng/g)	no.	OTA (ng/g)	no.	OTA (ng/g)	no.	OTA (ng/g)
33	0.85	73	0.57	113	2.14	153	1.67	193	1.35
34	1.11	74	2.30	114	1.66	154	0.83	194	1.48
35	0.38	75	1.86	115	4.56	155	3.31	195	1.51
36	0.84	76	1.93	116	3.03	156	0.93	196	0.68
37	1.26	77	0.66	117	2.22	157	0.21	197	1.33
38	1.26	78	1.21	118	1.64	158	0.73	198	1.30
39	1.19	79	1.27	119	1.46	159	1.02	199	1.06
40	1.02	80	3.29	120	2.20	160	1.67	200	0.54

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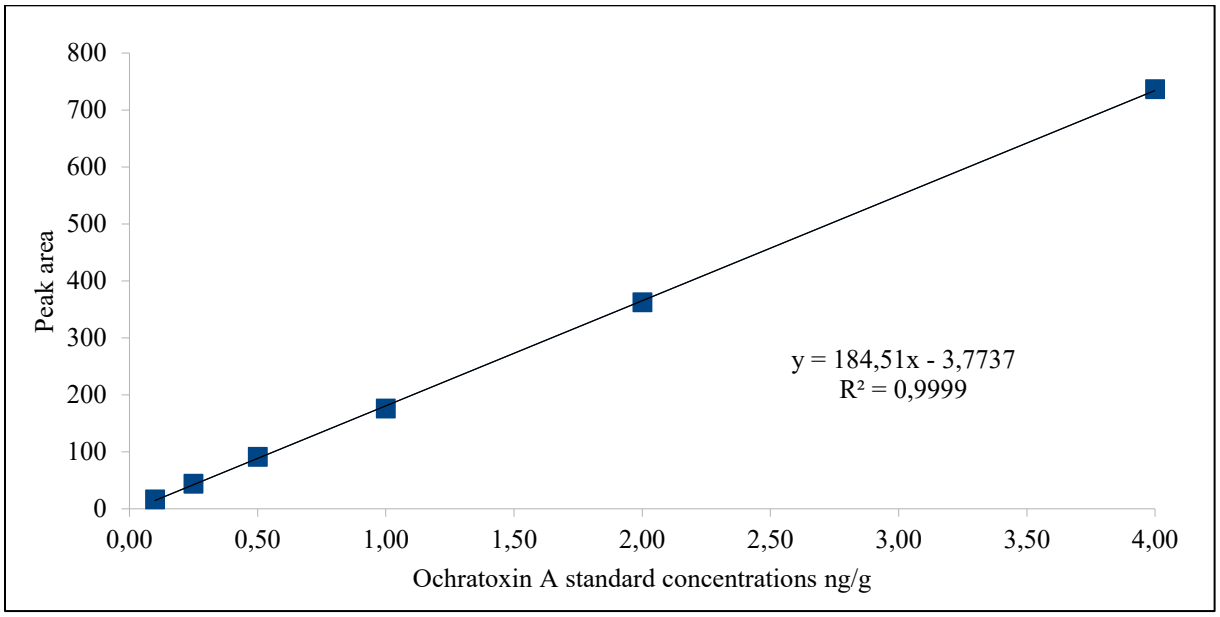
Figure S1 Ochratoxin A standard calibration curve



Příloha 7

Analyses of biomarkers of exposure to nephrotoxic mycotoxins in a cohort of patients with renal tumours

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ORIGINAL ARTICLE

Analyses of biomarkers of exposure to nephrotoxic mycotoxins in a cohort of patients with renal tumours

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Abstract

The Czech Republic occupies the first place in the world in the frequency of renal and other urinary tract tumours, but their aetiology is unknown. To explore whether carcinogenic and nephrotoxic mycotoxins may contribute to kidney diseases in the Czech population, biomarkers of ochratoxin A (OTA) and citrinin (CIT) exposure were determined in biological specimens from a cohort of 50 patients with malignant renal tumours. Biomarker analyses in blood and urine samples used validated targeted methods for measuring OTA and CIT plus dihydrocitrinone (DH-CIT) after enrichment of analytes by specific immunoaffinity clean-up. OTA and CIT plus its metabolite DH-CIT were frequently detected in patient urine samples (OTA 62%; CIT 91%; DH-CIT 100%). The concentration ranges in urine were 1–27.8 ng/L for OTA, 2–87 ng/L for CIT and 2–160 ng/L for DH-CIT. The analyses of blood samples revealed also a frequent co-occurrence of OTA and CIT, in the ranges of 40–870 ng/L serum for OTA and 21–182 ng/L plasma for CIT. This first analysis of biomarkers in blood and urine samples of Czech patients revealed no major differences in comparison with published data for the general healthy Czech and European populations. Nonetheless, a frequent co-occurrence of CIT and OTA biomarkers in patient samples may be of interest with regard to potential interactions with other risk factors for renal disease.

Keywords Ochratoxin A · Citrinin · Dihydrocitrinone · Biomarkers · Renal carcinogenicity

Introduction

Based on epidemiological information on malignant diseases, the incidence in the Czech Republic of renal tumours and other urinary tract tumours is very high in comparison with other countries (e.g. Germany, see Robert Koch-Institut 2017). The incidence of malignant neoplasms of the kidney and of renal pelvis and ureter (diagnosis C64–C66) is 29.5 renal tumours/100,000 inhabitants of the Czech Republic (Dusek et al. 2017). The tumours diagnosed are mainly renal carcinoma/adenocarcinoma arising from the proximal tubule cells (clear cell renal cell carcinoma, ccRCC, formerly called Grawitz tumour) and papillary renal carcinoma of the renal pelvis (Tesar et al. 2015). Balkan endemic nephropathy (BEN), a unique chronic renal disease, is often associated with upper urinary tract tumours arising from the urothelium

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(Grollman and Jelakovic 2007). Factors suspected of inducing this disease are nephrotoxins, primarily mycotoxins and aristolochic acid, metals and metalloids as well as possibly an individual genetic predisposition (Pfohl-Leszkowicz et al. 2002; Peraica et al. 2008; Pfohl-Leszkowicz 2009; Stiborova et al. 2016; Chan et al. 2018).

Different factors can play a role in the incidence of ccRCCs and other kidney tumours, e.g. hypertension and obesity (Sanfilippo et al. 2014), lifestyle and health behaviours such as physical activity, alcohol consumption and smoking (Chow et al. 2010) but also genetic factors (Reaume et al. 2013; Schmidt and Linehan 2016). Other, mainly occupational risk factors for renal cancer include high exposure to cadmium, lead and solder fumes as well as paints, mineral oils, cutting fluids, benzene, polycyclic aromatic hydrocarbons, trichloroethylene and tetrachloroethylene (Pesch et al. 2000; Radford et al. 2013). In short, the aetiology of renal cancer is not well understood, and the impact of various risk factors on disease may vary in different cohorts. In a Czech cohort of patients with kidney tumours (main diagnosis C64), we have studied their exposure to carcinogenic and nephrotoxic mycotoxins, namely ochratoxin A (OTA) and citrinin (CIT). The contamination of food commodities with OTA is quite frequent in Europe including the Czech Republic (EFSA 2006; Ostry et al. 2013; Ostry et al. 2015), whereas available data on CIT levels in food and feed is rather scarce (EFSA 2012).

Since CIT has similar toxic properties as OTA, their co-occurrence has raised concerns regarding possible combined effects on animals and humans, in particular porcine nephropathy and BEN (Pfohl-Leszkowicz et al. 2002; Pfohl-Leszkowicz et al. 2008; Peraica et al. 2008; Ostry et al. 2013). An experimental co-administration of CIT and OTA in rodents or in vitro can increase OTA-DNA adducts in kidney (Manderville and Pfohl-Leszkowicz 2008; Pfohl-Leszkowicz et al. 2008) and also oxidative DNA damage (Segvic-Klaric et al. 2013). Depending on doses and the relative proportion of CIT and OTA, either antagonism or synergy has been observed (Pfohl-Leszkowicz et al. 2008; Föllmann et al. 2014). Thus, it is of interest to assess the exposure to both mycotoxins in humans.

A valuable tool to investigate human exposure to mycotoxins is biomonitoring, i.e. the analysis of parent compounds and/or metabolites in biological fluids such as blood, urine or breast milk samples. It has served to study exposure to mycotoxins in different countries and cohorts, and to study the success of intervention measures aimed to reduce dietary intake (Duarte et al. 2011; Turner et al. 2012). Analysis by means of suitable biomarkers of exposure is considered to be the preferable tool for human exposure assessment as it covers mycotoxin intake from all sources and routes, and better reflects the individual exposure situation, toxicokinetics and bioavailability (Duarte et al. 2011; Malir et al. 2012; Fromme et al. 2016).

In this study, biomarkers of OTA and CIT exposure have been determined in 50 patients with renal tumours from the Czech Republic. We applied validated specific methods for analyses of OTA, CIT and its metabolite dihydrocitrinone (DH-CIT) in blood and urine samples collected prior to surgery. The structures of the analytes are depicted in Fig. 1. Their levels in the patient cohort are compared with those of healthy populations to explore if exposure to nephrotoxic mycotoxins may be a contributing factor to the high frequency of renal tumours observed in the Czech Republic. This first biomarker analyses in Czech tumour patients revealed current mycotoxin exposures well below the tolerable daily intake values for OTA and CIT. The results will be also discussed in relation to remaining uncertainties such as potential risks from past exposure, not reflected in our present study, or combined exposures.

Materials and methods

Chemicals and materials

Glacial acetic acid, hydrochloric acid, *ortho*-phosphoric acid 85%, magnesium sulphate hexahydrate, sodium chloride, anhydrous sodium acetate (all in p.a. purity), methanol and acetonitrile (both gradient grade for HPLC) were obtained from Merck KGaA (Prague, Czech Republic), and acetic acid (96%) from Merck KGaA (Darmstadt, Germany). Acetonitrile and methanol (LC-MS grade) were from Promochem (Wesel, Germany). Chloroform and sodium hydrogen carbonate (both p.a.) were purchased from Riedel-de Haen (Prague, Czech Republic). OTA standard material (1 mg, purity > 98%) and phosphate-buffered saline of pH 7.4 were obtained from Sigma-Aldrich (Prague, Czech Republic). Ultrapure water was prepared by Milli-Q Plus (Millipore, Billerica, MA, USA).

OTA was dissolved in methanol and spectrophotometrically calibrated at 333 nm using the molar extinction coefficient (ε) of 6400 (Reinhard and Zimmerli 1999). A basic OTA solution was prepared by dissolving 1 mg of OTA in 5 mL

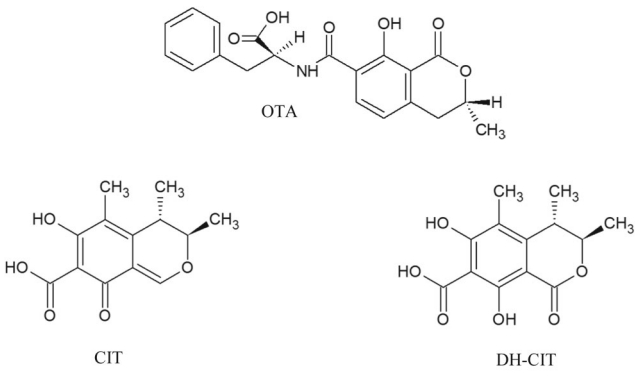

Fig. 1 Structural formulas of the analytes

of methanol, and an OTA stock standard solution (40 ng/mL) was prepared by further dilution in methanol. The working standard solutions of OTA in a range of 0.1–20.0 ng/mL were prepared weekly from the OTA stock solution as dilutions in the mobile phase (methanol/acetonitrile/0.005 mol/L sodium acetate/acetic acid, 300/300/400/14, *v*/*v*/*v*/*v*).

CIT standard material (5 mg, purity > 98%) was from Sigma-Aldrich (Taufkirchen, Germany); CIT stock solution in methanol was calibrated at 321 nm using the absorptivity $\varepsilon = 5490$ (Reinhard and Zimmerli 1999). The CIT metabolite DH-CIT (purity 98.9%) was obtained from AnalytiCon Discovery GmbH (Potsdam, Germany). CIT and DH-CIT working standard solutions were prepared weekly from their stock solutions (CIT, 200 μg/mL acetonitrile; DH-CIT, 500 μg/mL acetonitrile) by dilution in methanol in a range of 1–20 ng/mL (1000–20,000 ng/L).

Cohort and collection of samples

Biological samples (blood and urine) were collected from November 2015 to April 2017 from 50 randomly selected patients (a group of 39 men and 11 women) with diagnosis of kidney cancer and aged between 40 and 81 years (see Table 1). For the purposes of this study, on the basis of the previous standard laboratory and medical examinations, only those patients without overall metabolic disruption and cardiovascular complications were randomly selected. Blood and urine samples for this study were collected just before surgical operation as part of standard clinical sampling so that patients were not excessively burdened (one of the conditions of approval by the Ethics Commission). Samples of urine were collected from the patients in all standard preoperative examinations, starting from their arrival at the Department of Urology until the morning of the next day when the operation was performed. Blood samples were divided in three containers from Sarstedt: (i) two S-Monovettes (2.7 mL, K_2EDTA—for obtaining optimally about 4 mL of plasma overall) were centrifuged (at 3000 rpm, approximately about 1620×*g*) for 15 min for separation of plasma using a B4i Jouan (France) centrifuge, and then the separated plasma was placed into a vial; (ii) another container marked S-Monovette (5.5 mL Z, Clotting Activator/serum) was used for obtaining the serum, and then the sample of separated serum was placed into a vial. Urine samples were collected into a non-sterile container (1.5 L) stored in the refrigerator. Each urine sample was mixed, and from it approximately 100 mL of urine after centrifugation (at 3000 rpm) was placed into a polypropylene container, and these samples were immediately stored at − 80 °C until analysis. All samples were sent anonymously (using a numerical code) to the laboratories. Personal data on gender, the year of birth and the body weight of the patient were recorded.

OTA extraction, detection and quantification

Prior to detection and quantification of OTA in serum and OTA in urine, aliquoted samples of 3 mL of acidified blood serum (Zimmerli and Dick 1995; Dohnal et al. 2013) and 20 mL of filtered urine sample (Ostry et al. 2010) were extracted and cleaned on Ochraprep® R immunoaffinity columns (Biopharm Rhone Ltd., Great Britain).

As in previous studies of other cohorts (Malir et al. 2006; Ostry et al. 2010; Dohnal et al. 2013), the validated and accredited method (CSN EN ISO/IEC 17025) of reversed-phase high-performance liquid chromatography with fluorescence detection was employed for purposes of OTA detection and quantification. OTA was analysed on a liquid chromatograph consisting of a vacuum degasser SCM400, gradient pump P2000, autosampler AS 3000 (all from Spectra System, USA), fluorescence detector 920 FP (Jasco, Japan) and Solvent Saver 2907 (Jour Research, Sweden) coupled with the analytical column Inertsil ODS-3V (5 μm × 150 mm × 4.6 mm; Hichrom Ltd., UK) with a guard column (3.0 × 4.0 mm filled with C_{18} material of particle size 5 μm, Phenomenex, USA) and—for calculations and evaluations—equipped with a computer and CSW 32 (DataApex, Prague, Czech Republic) software. OTA fluorescence was measured at an excitation wavelength of 333 nm, and an emission wavelength of 465 nm for serum analysis or 443 nm for urine analysis. The injection volumes were 50 μL for serum samples and 100 μL for urine samples. The mobile phase for OTA analysis consisted of methanol/acetonitrile/0.005 mol/L sodium acetate/acetic acid (300/300/400/14, *v*/*v*/*v*/*v*). The flow rate was 1.5 mL/min (Dohnal et al. 2013). Under these chromatographic conditions, the retention time of OTA for serum samples was about 7.1 min and for urine samples about 7.9 min. For OTA in serum, the LOD was 40 ng/L and LOQ was 100 ng/L. For OTA in the urine, the LOD was 1.0 ng/L and LOQ was 2 ng/L. The linear calibration curve was constructed by measurement of OTA peak areas of standard solutions in mobile phase with concentrations of 0.125 to 4.000 ng OTA/mL for serum analysis, and of 0.1 to 20 ng OTA/L for urine analysis. Blank samples were mobile phases. Each point of the calibration curve was measured in triplicate. The recoveries for OTA were 82–86% in the range of 0.5–1.0 μg/L (500–1000 ng/L) in spiked blood serum samples and 92.6–85.1% in the range of 20–50 ng/L in spiked urine samples. The average relative standard deviations of repeatability (RSD_r) for OTA were 4.5% at 0.5 ng/mL (500 ng/L) and 1.5% at 1 ng/mL (1000 ng/L) for serum and 4.2% at 20 ng/L and 2% at 50 ng/L for urine.

Table 1 Characteristics of male and female Czech patients with diagnosis of kidney cancer

Characteristics of patients	Subgroup of men	Subgroup of women	Whole cohort
Subject-gender (*n*)	39	11	50
Age (years)			
Mean ± SD	65 ± 10	58 ± 12	63 ± 11
Range	40–81	40–74	40–81
Weight of patients (kg)			
Mean ± SD	93 ± 11	80 ± 31	90 ± 18
Range	68–118	57–155	57–155
Area of residence			
Rural	6	1	7
Urban	33	10	43
Diagnosis			
Malignant neoplasm of kidney, outside renal pelvis (C64)	38	10	48
Malignant neoplasm on urine bladder neck (C675)	1	–	1
No-malignant neoplasm of kidney (D410)	–	1	1
Urinary parameters			
Creatinine (mg/L)*			
Mean ± SD	794 ± 592	701 ± 353	773 ± 547
Range**	100–3290	260–1236	100–3290
pH of urine			
Mean ± SD	6.04 ± 0.54	5.89 ± 0.50	6.01 ± 0.53
Range	5.03–7.08	5.38–7.13	5.03–7.13
Volume of urine (mL)			
Mean ±SD	444 ± 64	459 ± 131	447 ± 82
Range	252–560	110–586	110–586

Biomarker values determined in patient urines (see Tables below) are expressed both as concentration (ng/L), and also adjusted for urine creatinine content

* Range of creatinine reference value in healthy adults: 500–2500 mg/L (Kommission Humanbiomonitoring des Umweltbundesamtes 2005)

** In one case i.e. 2% from the whole cohort, urinary creatinine was > 2500 mg/L and in 15 cases i.e. 30% of the cohort it was < 500 mg/L, i.e. higher or lower than the range of creatinine reference values in healthy adults (Kommission Humanbiomonitoring des Umweltbundesamtes 2005)

CIT and DH-CIT extraction, detection and quantification

CIT and DH-CIT in urine were analysed by the validated method of Blaszkewicz et al. (2013) after extraction and cleanup with CitriTest® columns (Vicam provided by Ruttmann, Hamburg, Germany), with minor modifications. A mixture of 5 mL urine diluted with 5 mL of 1 mM acetic acid was loaded on a CitriTest® column; see Ali et al. (2015a, 2015b). CIT and DH-CIT in plasma were analysed by the method of Blaskewicz et al. (2013) and Ali et al. (2018). In short, for protein precipitation, 1 mL plasma was mixed with 1 mL acetonitrile (1/1, *v*/*v*) and then centrifuged at 9800×*g* for 3 min; 1 mL of the upper layer was transferred into a vial and evaporated to dryness under a gentle stream of nitrogen at a temperature of 40 °C. Then, the sample was reconstituted in 350 μL methanol, vortexed, and centrifuged at 9800×*g* for 3 min; the extract was filtered through a 0.45-μm pore size Teflon syringe filter (WICOM, Germany) before LC-MS/MS analysis.

Detection of CIT and DH-CIT was performed with a Varian 1200L Quadrupole MS/MS equipped with an electrospray ionization (ESI) source and a Prostar® Varian HPLC system and Varian MS Workstation version 6.9.1 data system (Agilent Technologies, Germany) after separation on a Nucleosil® 100-5 C18 HD column (125 × 3 mm, Macherey-Nagel, Düren, Germany). The mobile phase consisted of water containing 1 mmol/L ammonium formate and methanol containing 1 mmol/L ammonium formate. Instrumental settings and chromatographic conditions were identical with those used before for urine and plasma extract analyses (Ali et al. 2015a, 2015b; Ali et al. 2018). A gradient elution was performed, and the retention times of CIT and DH-CIT were 9.3 and 8.7 min, respectively.

The method was validated using spiked blank urine and plasma samples. Recoveries were 79% and 82% in the urine, and 82% and 84% in plasma, for CIT and DH-CIT, respectively. The LOD and LOQ were determined by an external calibration curve in the urine and plasma matrix. The LOD and LOQ were 0.02 ng/mL (20 ng/L) and 0.05 ng/mL (50 ng/L) for CIT, and those for DH-CIT were 0.05 ng/mL (50 ng/L) and 0.1 ng/mL (100 ng/L) in the urine matrix. In plasma samples, the LODs of CIT and DH-CIT were 0.07 ng/mL (70 ng/L) and 0.15 ng/mL (150 ng/L), respectively, and their LOQs were 0.15 ng/mL (150 ng/L) and 0.30 ng/mL (300 ng/L), respectively. Reproducibility was determined by inter-day assays on three different days at a level of 0.25 ng/mL (250 ng/L) in urine and 0.5 ng/mL (500 ng/L) in plasma for the analytes; the RSD_r range was 4.2 to 7.4% for the analytes. Calibration curves for quantification were done by spiking urine and plasma matrix that showed no detectable analyte background.

Creatinine analysis in urines

Urinary creatinine was determined by the Jaffe reaction method with alkaline picrate at a wavelength of 520 nm using the spectrometer Cintra 101 (GBC Scientific Equipment Ltd., Australia). Creatinine levels were controlled by Lyphochek® Quantitative Urine Control, levels 1 and 2 (Bio-Rad, Prague, Czech Republic). Levels of OTA, CIT and DH-CIT in urines (ng/L) were then adjusted for creatinine content and expressed as nanograms per gram of creatinine.

OTA exposure calculation

The average OTA daily intake in patients with malignant neoplasms was calculated on the basis of OTA serum concentrations as done in previous assessments (e.g. Märtlbauer et al. 2009; Coronel et al. 2009; Duarte et al. 2011) by means of the Klaassen equation:

$K_0 = Cl_p \times C_p/A$ and in the version $K_0 = 0.99 \times C_p/0.5 = 1.97$where K_0 is the continuous dietary intake (ng/kg b.w./day), Cl_p is the plasma clearance (0.99 mL/kg b.w./day), C_p is the plasma OTA concentration (ng/mL) and A is the toxin bioavailability, estimated at 50%. We opted also to use the more conservative conversion factor of 1.97 (Miraglia et al. 1996), since this version of the Klaassen equation has resulted in a better match of biomarker-based intake assessments for OTA with estimates based on food analysis data (Märtlbauer et al. 2009).

$$\text{OTA intake (ng/kg b.w.)} \cong 1.97 \times C_{OTA},$$

where C_{OTA} is the OTA concentration measured in serum (ng/mL).

CIT exposure calculation

For CIT exposure assessment, the urine concentrations for both CIT and its main metabolite DH-CIT were summed up ("total CIT") for each individual and then converted to CIT intake. It was calculated as follows (Degen et al. 2018):

$$PDI(\mu g/kg\ b.w./day) = C/W*V*100/fraction\ excreted\ in\ urine\ in\%,$$

where PDI is the provisional daily intake, C is the concentration measured in the urine sample, W is the standard body weight of 70 kg (EFSA 2012) and V is the average daily urine volume of 1.6 L excreted by adults. As for the assumption for V, one favours a higher i.e. conservative intake estimate, although the urine volume excreted by patients who are kept from drinking or eating 12 h before surgery was lower. The fraction excreted in the urine is the percentage of an oral CIT dose found in the urine within 24 h and set here to 40%, the median value in the study of Degen et al. (2018).

Statistical analysis

Obtained data were processed using the universal statistical software Statistica version 11 (StatSoft). The results as mean ± standard deviation, median and ranges are presented on the basis of descriptive analysis. The samples with OTA concentrations below the limit of detection (LOD) were calculated as one-half the detection limit for calculation of mean and median values (Hornung and Reed 1990).

Results

Biomarkers in blood and urine samples: OTA, CIT and DH-CIT

OTA was frequently detected in body fluids (serum 48%; urine 62%) from the patient cohort. OTA amounts measured in blood serum and urine are summarized in Table 2. The OTA serum concentrations ranged from 40 ng/L (LOD) to 830 ng/L, with a mean value 145 ± 213.8 ng/L, median 20 ng/L. OTA concentrations in the corresponding urines ranged from 1 ng/L (LOD) to 27.8 ng/L, with a mean value for all urines of 5.9 ± 5.97 ng/L, median 5.4 ng/L. OTA concentrations in both matrices are higher in the male than the smaller subgroup of female patients (Table 2), yet the differences did not reach statistical significance. This is also the case for creatinine-adjusted OTA levels in urine.

Determining OTA in blood and urine is very useful for a comparison with published data from other cohorts. Yet, biomarker analyses data in the two matrices of an individual are

Table 2 Serum and urinary concentrations of OTA in the Czech patient cohort

			Serum			Urine			
		n	Positive *n* (%)	Mean ± SD (ng/L)	Median (range) (ng/L)	Positive *n* (%)	Mean ± SD (ng/L)	Median (range) (ng/L)	Mean ± SD (ng/g creatinine)
OTA	Men	39	21 (54)	175.8 ± 232.8	47.3 (*nd*–830)	24 (62)	6.53 ± 6.41	6.41 (*nd*–27.8)	8.0 ± 6.0
	Women	11	3 (27)	35.7 ± 35.1	20.0 (*nd*–112.8)	7 (64)	3.66 ± 3.44	2.74 (*nd*–10.0)	5.0 ± 4.0
	All	50	24 (48)	145 ± 213.8	20.0 (*nd*–830)	31(62)	5.9 ± 5.97	5.41 (*nd*–27.8)	7.0 ± 6.0

Note: positive sample refers to urines containing the analyte ≥ the limit of detection (LOD). LOD of OTA in blood was 40 ng/L of serum and in urine 1.0 ng/L. Samples that contained analyte levels below LOD were assigned a value of one half of the LOD for calculation of mean and median values. See also individual data in Fig. 2 and Fig. 3

nd level below LOD

not necessarily strictly correlated with each other due to the rather complex kinetics of OTA in the human organisms. Figure 2 and Fig. 3 depict the OTA concentrations determined in serum and urine samples of all individuals. Patients with higher OTA serum amounts generally excreted more OTA aglycone, the unconjugated OTA form (which was analysed) in urine than those with lower circulating amounts of OTA.

In blood and urine of the same cohort, CIT is detected in almost all patients. Table 3 summarizes the data. The CIT plasma concentrations of all 50 Czech patients ranged from 20 (LOD) to 182 ng/L, median of 51 ng/L, a mean value 61 ± 35 ng/L and similar concentrations in both subgroups of men and women. Due to a high LOD for DH-CIT in plasma (200 ng/L), the metabolite was not detected in any of the patient blood samples. CIT concentrations in urine ranged from 2 (LOD) to 87 ng/L, with a mean value for all patients of 16 ± 20 ng/L, median 8 ng/L and higher mean concentrations for the metabolite DH-CIT of 48 ± 35 ng/L, median 38.5 ng/L (for 100% of samples in a range of 6 to 160 ng/L), with similar amounts in males and females (see Table 3).

Figures 4 and 5 illustrate the variability in biomarker concentrations among this cohort for biomarkers measured in plasma and in urine samples of individuals. The biomarker pattern in both matrices, with higher CIT concentrations in blood than in urine of patients, and higher urinary levels of DH-CIT than CIT, resembles that observed in reference cohorts.

Exposure assessments for OTA and CIT

These data have been used to estimate the exposure to OTA and CIT of the patients before surgery, and have been compared to the tolerable intake (TDI) for OTA and CIT. Using the

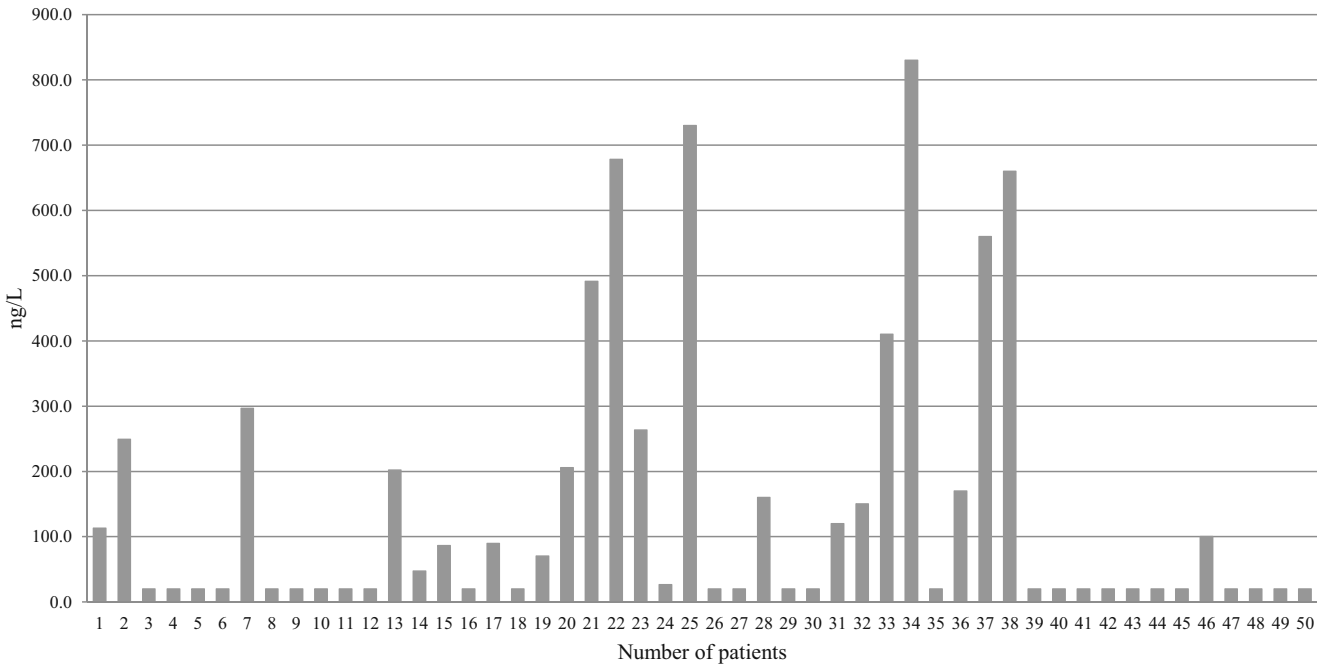

Fig. 2 OTA in blood serum samples (ng/L) of individual Czech patients

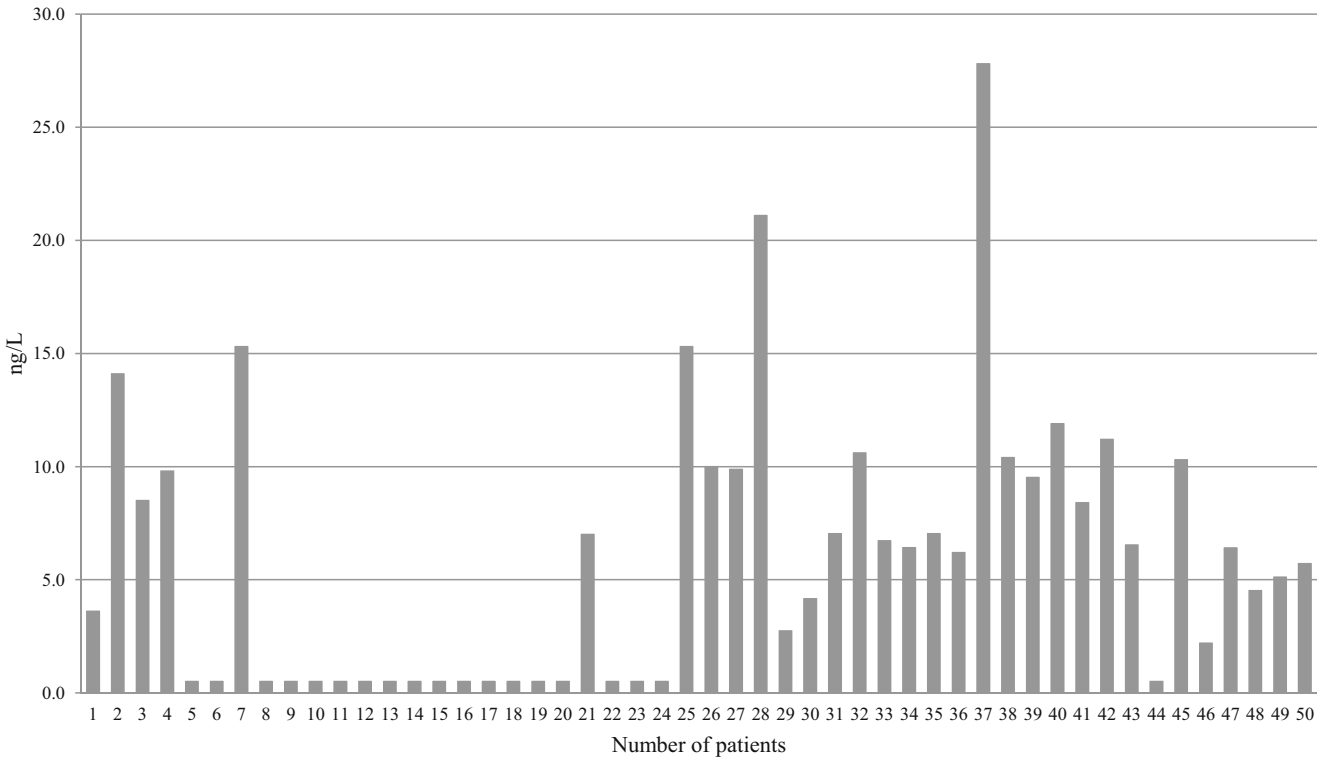

Fig. 3 OTA in urine samples (ng/L) of individual Czech patients

OTA serum concentrations of Czech patients, we calculate a probable daily intake, using a conversion factor of 1.97 for the Klaassen equation as applied in previous exposure assessments for this mycotoxin. The mean blood OTA concentrations of 145 ± 214 pg/mL (or ng/L) correspond to a mean daily dietary OTA intake for the entire cohort of 286 ± 421 pg/kg b.w/day (i.e. 0.29 ± 0.42 ng/kg b.w./day). This daily intake is lower than the TDI value which was set by several regulatory bodies for OTA.

For CIT exposure, the urine concentrations for CIT and its main metabolite DH-CIT were summed up ("Total CIT") for each individual (see Fig. 4) and then converted to CIT intake as explained in "Materials and methods". The probable daily CIT intake for the cohort is 3.5 ± 2.3 ng/kg b.w./day, with no

Table 3 Plasma and urinary concentrations of CIT and its metabolite DH-CIT Positive

			Plasma			Urine			
			Positive n (%)	Mean ± SD (ng/L)	Median (range) (ng/L)	Positive n (%)	Mean ± SD (ng/L)	Median (range) (ng/L)	Mean ± SD (ng/g creatinine)
CIT	Men	39	39 (100)	63 ± 32	58 (21–170)	35 (90)	17 ± 21	9 (6–87)	24.0 ± 23.0
	Women	11	10 (91)	53 ± 46	45 (*nd*–182)	10 (91)	10 ± 11	6 (*nd*–31)	14.0 ± 12.0
	All	50	49 (98)	61 ± 35	51 (*nd*–182)	45 (90)	16 ± 20	8 (*nd*–87)	22.0 ± 21.0
DH–CIT	Men	39	*nd*			39 (100)	48 ± 35	70 (6–160)	87.0 ± 85.0
	Women	11	*nd*	–	–	11 (100)	49 ± 30	39 (6–114)	74.0 ± 37.0
	All	50	*nd*			50 (100)	48 ± 34	38 (6–160)	84.0 ± 77.0
Total CIT	Men	39	*NC*	–	–	35 (90)	66 ± 42	62 (*nd*–206)	111.0 ± 91.0
	Women	11	*NC*	–	–	10 (91)	59 ± 40	41 (*nd*–145)	88.0 ± 45.0
	All	50	*NC*	–	–	45 (90)	64 ± 42	57 (*nd*–206)	106.0 ± 83.0

Positive sample refer to urines containing the analyte ≥ limit of detection (LOD). Samples with analyte levels below LOD were assigned a value of one half the LOD for calculation of mean and median values. The LOD in urine were 0.002 ng/mL (2.0 ng/L) for both CIT and its metabolite; in plasma the LOD for CIT was 0.02 ng/mL (20 ng/L), but for DH-CIT the LOD was 0.2 ng/mL (200 ng/L). Thus, the total CIT (sum of CIT plus DH-CIT) was not calculated (NC) for plasma, but for urines; see also individual data in Fig. 4 and Fig. 5

nd level below LOD

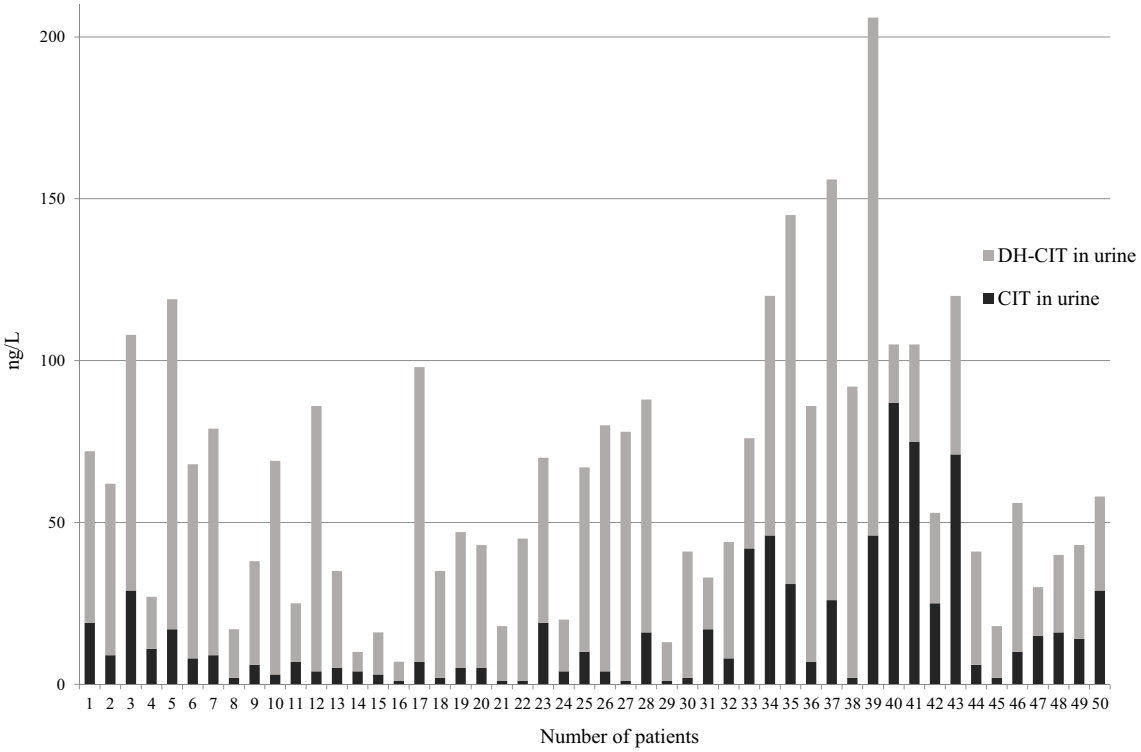

Fig. 4 CIT and DH-CIT in urine samples (ng/L) of individual Czech patients

significant difference between males and females. The estimated exposure is far lower than the "level of no concern for nephrotoxicity" (a provisional TDI) derived by EFSA (2012) for this mycotoxin.

Comparisons with healthy populations

Results of this first biomarker analysis for nephrotoxic mycotoxins in Czech patients with renal tumours are compared with

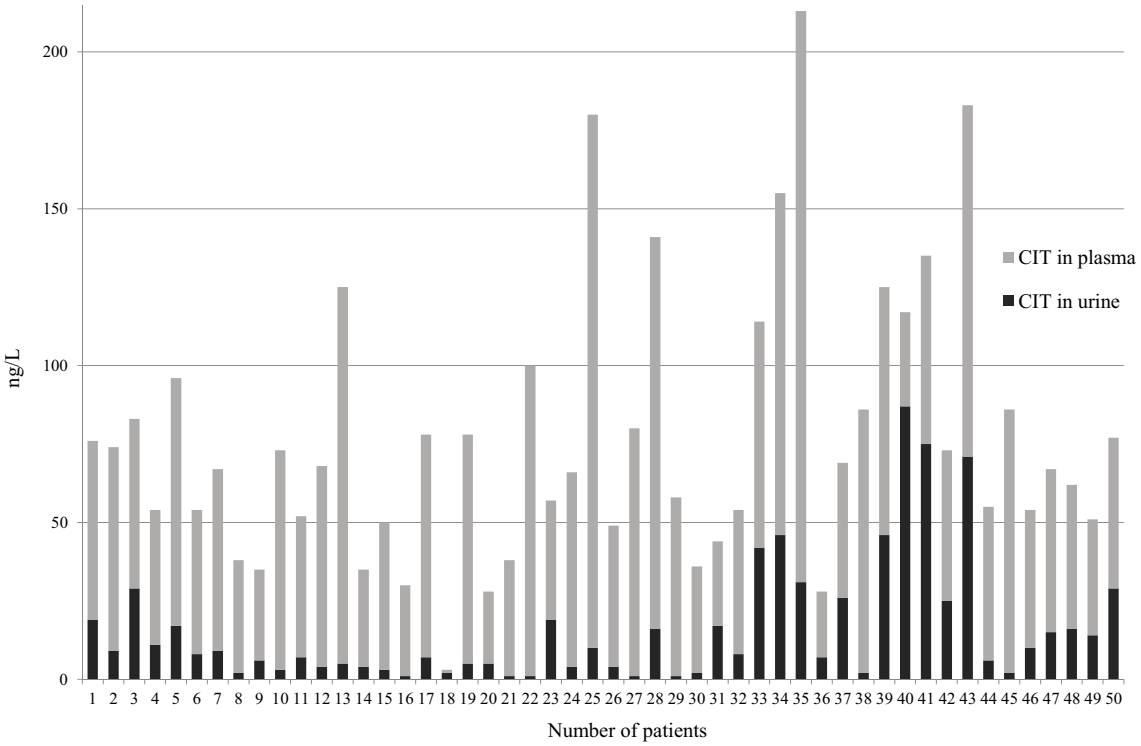

Fig. 5 CIT biomarkers in blood plasma and urine samples (ng/L) of individual Czech patients

data for OTA and CIT biomonitoring in reference cohorts; more details on the latter studies are provided in "Discussion".

OTA concentrations both in urine and in serum of Czech patients in this study and the group representing the Czech healthy population—reference cohort (236 examined individuals; male/female, 45–60 years old, blood donors) (Ostry et al. 2010)—were compared by the independent sample *t* test. No statistically significant difference was observed ($p > 0.05$).

Comparisons of CIT and DH-CIT urinary concentrations were made by independent sample *t* test between Czech patients and those reported in a cohort of German healthy adults (23 males, 27 females) (Ali et al. 2015a), because similar data were not available in a healthy cohort in the Czech Republic. CIT and DH-CIT concentrations in urine of Czech patients were statistically significantly lower ($p < 0.05$) compared with the German control group, but no significant difference was observed in their blood levels.

Discussion

The aim of this collaborative study was to estimate the exposure of kidney cancer patients (clinical diagnoses: C64–C66) to two nephrotoxic mycotoxins (OTA and CIT) by sensitive targeted biomarker analyses in both blood and urine samples. As sample collection was made in hospital before surgery, the measured OTA and CIT biomarkers reflect only recent exposure but not the past exposure. Yet, analysis of paired blood and urine samples from patients allows to consider biomarker levels in both matrices and compare the outcome to studies in non-diseased cohorts (e.g. MacDonald et al. 2001; Ostry et al. 2010; Ali et al. 2015a). Table 4 summarizes data from some studies that used the same or very similar methodology as applied in the present investigation.

OTA biomarker concentrations in Czech kidney tumour patients and in other cohorts

For the Czech patients, the mean OTA serum concentration and the range are similar to those found in a large survey of serum samples obtained between 1994 and 2002 from healthy Czech blood donors (Malir et al. 2006), and from another more recent survey on healthy Czech persons (Ostry et al. 2010). The data for Czech adults are also similar to OTA values found in a large German study (Rosner et al. 2000) and a retrospective study of 102 serum samples from 36 healthy persons (Märtlbauer et al. 2009). Higher OTA mean blood plasma levels and ranges than in the Czech and German adults have been reported in a 1-month diet duplicate study carried out in the UK some years ago (MacDonald et al. 2001). This study analysed also unconjugated OTA in 24-h urines collected by the volunteers. OTA concentrations in urine were far lower than those measured in the blood plasma, yet showed a good correlation with dietary OTA intake of UK adults (Gilbert et al. 2001; MacDonald et al. 2001).

OTA levels now determined in urines from 50 Czech patients are close to the mean concentration and range were found previously for the healthy Czech population (Ostry et al. 2010). In the patient cohort, OTA concentrations in urine were also much lower than in blood. This is in line with data from the UK (Table 4) and further biomonitoring data in healthy persons (reviewed in Fromme et al. 2016; Malir et al. 2016; Ali et al. 2017).

The fact that both blood and urinary OTA levels in patients suffering from kidney cancer are not significantly different from the healthy Czech population or lower than in healthy persons from other countries is in line with the present knowledge on OTA kinetics. Only a small fraction of the circulating OTA is excreted in a given time, due to its pronounced binding to serum proteins (about 99%) which hinders its glomerular filtration (Gekle et al. 2005). The free (unbound) OTA fraction is filtered, but reabsorption of the non-ionized form along all nephron segments delays its elimination (Castegnaro et al. 2006; Ringot et al. 2006). The fraction of OTA bound to serum proteins constitutes a mobile OTA reserve that can be released as soon as the fraction of free OTA decreases, e.g. when more polar metabolites are formed and excreted (Pfohl-Leszkowicz and Manderville 2007; Ali et al. 2017). An increase in OTA blood concentrations after high intake can be compensated by increasing urinary OTA excretion, which brings the OTA concentration back to the former steady-state level in blood (Castegnaro et al. 2006; Pfohl-Leszkowicz et al. 2006). In patients with impaired renal function and decreased filtration capacity, one would expect an increase in OTA concentration in blood compared to healthy persons with a similar OTA intake (Duarte et al. 2011). Yet, this was not the case, except for 30% of patients having a low creatinine excretion, probably because the steady state was not reached.

Estimation of the OTA intake before surgery, based on OTA blood concentrations, is low with about 0.3 ng/kg b.w./day: This is about 10-fold lower than the most conservative limit value of 4 ng/kg b.w./day proposed by Health Canada for a negligible cancer risk intake (Kuiper-Goodman et al. 2010) and 50 times lower than the tolerable weekly intake (TWI) of 120 ng/kg b.w. proposed by EFSA (2006). OTA biomarker analysis in urine seems to better reflect short-term variations in OTA exposure of adults and children (Gilbert et al. 2001; Castegnaro et al. 2006; Muñoz et al. 2014). Whilst urines can be obtained by non-invasive sampling, one must keep in mind that only a very small fraction of the ingested OTA is excreted with urine, less than 3% within a day (Studer-Rohr et al. 2000; Degen 2016). Based on this information, the estimated daily intake is about 2.37 ng/kg b.w./day, which corresponds to half of the most conservative limit value for cancer risk.

Table 4 Comparison of biomarker levels in Czech patient cohort and some reference cohorts

Cohort (samples; year of collection) (*reference*)	OTA		CIT		DH-CIT
	Serum	Urine	Plasma Mean ± SD (range) (ng/L)	Urine	Urine
Czech kidney tumour patients ($n = 50$; 2015–2017) *this study*	145 ± 214 (nd–830)	5.9 ± 6 (nd–27.7)	61 ± 35 (20–182)	16 ± 20 (nd–87)	48 ± 34 (6–160)
Czech blood donors ($n = 2206$; 1994–2002) Malir et al. 2006	280 (nd–13,700)	Not analysed	Not analysed	Not analysed	Not analysed
Czech adults healthy volunteers ($n = 236$; 2007–2008) Ostry et al. 2010	180 ± 146 (nd–660)	7.3 ± 6.5 (nd–27.8)	Not analysed	Not analysed	Not analysed
Czech non-pregnant women ($n = 115$; 2012) Dohnal et al. 2013	165 (50–1130)	Not analysed	Not analysed	Not analysed	Not analysed
German adults ($n = 102$; 1990–1997) *Märtlbauer* et al. 2009	368 ± 217 (50–1290)	Not analysed	Not analysed	Not analysed	Not analysed
German adults ($n = 927$; 1996–1998) Rosner et al. 2000	270 (60–2030)	Not analysed	Not analysed	Not analysed	Not analysed
German adults ($n = 8$; 2010) Blaszkewicz et al. 2013	– (210–1500)	Not analysed	– (110–260)	–	–
German adults ($n = 50$; 2013) *Ali* et al. 2015a, 2015b	Not analysed	Not analysed	Not analysed	30 ± 20 (20–80)	100 ± 100 (50–510)
UK adults (n = 50; 1997) MacDonald et al. 2001	1090 (*plasma*) (400–3111)	21 (10–58)	Not analysed	Not analysed	Not analysed

nd level below LOD or LOQ

Due to limited financial sources, the diet of the patients was not tested for OTA and CIT. But the dietary regimen (which can influence OTA and CIT levels) of all patients was assessed on the basis of a special questionnaire. There were no apparent differences in the consumption of OTA-containing foodstuffs in comparison to the referent Czech population (Ostry et al. 2015). As the average calculated OTA intake in the Czech Republic was about 3.9 ng/kg b.w./day (Ostry et al. 2015), the above urine OTA-based intake estimate is in accord with this value.

CIT biomarker concentrations in Czech kidney tumour patients and in other cohorts

In contrast with rather good databases on the dietary intake of certain mycotoxins such as aflatoxins or OTA, data on the occurrence of CIT in food commodities are still too limited to reliably estimate human exposure (EFSA 2012). Hence, there is a need to assess human exposure to CIT to enable a better characterization of related risks, e.g. by biomarker-based analysis of its intake (Degen et al. 2018). Recent studies that applied targeted methods for detection of CIT biomarkers revealed quite frequent exposure to this food contaminant in cohorts from different countries and also concurrent exposure to OTA (Pfohl-Leszkowicz 2009; Ali et al. 2015a; Ali et al. 2016a, 2016b; Ali et al. 2018). Yet, with biomarker analysis for CIT being more recently established, there are only few data compared to OTA data in European cohorts (Table 4).

The new results in plasma and urine samples from the Czech patients confirm dietary exposure to CIT. CIT was detected in 98% of blood plasma from 50 Czech patients in a similar, yet somewhat lower range of concentrations than those found in some healthy German volunteers (Blaszkewicz et al. 2013). Also in urines of the Czech patients, their CIT and DH-CIT levels indicate a lower exposure to this mycotoxin than in a cohort of healthy German adults (Ali et al. 2015a). This pertains to unadjusted concentrations (ng/L) as well as creatinine-adjusted biomarker levels in Czech and German urine samples. Average CIT biomarker levels in urines of Czech patients are significantly ($p < 0.05$) lower than those of German adults, but no significant difference exists between blood samples. The biomarker pattern in paired samples, with higher CIT concentrations in blood than in urine of patients, and higher urinary levels of DH-CIT than CIT, is similar to reference cohorts (Ali et al. 2015a, 2015b; Ali et al. 2018). A recent kinetic study in volunteers found that a high fraction (about 40%) of an ingested dose of CIT is excreted in the urine as the sum of CIT and its metabolite DH-CIT ('total CIT') within a day (Degen et al. 2018). This allows to estimate human dietary CIT exposure based on urine biomarker data, and compare it with the provisional tolerable daily intake ('TDI') proposed by the EFSA (2012). The CIT daily intake derived from urine analyses of the Czech patients

is 3.5 ± 2.3 ng/kg bw/day; this corresponds to 1.8 ± 1.1% of the 'TDI' set for this mycotoxin whereas the German exposure is slightly higher, but nevertheless represents only 3.7 ± 3.0% of the 'TDI' (Degen et al. 2018).

Concluding remarks: Overall, the presented data indicate a frequent, but low dietary exposure to CIT and OTA, although we cannot exclude that higher exposures to nephrotoxic mycotoxins may have occurred in previous years in the Czech patient cohort. Biomarker-based intake estimates for CIT and OTA are well below the respective health-based guidance values. Thus, we consider combinatory effects, found in rodents or in vitro at rather high mycotoxin doses (see "Introduction"), to be of low concern for the present human exposure scenario. Due to the short half-life of CIT in human blood (about 9 h), CIT is unlikely to accumulate in the organism (Degen et al. 2018). In contrast, OTA has a rather long half-life in human blood (about 35 days) (Studer-Rohr et al. 2000), and accumulates in the kidneys. As discussed elsewhere, there is the possibility of OTA uptake in proximal tubules leading to accumulation in kidney target cells (Gekle et al. 2005; Mally 2012). An analysis of OTA in renal adenocarcinoma samples is thus of interest, also regarding the OTA ratio between kidney and serum. Tissue samples of the Czech tumour patient cohort could also provide a chance for tracing changes related to past exposures and for further research on other factors involved in this multifactorial disease.

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Compliance with ethical standards

The study design was in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Conflict of interest The authors confirm that there are no conflicts of interest associated with this publication.

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Ethical approval Approval for the biomonitoring study protocol was obtained from the Ethics Commission of the University Teaching Hospital, Sokolska 581, CZ–500 05 Hradec Kralove (Reg. No. 201406 S02P). Written informed consent was obtained from all patients included in the study.

References

Ali N, Blaszkewicz M, Degen GH (2015a) Occurrence of the mycotoxin citrinin and its metabolite dihydrocitrinone in urines of German adults. Arch Toxicol 89:573–578. https://doi.org/10.1007/s00204-014-1363-y

Ali N, Blaszkewicz M, Mohanto NC, Rahman M, Alim A, Hossain K, Degen GH (2015b) First results on analysis of citrinin biomarkers in urines from two cohorts in Bangladesh. Mycotoxin Res 31:9–16. https://doi.org/10.1007/s12550-014-0217-z

Ali N, Blaszkewicz M, Alim A, Hossain K, Degen GH (2016a) Urinary biomarkers of ochratoxin A and citrinin exposure in two Bangladeshi cohorts: follow-up study on regional and seasonal influences. Arch Toxicol 90:2683–2697. https://doi.org/10.1007/s00204-015-1654-y

Ali N, Blaszkewicz M, Manirujjaman M, Degen GH (2016b) Biomonitoring of concurrent exposure to ochratoxin A and citrinin in pregnant women in Bangladesh. Mycotoxin Res 32:163–172. https://doi.org/10.1007/s12550-016-0251-0

Ali N, Muñoz K, Degen GH (2017) Ochratoxin A and its metabolites in urines of German adults – an assessment of variables in biomarker analysis. Toxicol Lett 275:19–26. https://doi.org/10.1016/j.toxlet.2017.04.013

Ali N, Hossain K, Degen GH (2018) Blood plasma biomarkers of citrinin and ochratoxin A exposure in young adults in Bangladesh. Mycotoxin Res 34:59–67. https://doi.org/10.1007/s12550-017-0299-5

Blaszkewicz M, Muñoz K, Degen GH (2013) Methods for analysis of citrinin in human blood and urine. Arch Toxicol 87:1087–1094. https://doi.org/10.1007/s00204-013-1010-z

Castegnaro M, Canadas D, Vrabcheva T, Petkova-Bocharova T, Chernozemsky I, Pfohl-Leszkowicz A (2006) Balkan endemic nephropathy: role of ochratoxins A through biomarkers. Mol Nutr Food Res 50:519–529. https://doi.org/10.1002/mnfr.200500182

Chan C-K, Liu Y, Pavlovic NM, Chan W (2018) Etiology of Balkan endemic nephropathy: an update on aristolochic acids exposure mechanisms. Chem Res Toxicol 31:1109–1110. https://doi.org/10.1021/acs.chemrestox.8b00291

Chow WH, Dong LM, Devesa SS (2010) Epidemiology and risk factors for kidney cancer. Nat Rev Urol 7:245–257. https://doi.org/10.1038/nrurol.2010.46

Coronel MB, Sanchiz A, Ramos J, Marin S (2009) Assessment of the exposure to ochratoxin A in the province of Lleida, Spain. Food Chem Toxicol 47:2847–2852. https://doi.org/10.1016/j.fct.2009.09.005

Degen GH (2016) Are we ready to estimate daily ochratoxin A intake based on urinary concentrations? Environ Int 97:254–255. https://doi.org/10.1016/j.envint.2015.10.010

Degen GH, Ali N, Gundert-Remy U (2018) Preliminary data on citrinin kinetics in humans and their use to estimate citrinin exposure based on biomarkers. Toxicol Lett 282:43–48. https://doi.org/10.1016/j.toxlet.2017.10.006

Dohnal V, Dvorak V, Malir F, Ostry V, Roubal T (2013) A comparison of ELISA and HPLC methods for determination of ochratoxin A in human blood serum in the Czech Republic. Food Chem Toxicol 62:427–431. https://doi.org/10.1016/j.fct.2013.09.010

Duarte SC, Pena A, Lino CM (2011) Human ochratoxin A biomarkers–from exposure to effect. Crit Rev Toxicol 41:187–212. https://doi.org/10.3109/10408444.2010.529103

Dusek L, Muzik J, Kubasek M, Koptikova J, Zaloudik J, Vyzula R (2017) Epidemiology of malignant tumours in the Czech Republic. Available online: http://www.svod.cz/?sec=aktuality&lang=en (accessed on 29 June 2018)

European Food Safety Authority [EFSA] (2006) Opinion of the scientific panel on contaminants in the food chain on request from the commission related to ochratoxin A in food. EFSA J 365:1–56

European Food Safety Authority [EFSA] (2012) Scientific opinion on the risks for public and animal health related to the presence of citrinin in food and feed. EFSA J 10:2605–2687 Available online: www.efsa.europa.eu/efsajournal (accessed on 25 June 2018)

Föllmann W, Behm C, Degen GH (2014) Toxicity of the mycotoxin citrinin and its metabolite dihydrocitrinone and mixtures of citrinin and ochratoxin A in vitro. Arch Toxicol 88:1097–1107. https://doi.org/10.1007/s00204-014-1216-8

Fromme H, Gareis M, Völkel W, Gottschalk C (2016) Overall internal exposure to mycotoxins and their occurrence in occupational and residential settings. Int J Hyg Environ Health 219(2):143–165. https://doi.org/10.1016/j.ijheh.2015.11.004

Gekle M, Sauvant C, Schwerdt G (2005) Ochratoxin A at nanomolar concentrations: a signal modulator in renal cells. Mol Nutr Food Res 49:118–130. https://doi.org/10.1002/mnfr.200400062

Gilbert J, Brereton P, MacDonald S (2001) Assessment of dietary exposure to ochratoxin A in the UK using a duplicate diet approach and analysis of urine and plasma samples. Food Addit Contam 18:1088–1093. https://doi.org/10.1080/02652030110070030

Grollman AP, Jelakovic B (2007) Role of environmental toxins in endemic (Balkan) nephropathy. J Am Soc Nephrol 18:2817–2823. https://doi.org/10.1681/ASN.2007050537

Hornung RW, Reed LD (1990) Estimation of average concentration in the presence of nondetectable values. Appl Occup Environ Hyg 5:46–51. https://doi.org/10.1080/1047322X.1990.10389587

Koch-Institut R (2017) Krebs in Deutschland für 2013/2014. Robert Koch-Institut, Berlin available online: https://www.krebsdaten.de/Krebs/DE/Content/Publikationen/Krebs_in_Deutschland/krebs_in_deutschland_node.html

Kommission Humanbiomonitoring des Umweltbundesamtes (2005) Normierung von Stoffgehalten in Urin - Kreatinin. Bundesgesundheitsbl Gesundheitsforsch Gesundheitsschutz 48:616–618. https://doi.org/10.1007/s00103-005-1029-2

Kuiper-Goodman T, Hilts C, Billiard SM, Kiparissis Y, Richard ID, Hayward S (2010) Health risk assessment of ochratoxin A for all age-sex strata in a market economy. Food Addit Contam Part A 27:212–240. https://doi.org/10.1080/02652030903013278

MacDonald SJ, Langton S, Brereton PA (2001) Assessment of human exposure to ochratoxin A in the UK-relationship between dietary intake and plasma and urine levels. In: de Koe WJ, Samson RA, van Egmond HP, Gilbert J, Sabino M (eds) Mycotoxins and Phycotoxins in Perspective at the Turn of the Millenium. Ponsen and Looyen, Wageningen, pp 181–188 ISBN 90–9014801-9

Malir F, Ostry V, Grosse Y, Roubal T, Skarkova J, Ruprich J (2006) Monitoring the mycotoxins in food and their biomarkers in the Czech Republic. Mol Nutr Food Res 50:513–518. https://doi.org/10.1002/mnfr.200500175

Malir F, Ostry V, Pfohl-Leszkowicz A, Roubal T (2012) Ochratoxin A exposure biomarkers in the Czech Republic and comparison with foreign countries. Biomarkers 17:577–589. https://doi.org/10.3109/1354750X.2012.692392

Malir F, Ostry V, Pfohl-Leszkowicz A, Malir J, Toman J (2016) Ochratoxin A: 50 years of research. Toxins 8:1–49. https://doi.org/10.3390/toxins8070191

Mally A (2012) Ochratoxin A and mitotic disruption: mode of action analysis of renal tumor formation by ochratoxin A. Toxicol Sci 127:315–330. https://doi.org/10.1093/toxsci/kfs105

Manderville RA, Pfohl-Leszkowicz A (2008) Bioactivation and DNA adduction as a rationale for ochratoxin A carcinogenesis. World Mycotoxin J 1:357–367. https://doi.org/10.3920/WMJ2008.x039

Märtlbauer E, Usleber E, Dietrich R, Schneider E (2009) Ochratoxin A in human blood serum- retrospective long-term data. Mycotoxin Res 25:75–186. https://doi.org/10.1007/s12550-009-0025-z

Miraglia M, Brera C, Colatosti M (1996) Application of biomarkers to assessment of risk to human health from exposure to mycotoxins. Microchem J 54:472–477. https://doi.org/10.1006/mchj.1996.0124

Muñoz K, Blaszkewicz M, Campos V, Vega M, Degen GH (2014) Exposure of infants to ochratoxin A with breast milk. Arch Toxicol 88:837–846. https://doi.org/10.1007/s00204-013-1168-4

Ostry V, Skarkova J, Malir F, Ruprich J (2010) An occurrence of ochratoxin A, a biomarker of dietary exposure, in human biological materials. In: Mykotoxíny 2010, 14–15 October 2010. Prague, Czech Republic. Proceedings, 32–40. ISBN: 978-80-7080-764-4

Ostry V, Malir F, Ruprich J (2013) Producers and important dietary sources of ochratoxin A and citrinin. Toxins 5:1574–1586. https://doi.org/10.3390/toxins5091574

Ostry V, Malir F, Dofkova M, Skarkova J, Pfohl-Leszkowicz A, Ruprich J (2015) Ochratoxin A dietary exposure of ten population groups in the Czech Republic: comparison with data over the world. Toxins 7:3608–3635. https://doi.org/10.3390/toxins7093608

Peraica M, Domijan AM, Sarić M (2008) Mycotoxic and aristolochic acid theories of the development of endemic nephropathy. Arh Hig Rada Toksikol 59:59–65. https://doi.org/10.2478/10004-1254-59-2008-1865

Pesch B, Haerting J, Ranft U, Klimpel A, Oelschlägel B, Schill W (2000) Occupational risk factors for renal cell carcinoma: agent-specific results from a case-control study in Germany. MURC Study Group Multicenter urothelial and renal cancer study. Int J Epidemiol 29:1014–1024

Pfohl-Leszkowicz A (2009) Ochratoxin A and aristolochic acid in the nephropathies and associated urothelial tract tumours development. Arh Hig Rada Toksikol 60:465–483. https://doi.org/10.2478/10004-1254-60-2009-2000

Pfohl-Leszkowicz A, Manderville RA (2007) Ochratoxin A: an overview on toxicity and carcinogenicity in animals and humans. Mol Nutr Food Res 51:61–99. https://doi.org/10.1002/mnfr.200600137

Pfohl-Leszkowicz A, Petkova-Bocharova T, Chernozemsky IN, Castegnaro M (2002) Balkan endemic nephropathy and the associated urinary tract tumours: review on etiological causes, potential role of mycotoxins. Food Addit Contam 19:282–302. https://doi.org/10.1080/02652030110079815

Pfohl-Leszkowicz A, Vrabcheva T, Petkova-Bocharova T, Garren L, Grosso F, Nikolov I, Dragacci S, Chernozemsky IN, Castegnaro M (2006) Analysis of ochratoxin A in serum, urine and food consumed by inhabitants from an area with Balkan endemic nephropathy: a one month follow up study. In: Njapau H, Trujillio S, Van Egmond HP, Park DL editors. Mycotoxins and Phycotoxins. Proceeding of the XIth International IUPAC Symposium on Mycotoxins and Phycotoxins, May 17–21, 2004, Bethesda, Maryland, USA: Wageningen Academic Publishers, 217–224

Pfohl-Leszkowicz A, Moliniè A, Tozlovanu M, Manderville RA (2008) Combined toxic effects of ochratoxin A and citrinin in vitro and in vivo. In: Siantar DP, Trucksess MW, Scott PM, Herman EM (eds) Food contaminants: mycotoxins & food allergens, ACS Symposium series, vol 1001. American Chemical Society, Washington, DC, pp 56–80. https://doi.org/10.1021/bk-2008-1001.ch003

Radford R, Frain H, Ryan MP, Slattery C, McMorrow T (2013) Mechanisms of chemical carcinogenesis in the kidneys. Int J Mol Sci 14:19416–19433. https://doi.org/10.3390/ijms141019416

Reaume MN, Graham GE, Tomiak E, Kamel-Reid S, Jewett MAS, Bjarnason GA, Bleis N, Care M, Drachenberg D, Gedye C, Grant R, Heng DYC, Kapoor A, Kollmannsberger C, Lattouf JB, Maher ER, Pause A, Ruether D, Soulieres D, Tanguay S, Turcotte S, Violette PD, Lori W, Basiuk J, Pautler SE (2013) Canadian guideline on genetic screening for hereditary renal cell cancers. Can Urol Assoc J 77:319–323. https://doi.org/10.5489/cuaj.1496

Reinhard H, Zimmerli B (1999) Reversed-phase liquid chromatographic behaviour of the mycotoxins citrinin and ochratoxin A. J Chromatogr A 862:147–159

Ringot D, Chango A, Schneider YJ, Larondelle Y (2006) Toxicokinetics and toxicodynamics of ochratoxin A. Chem Biol Interact 159:18–46. https://doi.org/10.1016/j.cbi.2005.10.106

Rosner H, Rohrmann B, Peiker G (2000) Ochratoxin A in human serum. Arch Leb 51:104–107

Sanfilippo KM, McTigue KM, Fidler CJ, Neaton JD, Chang Y, Fried LF, Liu S, Kuller LH (2014) Hypertension and obesity and the risk of kidney cancer in 2 large cohorts of US men and women. Hypertension 63:934–941. https://doi.org/10.1161/HYPERTENSIONAHA.113.02953

Schmidt LS, Linehan WM (2016) Genetic predisposition to kidney cancer. Semin Oncol 43:566–574. https://doi.org/10.1053/j.seminocol.2016.09.001

Stiborova M, Arlt VM, Schmeiser HH (2016) Balkan endemic nephropathy: an update on its aetiology. Arch Toxicol 90:2595–2615. https://doi.org/10.1007/s00204-016-1819-3

Studer-Rohr I, Schlatter J, Dietrich DR (2000) Kinetic parameters and intraindividual fluctuations of ochratoxin A plasma levels in humans. Arch Toxicol 74:499–510. https://doi.org/10.1007/s002040000157

Tesar V, Viklicky O, Bartonickova K, Boucek P, Bürgelova M, Certikova-Chabova V, Dusilova Sulkova S, Honsova E, Hruskova Z, Jancova E, Lyerova L, Matejovic M, Monhart V, Parikova A, Reiterova J, Rychlik I, Rysava R, Vachek J (2015) Klinická nefrologie (Clinical nephrology). Grada Publishing, Praha ISBN: 978–80–247-4367-7

Turner PC, Flannery B, Isitt C, Ali M, Pestka J (2012) The role of biomarkers in evaluating human health concerns from fungal contaminants. Nutr Res Rev 25:162–175. https://doi.org/10.1017/S095442241200008X

Zimmerli B, Dick R (1995) Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. J Chromatogr B 666:85–99. https://doi.org/10.1016/0378-4347(94)00569-Q

Příloha 8

Investigation of ochratoxin A biomarkers in biological materials obtained from patients suffering from renal cell carcinoma

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Investigation of ochratoxin A biomarkers in biological materials obtained from patients suffering from renal cell carcinoma

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ABSTRACT

Ochratoxin A (OTA) exposure can result in chronic renal diseases and cancer. The incidence of kidney, renal pelvis, and ureter malignant neoplasms in the Czech Republic is approximately 29.5 renal tumours per 100,000 inhabitants. The question arises whether mycotoxins are also involved in kidney disease and cancer. A sensitive validated analytical methodology, based on an immunoaffinity clean-up followed by HPLC with fluorescence detection, was developed to explore whether OTA accumulates in clear renal cell carcinoma-adenocarcinoma in Czech patients. Simultaneously, DNA-adducts and OTA metabolites were qualitatively analysed in tissues and urine. OTA was analysed in 33 kidney and tumour samples from 26 men and 7 women collected during nephrectomy from patients of the East Bohemian region from 2015 to 2017. OTA was found in 76% of the analysed samples. Its concentrations ranged from not detectable to 390 ng/kg with a median of 167 ng/kg in kidney samples and from not detectable to 430 ng/kg with a median of 122 ng/kg in tumour samples. Urinary OTA metabolites and DNA adducts were qualitatively analysed for the corresponding 20 patients. The presence of some OTA metabolites such as ochratoxin A hydroquinone and/or decarboxylated ochratoxin A hydroquinone correlate with the presence of OTA-DNA adducts.

1. Introduction

Approximately 84,000 new cases of kidney cancer and 35,000 deaths occur in Europe per year (Ferlay et al., 2015). Men are affected more frequently than women at a ratio of 2:1 (new diagnoses) (Hsieh et al., 2017). Based on epidemiological data, the incidence of malignant neoplasms of the kidney, renal pelvis, and ureter (diagnoses C64-C66) is in the Czech Republic set at 29.5 kidney tumours per 100,000 inhabitants (Dusek et al., 2012). This unfavourable number is very high compared to other countries (CanCon. (Cancer Control J, 2014; Robert Koch Institut, 2019). The very high incidence of kidney cancer in the Czech Republic is truly striking. The highest incidence in the Czech Republic is observed in the Pilsen region, and the situation is similar in neighbouring Bavaria in Germany. No clear explanation exists for this fact. Unfortunately, no industrial, nutritional, and genetic data are available that could reveal the real cause (personal communication, Prof. Pacovsky, 2021). The

Abbreviations: 10-OH-OTA, 10-hydroxy ochratoxin A; 4-R–OH–OTA, 4R-hydroxy ochratoxin A; 4-S–OH–OTA, 4S-hydroxy ochratoxin A; BEN, Balkan endemic nephropathy; $BMDL_{10}$, benchmark dose lower confidence limit; CIT, citrinin; ccRCC, clear renal cell carcinoma-adenocarcinoma; CT, computed tomography; OTHQ-GSH, conjugate ochratoxin A quinone–glutathione; OTB-GSH, conjugate ochratoxin B–glutathione; DA, Dark Agouti; DNA, deoxyribonucleic acid; DH-CIT, dihydrocitrinone; C-C8dG-OTA, Carbon bound DNA adduct ochratoxin A – deoxyguanosine; O6-OTA dG, DNA adduct ochratoxin A – deoxyguanosine; HE, hematoxylin eosin; HPLC – FLD, high-performance liquid chromatography with fluorescence detection; LOD, limit of detection; LOQ, limit of quantification; MRI, magnetic resonance imaging; OTA, ochratoxin A; OTHQ, ochratoxin A hydroquinone; OTHQ-NAC, ochratoxin A hydroquinone - N-acetylcysteine; DC-OTHQ, ochratoxin A hydroquinone decarboxylated; OP-OA, ochratoxin A open lactone; OTB-NAC, ochratoxin B - N-acetylcysteine; OTB, ochratoxin B (dechlorinated OTA); OTB-dG, ochratoxin B deoxyguanosine; OTC, ochratoxin C (ethyl ester ochratoxin A); OTα, ochratoxin α; OP-OTB, ochratoxin B open lactone; RCC, renal cell carcinoma; TDI, tolerable daily intake; UUC, upper urothelial cancer.

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Czech Republic has also the highest mortality rate from kidney cancer (Bosetti et al., 1970).

Renal cell carcinoma (RCC) designates a heterogeneous group of cancers derived from renal tubular epithelial cells (International Agency, 2020). Globally, RCC is one of the ten most common cancers and accounts for more than 90% of kidney cancers (Hsieh et al., 2017). So far, ten histological and molecular subtypes of RCC have been described (International Agency, 2020). Out of these, clear cell RCC (ccRCC) is the most common (Cancer Genome Atlas, 2013; Tesar et al., 2015). Unfortunately, adenocarcinoma ccRCC growing from proximal tubule cells (Tesar et al., 2015) is one of the most lethal types of cancer (Hsieh et al., 2017). The kidney cancer risk is increased by some factors including age (Tesar et al., 2015), smoking (Hunt et al., 2005), hypertension (Sanfilippo et al., 2014), obesity (Sanfilippo et al., 2014; Aurilio et al., 2019), in particular individual genetic predisposition (Chan et al., 2018; Pfohl-Leszkowicz, 2009), certain inherited syndromes, long-term dialysis (Tesar et al., 2015), aristolochic acids (Hoang et al., 2016), as well as professional exposure to coke (fuel) production, oil refining and gasoline, diesel engine exhaust, polycyclic aromatic hydrocarbons, asbestos, trichloroethylene, tetrachloroethylene, polychlorinated biphenyls, heavy metals (e.g. lead and cadmium) (Slack et al., 2012). The risks are also increased by the consumption of dairy products, red meat, and preserved vegetables (Hsu et al., 2007). The possible causes of carcinogenesis are virtually innumerable. The exposure to some chemical factors such as multi-mycotoxin is known to be a strong modulator of carcinogenic risk (Cohen and Arnold, 2011; De Ruyck et al., 2015).

The risk of kidney tumour can also be associated with dietary exposure to nephrotoxic mycotoxins (Malir et al., 2019). Excessive intake of nephrotoxic mycotoxins, e.g. OTA and citrinin (CIT) in food enhances the risk for human (Malir et al., 2001). This is also similar to renal insufficiency that impairs the physiological degradation and detoxification potential of the organism (Malir et al., 2001). Besides other mycotoxins such as aflatoxins, deoxynivalenol, nivalenol, fumonisins, zearalenone, and patulin, OTA and CIT attract attention due to their toxic effects and high prevalence in the agro-food commodities (Coppa et al., 2019; Ojuri et al., 2018; Selvaraj et al., 2015).

Biomarkers are essential tools used to measure the exposure to a toxic substance or the scope of any toxic reaction to such an agent, as well as to predict the likely response (Timbrell, 1998). They are categorized in markers of internal dose and markers of effective dose. Biomarkers of internal dose confirm the exposure to a toxic agent by measuring the compound or its metabolite(s) in body fluids or tissues (Timbrell, 1998; Malir et al., 2012), e.g. in milk, blood, urine, and kidney (target dose) (Malir et al., 2012).

The biomarkers of exposure to OTA find OTA in humans evidence the consumption and indicate that OTA and/or its metabolites are in body fluids, tissues, and fetuses. The changes in OTA contents in food are not directly mirrored by changes in blood OTA concentration in humans at a relatively low dietary OTA intake (Duarte et al., 2009). Using OTA levels in plasma or serum, the Klaassen equation has been applied with varying rates of success to calculate continuous dietary exposure to OTA (Malir et al., 2013a, 2019; Klaassen et al., 1986; Duarte et al., 2011).

Biomarkers of the effective dose provide information that exposure to a specific toxic compound has led to the compound or its metabolite (s) achieving a toxicologically important target. Due to many possible interindividual differences in the rate and pathway of the compound metabolism, the measurement of the effective dose at the target site is preferred to the measurement of the internal dose (Timbrell, 1998). In addition to individual differences in absorption and distribution, this occurs because the former reflects differences in activation versus detoxification metabolism and the extent of DNA damage repair (Pfohl-Leszkowicz et al., 2007a). The effective dose is usually determined by measuring specific adducts in body fluids and tissues. Electrophilic compounds that are reactive or are metabolized to reactive intermediates electrophiles and react with DNA are of interest and concern for genotoxicity due to their possible carcinogenicity. The presence of a chemically specific DNA adduct in human DNA is a good indicator that chemical exposure has occurred. Therefore, it is used as an exposure biomarker (Timbrell, 1998; Pfohl-Leszkowicz et al., 2007a). These adducts are considered markers of exposure and generally reflect recent exposure rather than that in the distant past.

OTA and CIT are nephrotoxins produced by microscopic fungi of the genera *Aspergillus* and *Penicillium* (Ostry et al., 2013) that often simultaneously contaminate a wide range of foodstuff of both plant and animal origins. OTA often contaminates cereal products, coffee, chocolate, cocoa, spices, liquorice, raisins, grape juice, wine, beer, as well as pork, pork blood products, poultry kidney, liver, crude meat, smoked and salted fish, and cheese (Malir et al., 2013b), while CIT is typically found in cereals, roasted nuts, black olives, spices, and cheese (Ostry et al., 2013).

The OTA and CIT co-occurrence in food represents an increased risk to human health, although contamination may differ year by year (Ostry et al., 2013). Cereals (about 58%), wine (15%) pork (3%), and coffee (about 1–10% depending on the actual consumption) are considered the main sources of daily OTA intake (JECFA FAO/WHO, 2007; Tozlovanu and Pfohl-Leszkowicz, 2010).

Generally, mycotoxins cause several diseases. OTA [PubChem CID: 442530 or http://www.t3db.ca/toxins/T3D3605] is associated with various nephropathies including chronic interstitial nephropathy causing renal dysfunction leading to renal failure (Pfohl-Leszkowicz et al., 2002a). It is also suspected to be involved in the development of Balkan Endemic Nephropathy (BEN) (Pfohl-Leszkowicz, 2009; Pfohl--Leszkowicz et al., 2002a) and related renal tumours (Castegnaro et al., 2006), in particular upper urothelial cancer (Tesar et al., 2015; Pfohl--Leszkowicz, 2009).

OTA is a potent nephrotoxic and nephrocarcinogenic mycotoxin (JECFA FAO/WHO, 2007; Castegnaro et al., 1998; Kathuria et al., 2018; Mantle, 2009; Pfohl-Leszkowicz and Manderville, 2012). *In vivo*, OTA induces genetic damage, particularly aberrant mitoses and karyomegaly (European Food Safety Authority, 2020). It is also thought to cause oxidative DNA damage leading to mutagenesis and potential carcinogenesis (Zepnik et al., 2001). Direct OTA genotoxic mechanisms of action have been proposed and a pathway described that metabolizes OTA into an electrophilic form capable of direct binding to specific nucleotide bases (Pfohl-Leszkowicz and Manderville, 2012; Pfohl-Leszkowicz and Castegnaro, 2005; Manderville and Pfohl-Leszkowicz, 2008).

In vivo study on mice showed that the tumour suppressor protein p53 is upregulated during OTA treatment and also investigated the extent to which p53 inhibits the progression of OTA-induced DNA damage (Kuroda et al., 2015). Conversely, it is believed that OTA induces carcinogenicity by disrupting mitosis and genetic instability (Mally, 2012). However, the genotoxicity of OTA displayed as the formation of OTA-DNA adducts, its role in oxidative stress, and the identification of epigenetic factors involved in OTA carcinogenesis suggested that carcinogenicity of OTA was mediated by a mechanism that also operates in humans (Ostry et al., 2017).

A long-term study using 12 weeks old male and female Dark Agouti (DA) rats demonstrated that 0.4 mg OTA/kg body weight administered 3 times per week induced a statistically significant increase in the incidence of renal adenocarcinoma. Male DA rats were affected more often by these tumours than their female counterparts (Castegnaro et al., 1998).

High OTA levels in slaughtered pigs fed by OTA contaminated feedstuffs (ranging from 149 μg/kg to 327 μg/kg) were observed to accumulate in the kidneys and bladder with subsequent development of macro- and microscopic renal lesions. Even precancerous changes were detected including large nuclei, hyperchromic nucleoli, and karyorrhexis (Ceci et al., 2007). In another study carried out on slaughtered pigs, the highest OTA level was also found in the kidney and the high correlation between nephritis severity and OTA level was confirmed (Milićević et al., 2008).

According to IARC/WHO, OTA was in 1993 classified as “probably

carcinogenic to humans" (group 2B) with sufficient evidence of carcinogenicity in animal models and insufficient evidence from human studies (IARC, 1993).

Levels of OTA and CIT in blood and urine of 50 Czech patients suffering from ccRCC- adenocarcinoma have been analysed in a previous study (Malir et al., 2019). Depending on the availability of the tissue, we determined OTA and/or DNA adducts in their kidney and tumour in 33 patients of this cohort. We compare findings obtained in this study with results from the Czech biological monitoring program that concerned persons without previous renal failures (Ostry et al., 2005) and with data from France (Pfohl-Leszkowicz et al., 2007a) and Poland (Grajewski et al., 2007) acquired from patients who suffered from kidney tumours.

2. Materials

2.1. Diagnosis of clear renal cell carcinoma-adenocarcinoma

The basic steps in the kidney cancer diagnosis at the cohort of selected patients at the Department of Urology consisted in assessing clinical symptoms or in ultrasound visualization of the tumour mass. The cancer diagnosis was always confirmed by computed tomography or magnetic resonance imaging. The surgery was the first choice in localized, locally advanced, and even in metastatic renal cancer. No tumour biopsy was indicated before planned surgery. The division between the nephron-sparing surgery and the radical nephrectomy depended on the local situation such as the tumour size, localization within a kidney, and lymphadenopathy, or general situation including renal functions and general health conditions (personal communication, Prof. Pacovsky, 2019).

Fig. 1 confirms ccRCC diagnosis and shows the interface of non-tumoural kidney tissue (A) and ccRCC (B). Fig. 2 then presents the histology of ccRCC.

2.2. Biological material sample collection

A total of 33 samples of kidneys and their corresponding tumour tissues were collected during the period of 2015–2017 from surgeries of 26 men and 7 women aged between 39 and 80 years with the ccRCC diagnosis.

All samples were processed anonymously using a numerical code, while only gender, the year of birth, and the bodyweight of the patient were recorded. The samples were frozen and stored at −80 °C until the analysis. More detailed anamnestic data of the patients are only available in the database of the Department of Urology at Hradec Kralove.

Based on the previous standard laboratory and medical examinations, only those patients that did not exhibit cardiovascular complications and overall metabolic disruption were randomly selected at the Department of Urology for this study. Standard clinical pre-operative blood analysis included glycaemia, liver and renal functions examinations, determinations of minerals (Na, K, Cl), C-reactive protein, blood count, haemostasis, chemical, and both microscopical urine, and microbiological analyses (personal communication, Prof. Pacovsky, 2019).

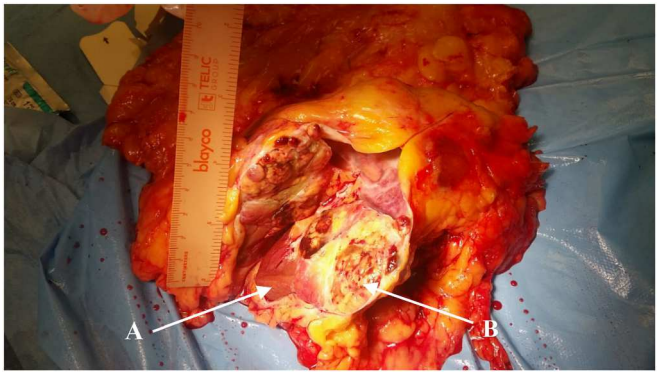

Fig. 1. ccRCC. The interface of non-tumoural kidney tissue (A) and clear cell kidney cancer (B). Photo: Miroslav Louda (April 2017).

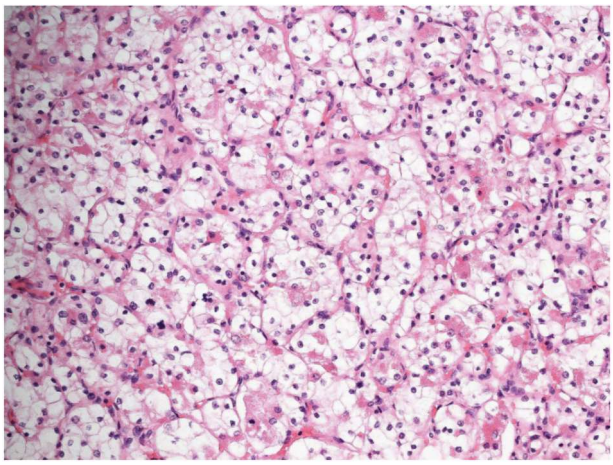

Fig. 2. Histology of ccRCC showing the detail of tumour cells (HE, 200×). Photo: Miroslav Louda (April 2017). Patient demographics and baseline characteristics of male and female patients with the kidney cancer diagnosis are shown in Table 1.

Table 1
Characteristics of male and female patients with the kidney cancer diagnosis.

Characteristics of patients[a]	Subgroup of men	Subgroup of women	Whole cohort
Subjects - gender (n)	26	7	33
Rural	5	1	6
Urban	21	6	27
Diagnosis	26	7	33
Malignant neoplasm of kidney, outside renal pelvis (C64)			
Smokers	10	2	12
Hypertension	11	1	12
Obesity (BMI >30)	10	1	11
Weight of patients (kg)	92 ± 11.2	60 ± 33.3	90 ± 20.0
Mean ± SD			
Range	68–118	57–155	57–155
Age (years)			
Median ± SD	67.5 ± 9.3	61 ± 12.9	67 ± 10.6
Range	43–80	39–73	39–80

[a] For about 20% of patients (4 males, 2 females) another cancer existed in their family (e.g. cancer of the oesophagus, or intestine).

Samples for determination of the OTA and CIT presence in urine and blood were collected immediately before surgery as a part of standard clinical sampling to avoid overburdening the patients and to accept only those with their explicit consent. These results were published elsewhere and are not mentioned in this study (Malir et al., 2019).

2.3. Control group

Due to the difficulties in obtaining reference kidney samples from healthy individuals, we used for comparison results published in 2005 concerning OTA concentration in the human kidney (Ostry et al., 2005). These data were collected during human biomonitoring in 2001 within the framework of the System of Environmental Health Monitoring in the Czech Republic.

2.4. Chemicals and materials

Glacial acetic acid, formic acid (85%) (both in p.a. purity),

orthophosphoric acid (suprapur), chloroform, methanol, and acetonitrile (gradient grade for HPLC) were obtained from VWR International s. r.o. (Stribrna Skalice, Czech Republic). Phosphate buffered saline, powder, pH 7.4 (for preparing 1 L of solution), sodium hydrogen carbonate p.a., and OTA standard were obtained from Sigma–Aldrich spol. s r.o., (Prague, Czech Republic). Immunoaffinity columns OCHRAPREP® for sample cleaning before the quantitative determination of OTA by HPLC were produced by R-Biopharm Rhone Ltd. (Glasgow, Great Britain) and delivered by Jemo Trading spol. s r.o. (Bratislava, Slovak Republic). Paper filters for sample filtration Whatman No. 4 were from Merck (Prague, Czech Republic) or KA-2M from Papirna Pernstejn spol. s r.o. (Pernstejn, Czech Republic). Sample extracts were evaporated under nitrogen 4.7 from SIAD spol. s r.o. (Branany u Mostu, Czech Republic). All solutions were prepared in ultrapure water produced by Milli Q Plus system from Merck Millipore (Billerica, MA, USA).

OTA (benzene free, CAS# 303-47-9), 3-morpholinopropanesulfonic acid, ammonium formate, 5-bromosalicylic acid, tris(3-sulfophenyl) phosphine trisodium salt, and quinine bisulfate, sulphuric acid were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France) and 2′-deoxyguanosine from ChemGenes (Wilmington, USA). LC grade solvents acetonitrile, dimethyl sulfoxide, methanol, and chloroform were purchased from ICS (Lapeyrouse-Fossat, France) and used without any purification. 4-S–OH–OTA, 4-R–OH–OTA, 10-OH-OTA, OTHQ, OTB, and OTB-methyl ester were prepared in the laboratory using previously published procedures (Faucet-Marquis et al., 2006; Frenette et al., 2008). OTHQ, OTB, and OTB-methyl ester were mixtures of stereoisomers.

3′- dGMP was purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). The enzymes proteinase K (used as received), RNase A, RNase T1 (boiled 10 min at 100 °C to destroy DNases), and micrococcal nuclease (dialyzed against deionized water) were from Sigma-Aldrich (Saint Quentin Fallavier, France), spleen phosphodiesterase (centrifuged before use) was from Calbiochem (Sandhausen, Germany), and nuclease P1 and T4 polynucleotide kinase were from Roche Diagnostics (Meylan, France). [γ^{32}P-ATP] (444 Tbq/mmol, 6000 Ci/mmol) was from Amersham (Les Ullis, France), rotiphenol (phenol saturated with Tris-HCl, pH 8) was from Rothsichel (Lauterbourg, France), cellulose MN 301 was from Macherey Nagel (Düren, Germany), polyethyleneimine (PEI) was from Corcat (Portsmouth, Great Britain), Whatman no. 1 paper was from VWR (Saint-Prix, France). PEI/cellulose TLC plates used for 32P-postlabeling analyses were prepared in the laboratory. Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Strasbourg, France).

2.5. Determination of OTA in kidney and tumour tissue

2.5.1. OTA standard preparation

OTA was dissolved in methanol and spectrophotometrically calibrated at 333 nm using a molar absorption coefficient (κ) of 544 m^2/mol according to EN ISO 15141-1. The basic OTA solution was prepared by dissolving 1 mg OTA in 5 mL methanol while the OTA stock standard solution (40 ng/mL) was prepared by further dilution with methanol (Dohnal et al., 2013).

2.5.2. Working standard solutions of OTA

A working standard OTA solution were prepared on each day of the measurement from a stock solution via dilution with the mobile phase. The blank sample, i.e. the plain mobile phase was also prepared fresh daily. The injection of 50 μL of these standards in HPLC led to the following standard range points: 0.05; 0.10; 0.20; 0.50; 1.00; 2.00; 4.00 ng OTA/mL.

2.5.3. Acidified chloroform

This solution was prepared from 50 mL of chloroform and 0.75 mL concentrated orthophosphoric acid.

2.5.4. Acidified methanol

This solution was prepared from 98 mL of methanol and 2 mL acetic acid (98/2; v/v).

2.5.5. Acetonitrile/acetic acid

This solution was prepared from 98 mL of acetonitrile and 2 mL acetic acid (98:2; v/v).

2.5.6. Water/acetic acid

This solution was prepared from 98 mL of water and 2 mL acetic acid (98:2; v/v).

3. Methods

3.1. OTA extraction and separation in tissues

3.1.1. Sample homogenization

Samples of kidney or tumour tissue were homogenized step by step on Ultra-Turrax T 25 digital (IKA®-Werke GmbH & Co. KG, Staufen, Germany), starting at 500 rpm for 10 s, then at 10,000 rpm for another 10 s, and finally at 20,000 rpm for 15 s.

3.1.2. OTA extraction

OTA was extracted from 5 g homogenized sample using 25 mL acidified chloroform and then re-extracted two times using 12.5 mL acidified chloroform to get a final volume of 50 mL. Note that the ratio 5 g of sample to 50 mL acidified chloroform was respected and adjusted to the actual weight of each sample. The homogenized solution was filtered and the exact volume of the filtrate was measured. Then, 20 mL filtrate was re-extracted twice using 10 mL of 0.5 mol/L sodium hydrogen carbonate solution according to Zimmerli and Dick (1995) and vigorously shaken for 3 min. Note that the efficiency of extraction was verified. The mixture was centrifuged at 3500 rpm for 5 min and the combined extracts were acidified with 0.5 mL formic acid and 1 mL chloroform, and then re-extracted two times using 2 mL chloroform. The mixture was centrifuged each time at 3500 rpm for 5 min to achieve a compact thin layer between two phases. The clear organic chloroform layer at the bottom of the tube was collected and evaporated to dryness under a nitrogen stream at 45 °C.

3.1.3. OTA separation

The residuum was gradually dissolved in 20 mL phosphate-buffered saline solution, pH 7.4 solution containing 15% methanol (*v/v*) and separated on OCHRAPREP® immunoaffinity column. This column was washed with 20 mL water and dried. The adsorbed compound was then eluted using acidified methanol and evaporated again (modified methods of OCHRAPREP® (R-Biopharm - Rhone Ltd. OCHRAPREP®, 2014) and Zimmerli and Dick (1995)).

3.2. Targeted OTA HPLC-FLD analysis

Chromatographic analyses were carried out using HPLC-FLD system comprising the Jasco PU-2085 Plus pump, the Jasco DG – 2080-54 degasser, the gradient unit Jasco LG-2080-04S gradient unit, the Jasco AS-2059-SF Plus autosampler, the Jasco FP-2020 Plus fluorescence detector, and the Jasco LC-Net II/ADC hardware interface from Jasco, Easton USA.

The separation was performed on a Kinetex C18 (50 × 2.1 mm) analytical column packed with 2.6 μm core-shell particles. The analytical column was coupled with a SecurityGuard™ column C18, 4 × 2.0 mm (Phenomenex, Torrance, CA, USA). The mobile phase gradient was formed from the mobile phase A acetonitrile/acetic acid (98:2; v/v) and the mobile phase B water/acetic acid (98:2; v/v). The gradient shape was 45% A in B for 1 min, ramped to 98% A in B in 5 min, held for 4 min, decreased to 45% A in B in 0.1 min, and held at 45% A in B for 8 min. The evaporated sample was dissolved in 500 μL starting mobile phase

mixture. The injected sample volume was 50 μL and the flow rate 0.2 mL/min. Fluorescence detection was achieved at an excitation wavelength of 333 nm and an emission wavelength of 465 nm, gain x 1,000, attenuation 16. The OTA retention time was around 7.3 min under these chromatographic conditions. The OTA concentrations were quantified using the calibration curve method. No internal standards were used.

All calculations and evaluations of the analyses were processed by a computer using the Jasco ChromPass Chromatography Data System Connection version 1.8.6.1 software (Jasco, Easton USA).

3.2.1. Performance characteristics

The calibration curve plotting the peak areas vs. the OTA concentrations was constructed and verified. The linearity of the calibration curve was assessed by 7-point calibration in a range of 50–4000 ng/L. The correlation coefficient was 0.999. The validation process revealed a limit of detection (LOD) of 8 ng/kg and a limit of quantification (LOQ) of 27 ng/kg. The certified reference material for the determination of OTA in human kidneys was not available. Therefore, recovery experiments were performed in triplicate spiked pig kidney samples at OTA concentration levels of 500 and 1000 ng/kg. Recovery was 69.8% and repeatability standard deviation (RSD) was 6.8%. Validations of this method were carried out according to the protocol approved by the IUPAC/AOAC/ISO (Horwitz, 1995) (Thompson et al., 2002).

3.2.2. The calculation of OTA concentration in tumour/kidney

The following equation was used for calculation of the OTA concentration Cs:

$$Cs\ (ng\ OTA\ /\ g) = \frac{100 \cdot (Pa + Iv)}{2 \cdot As \cdot Sv \cdot Rv}$$

Where Pa is the peak area of the sample, Iv is the intercept value of calibration curve function, As is the amount of processed sample (g), Sv is the slope value of calibration curve function, Rv is the recovery value of the method (69.8%), Cs is the real concentration of OTA in the sample (ng/g), number 2 is the multiplicator for the amount of dissolved sample (500 μL), and 100 is the multiplicator for real value (100%).

3.2.3. Statistical analysis

Data were processed using the universal statistical software Microsoft Excel (version 2019). The results are presented based on descriptive analysis (Hornung and Reed, 1990).

3.3. OTA metabolites separation and identification in urine

OTA metabolites OTB-GSH, OTHQ-NAC, OTHQ-GSH, OTB-NAC, DC-OTHQ, OP OA, 4S-OH-OTA, 4R–OH–OTA, OTHQs, OTBs, OP-OTB, OTC, OTα, and OTB were analysed on RP HPLC using C18 column PRONTOSIL (250 × 4 mm, 3 μm) using the gradient elution. The mobile phase A was MeOH/ACN/6.5 mM ammonium formate (200/200/600) adjusted to pH 3 with formic acid while the mobile phase B was MeOH/ACN/6.5 mM ammonium formate (350/350/300) adjusted to pH 3 with formic acid. Gradient shape adopted from Faucet-Marquis et al. (2006) was the following: 100% A for 10 min, ramp to 30% A in B in 15 min and held for 5 min, increase to 100% B in 5 min and held for 10 min, followed by decrease to 100% A in 3 min. Detection was performed with the programmable fluorimeter GTI Spectrovision (ex = 350, em = 510 nm) and allowed better detection of some OTA metabolites (Frenette et al., 2008).

Identification of the metabolites have been described elsewhere (Pfohl-Leszkowicz, 2009; Faucet-Marquis et al., 2006; Tozlovanu et al., 2012; Mantle et al., 2010). 4-S–OH–OTA, 4-R–OH–OTA, and 10 hydroxylated OTA (10–OH-OTA) were generous gifts of Dr. Frederik Størmer, Quinone OTA (OTHQ) was donated by Prof. Richard Manderville, Guelph University, Canada. All other metabolites were synthesized in our laboratories and identified by mass spectrometry by Dr Fréderic Pont.

3.4. DNA adduct identification

Kidney DNA isolation and purification as well as the method used for 32P-postlabeling we used were described by Faucet et al. (Faucet-Marquis et al., 2004) and in detail by Pfohl-Leszkowicz and Castegnaro (2005). In brief, an equivalent of 7 μg of DNA was dried *in vacuo*, dissolved in 10 μL mixture containing 1 μL micrococcal nuclease (2 mg/mL corresponding to 500 U), spleen phosphodiesterase (15 mU/μg DNA), 1 μL sodium succinate solution (200 mM), and 1 μL calcium chloride solution (100 mM, pH 6), and digested at 37 °C for 4 h. The digested DNA was then treated with 5 μL mixture containing 1.5 μL of nuclease P1 (4 mg/mL), 1.6 μL of $ZnCl_2$ solution (1 mM), and 1.6 μL sodium acetate solution (0.5 M, pH 5) at 37 °C for 45 min. The reaction was stopped by the addition of 3 μL Tris base solution (500 mM). The DNA adducts were labelled as follows: 5 μL reaction mixture containing 2 μL bicine buffer containing bicine (800 μM), dithiothreitol (400 mM), $MgCl_2$ (400 mM), and spermidine (400 mM) adjusted to pH 9.8 with NaOH, 10 U of polynucleotide kinase T4, and 100 μCi of [γ-32P]ATP (specific activity 6000 Ci/mmol) was added to the nuclease P1 digest and the mixture incubated at 37 °C for 45 min. Normal nucleotides, pyrophosphate, and excess ATP were removed overnight by chromatography on PEI/cellulose TLC plates (D1) in 2.3 M sodium dihydrogen phosphate buffer, pH 5.7. The original 2 cm areas containing labelled adducted nucleotides were cut out and transferred to another PEI/-cellulose TLC plate that was run (D2) in 4.8 M lithium formate and 7.7 M urea, pH 3.5, for 3 h. A further (D3) migration was performed after turning the plate 90° anticlockwise using 0.6 M sodium dihydrogen phosphate and 5.95 M urea, pH 6.4, for 3 h. Finally, the chromatogram was washed in the same direction in 1.7 M sodium dihydrogen phosphate, pH 6, for 2 h (D4). Adduct profiles were analysed qualitatively and semi-quantitatively by autoradiography of the plates carried out at −80 °C for 48 h in the presence of an intensifying screen using a radioanalytical system of image analysis (AMBIS, Lablogic).

Identification of the spots of O-C8dG-OTA and C-C8dG-OTA were achieved via comigration with the authentic DNA-adducts synthesized by Prof. Manderville as described in Mantle et al. (2010). The adducts corresponding to OTHQ were also determined via comigration with DNA samples incubated with OTHQ as described elsewhere (Tozlovanu et al., 2006).

4. Results

4.1. OTA in kidney, tumour tissue, serum, and urine

Table 2 summarizes OTA contents in kidney and tumour parts analysed in this study, blood serum and urine (biomarkers of exposure – markers of internal dose) from 33 patients suffering RCC. The combination of immunoaffinity chromatography and HPLC-FLD increased the sensitivity and selectivity of the method.

Only twenty-five samples could be analysed for their OTA content due to the limited availability of tissue. OTA was detected in all kidney samples (25/25) in concentrations ranging from 72 to 385 ng/kg, with a median value of 186 ng/kg. It has also been detected in all the corresponding tumour samples (25/25) in the range from 54 to 431 ng/kg with a median value of 191 ng/kg. In 12 patients (No. 8, 13, 15, 19, 23, 24, 25, 26, 27, 30, 31, 32) the quantity of OTA found in the tumour was by 17–75% lower than that found in the kidney.

The amounts of OTA found in kidney tissues was compared to those found in previous studies published elsewhere. Table 3 summarizes the OTA concentration in the kidneys and tumours in the Czech Republic, France, and Poland.

Higher contents of OTA were found in the kidney tissue samples of Czech patients suffering from ccRCC compared to the samples originating from the healthy Czech population collected from 2000 to 2001

Table 2
Detailed overview of OTA contents in kidney, tumour, blood, and urine of patients with ccRCC.

N°	Gender	Area	Age	Kidney[a] ng/kg	Tumour[a] ng/kg	Serum[b] ng/L	Urine[b] ng/L
1	Female	urban	73	327	393	112.8	118.3
2	Male	urban	80	161	122	249.2	127.0
3	Female	urban	73	205	236	nd[c]	85.1
4	Male	rural	61	NA[d]	NA	nd	670.1
5	Male	rural	72	182	230	nd	nd
6	Male	urban	73	212	359	nd	nd
7	Male	urban	48	NA	NA	296.7	116.0
8	Female	urban	40	171	61	nd	nd
9	Male	urban	43	186	225	nd	nd
10	Male	urban	63	NA	NA	nd	nd
11	Male	urban	73	NA	NA	nd	nd
12	Male	urban	68	NA	NA	201.9	44.5
13	Male	rural	51	245	197	86.3	nd
14	Female	urban	61	163	296	nd	nd
15	Male	urban	59	347	215	70.3	nd
16	Male	urban	71	158	173	205.7	40.2
17	Male	urban	69	NA	NA	491.1	50.5
18	Male	rural	71	NA	NA	678.2	nd
19	Male	urban	73	385	198	263.3	45.6
20	Male	rural	66	NA	NA	26.5	156.2
21	Male	urban	77	121	191	730.0	15.3
22	Male	urban	76	115	226	nd	9.9
23	Male	urban	67	223	120	160.0	21.1
24	Female	urban	62	213	54	nd	2.7
25	Female	rural	39	163	67	nd	4.1
26	Male	urban	68	272	114	150.0	10.6
27	Male	urban	60	141	117	410.0	6.7
28	Male	urban	53	167	431	660.0	10.4
29	Male	urban	51	72	167	nd	11.9
30	Male	urban	69	233	180	nd	11.2
31	Male	urban	67	215	122	nd	6.5
32	Male	urban	62	267	110	nd	10.3
33	Female	urban	59	179	193	100.0	2.2

[a] Positive sample of kidney and tumour containing OTA exceeding the limit of detection (LOD 8 ng/kg).
[b] Positive sample containing OTA exceeding the limit of detection (LOD in urine 1.0 ng/L; LOD in serum 40 ng/L).
[c] nd is a level below LOD, see Malir et al. (2019)
[d] NA: not analysed.

Table 3
Comparison of OTA levels in kidney and tumour in Czech, Poland, and French cohorts.

Cohort (samples; year of collection; reference)	OTA mean/± SD (ng/kg)/ range (ng/kg)		References
	kidney	tumour	
1. Czech kidney tumour samples; dg.ccRCC (mean ± SD) (n = 33; 2015–2017)	160 ± 110 (nd[b] – 390)	150 ± 100 (nd[b] – 430)	this study
men (n = 26)	150 ± 190 (nd – 390)	150 ± 120 (nd – 430)	
women (7)	180 ± 20 (160–210)	150 ± 90 (50–300)	
2. Czech adults[a] *post mortem* (n = 30; 2000–2001) (men; women not specified, *see the explanation below)*	70 (nd - 200)	NA[c]	Ostry et al. (2005)
3. French patients with kidney carcinoma (n = 18; 2007)	280 (nd – 1760)	NA	Pfohl-Leszkowicz et al. (2007a)
men (n = 13)	240 (nd – 1760)	NA	
women (n = 5)	400 (nd – 1160)	NA	
4. Poland kidney samples after nephrectomy (due to kidney carcinoma) (n = 19; 2005)	220 (nd - 450)	NA	Grajewski et al. (2007)
men (n = 9)	230 (nd - 450)	NA	
women (n = 10)	200 (50–390)	NA	

[a] Cohort 2. "persons without previous renal failures", samples from Monitoring of National Institute of Public Health, Prague, Czech Republic.
[b] nd is the level below LOQ 27 ng/kg.
[c] NA: not analysed. Explanation: Czech study No. 2: The samples were supplied to the laboratory anonymously only under numbers without knowledge of the age or gender of the patients.

post mortem within the monitoring program carried out by the National Institute of Public Health in Prague (Ostry et al., 2005). Resting blood flow in kidney is 1.2 L/min. This means that 20% of the minute cardiac output passes through the renal tissue where OTA is metabolized. If renal function is impaired, OTA metabolism is also impaired, and it accumulates in the body. The Czech healthy population study included only kidney samples from healthy people after professional fatalities, fatal traffic accidents, etc.

In our study, a total of 9% of the kidney samples contained more than 300 ng/kg OTA. In the French study, 27% of the patients had OTA content in the kidney exceeding 400 ng/kg, and 22% had OTA level over 900 ng/kg (Pfohl-Leszkowicz et al., 2007a). The Polish study revealed that 21% of the kidney samples obtained after nephrectomy from the patients suffering from renal tumour also contained more than 300 ng/kg OTA (Grajewski et al., 2007).

In parallel, OTA was analysed in the blood and urine of 33 patients collected before surgery. OTA was found in 17/33 (51.5%) patients, ranging from less than LOD to 730 ng/L in blood serum, with a median value of 26.5 ng/L. OTA also ranged from less than LOD to 670.1 ng/L, with a median value of 10.3 ng/L, in urine of 23/33 (69.7%) patients. Five patients (No. 5, 6, 8, 9, 14) did not have any OTA in blood serum and urine although this compound was detected in both kidney and tumour samples. OTA was detected in urine of 9 patients (No. 3, 4, 22, 24, 25, 29, 30, 31, 32) but not in blood serum. On the contrary, 3 patients (No. 13, 15, 18) had a significant amount of OTA in their blood serum while no detectable amounts of OTA were found in their urine. No correlation can be established between a high concentration of OTA in blood and/or urine and the OTA contents found in kidney tissue. Indeed, 4 patients with the highest OTA blood serum concentration (No. 18, 21, 27, 28) did exhibit the highest quantities in kidney and tissue. Similarly, out of 4 individuals (No. 1, 2, 6, and 28) having the highest OTA contents of around 400 ng/kg in their tumour, individual No. 6 did not have any trace of OTA in blood serum and urine.

4.2. Determination of OTA metabolites and DNA adducts

The presence of OTA metabolites and DNA adducts were analysed in urine and kidney tissues of 20 patients including 4 females and 16 males. Samples from these patients were analysed since they were available. The retention time of OTA was 44.5 min. An example of the separation of OTA metabolites in urine of a patient is shown in Fig. 3.

Table 4 shows the results of qualitative analysis of the main OTA metabolites found in the urine of the patients.

No OTA metabolites were detected in 2 individuals' (No. 8, 14) blood serum and urine.

Decarboxylated hydroxyquinone (DC-OTHQ) was detected in 12 individuals (No. 1, 2, 4, 6, 7, 10, 11, 12, 13, 15, 17, 18, 20) generally associated with ochratoxin hydroquinone (OTHQ), glutathione-OTHQ (OTHQ-GSH), N-acetylcystein-OTHQ (OTHQ-NAC), and/or ochratoxin A open lactone (OP-OA) and dechlorinated OTA (OTB). Ethyl ester ochratoxin A (OTC) was found in 6 patients (No. 3, 4, 7, 10, 11, 20).

In parallel, specific OTA related DNA adducts were analysed in the kidneys of the same patients and results are presented in Table 5. The numbering of the DNA adducts is depicted in the scheme (Fig. 4.).

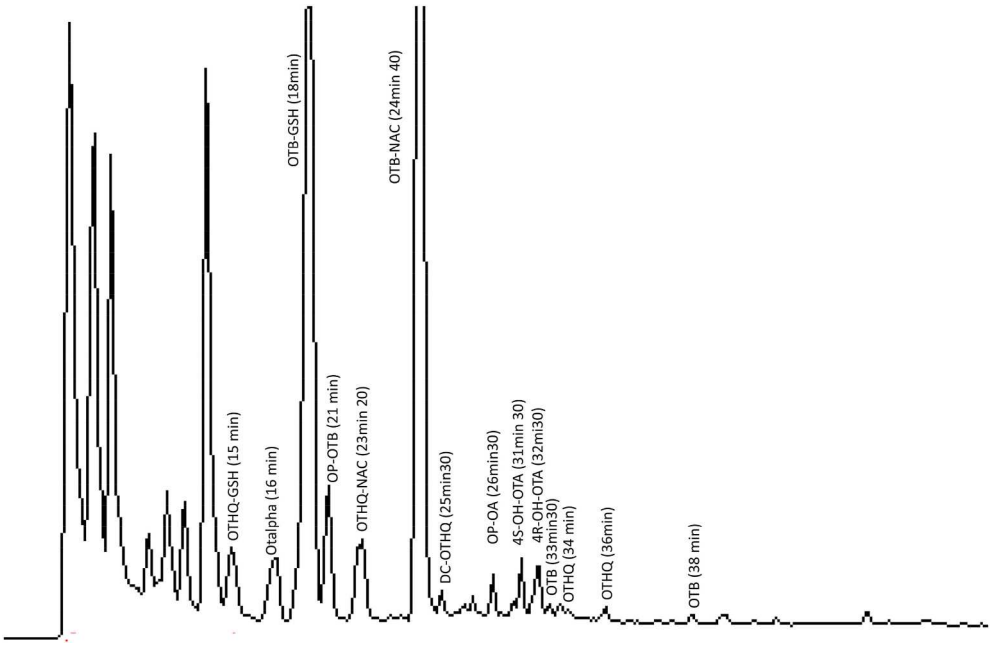

Fig. 3. HPLC-FLD chromatogram of OTA metabolites
OTB, dechlorinated OTA; OTHQ, ochratoxin hydroquinone; OTHQ-GSH, glutathione-OTHQ; OTB-GSH, glutathione OTB; OTHQ-NAC, N-acetylcystein OTHQ; OTB-NAC N-acetylcystein-OTB; OP-OA, ochratoxin A open lactone, OP OTB, ochratoxin B open lactone; OTC, ethylester OTA.

Some adducts were found in samples from five patients (No. 5, 8, 9, 14, 16). It should be emphasized that two of them (No. 8, 9) were younger at 40 and 43 years compared to the other patients. None of them had a significant amount of OTA in blood and urine. The content of OTA in the tumour of the female No. 8 was low. Only two metabolites OTB-GSH and 4R-OT-OTA were found in the male No. 9 sample. OTα, 4R–OH–OTA and two unknown metabolites eluting at 19 and 28 min were detected in urine of patient No. 16.

Kidney samples of all other patients contained the C-C8dG-OTA adduct. Four adducts C-C8dG-OTA, O6-OTA dG, and both quinone OTA related adducts were detected in the kidney samples of 7 patients (No. 2, 6, 7, 10, 11, 12, 13). They also had the main OTHQ metabolites (OTHQ-GSH; OTHQ-NAC, OTHQ1, OTHQ2), DC-OTHQ, and OTB derivatives OTB-GSH, OTB-NAC, OTB1, and OTB2 in their urine.

5. Discussion

The mycotoxin and food contaminant OTA is nephrotoxic and is one of the most potent rodent renal carcinogens studied by the National Cancer Institute/National Toxicological Program (NCI/NTP) (Boorman, 1989). It is also carcinogenic in chicks (R-Biopharm - Rhone Ltd. OCHRAPREP®, 2014) and is suspected to be involved in testicular cancer (Jennings-Gee et al., 2010; Schwartz et al., 2010; Schwartz, 2002). Several studies have shown that OTA can be one of the etiological factors supposed to be at the origin of the BEN, (Pfohl-Leszkowicz, 2009; Pfohl-Leszkowicz et al., 2002b, 2007a, 2007b) a tubulo-interstitial nephropathy with unknown origin described in the Balkan Peninsula and Romania (Stoev, 2017). Over the past decade, new studies have strengthened the argument that direct genotoxic effects contribute to OTA-induced tumour formation (Pfohl-Leszkowicz and Manderville, 2012; Hibi et al., 2013a, 2013b; Kuroda et al., 2013). Thus, OTA was included in Group 2B by IARC in 1993 (Ostry et al., 2017). However new pieces of evidence suggest that it should be included in a higher group of toxicity such as 2A (Ostry et al., 2017; Kuiper-Goodman, 1996). For these reasons, Health Canada recommends a more stringent tolerable daily intake (TDI) of 4 ng/kg bw/day based on the non-threshold model of risk assessment that is generally applied to carcinogens that cause tumours through direct genotoxicity mechanisms (Kuiper-Goodman et al., 2010; Lock and Hard, 2004) in comparison with the benchmark dose lower confidence limit ($BMDL_{10}$) for neoplastic effects of 14.5 μg/kg bw/day on the basis of kidney tumours in laboratory rats (European Food Safety Authority, 2020).

Biomarkers are useful tools allowing evaluation of aetiology and biological mechanism involved along the chain from exposure to pathology (Pfohl-Leszkowicz, 2008). Some xenobiotics are excreted without any modification but they are often biotransformed in the organism in a more polar compound that is easily excreted via bile, urine, and milk, while lipophilic compounds are stored in the tissues. Measurement of xenobiotic or one of its metabolites in biologic fluids gives a notion of the internal dose. Interaction between metabolites and macromolecules such as DNA and proteins reflects biological effective dose (Pfohl-Leszkowicz, 2008).

In the previous study Malir et al. (2019), the presence of OTA in blood and urine of Czech patients suffering from ccRCC has been first measured. This second study concerns analysis of OTA in tissues, its metabolites in urine, and DNA adducts in tissues in 20 patients.

The concentrations of OTA and its metabolites in biological material depend on multiple factors including dose, route of intake, duration of administration, and the degree of serum binding (Pfohl-Leszkowicz et al., 2007b) (Studer-Rohr et al., 2000; Ringot et al., 2006).

No correlation could be drawn between OTA in blood, urine, and tissue. Indeed, OTA was found in all kidney and tumour samples, even in those originating from individuals without OTA in blood and urine (No. 5, 6, 8, 9, 10, 11, 14). Conversely, the largest amounts of OTA in the blood do not correspond to individuals with the highest amounts of OTA in their tissue (No. 2, 16, 21, 23, 26, 27). OTA has a very high affinity for plasma proteins. Thus, 99.9% of the circulating OTA is bound to plasma proteins and only a small fraction of OTA occurs in free form in blood (Zepnik et al., 2003). OTA bound to proteins cannot be excreted directly by glomerular filtration (Castegnaro et al., 2006) and remains stored in tissues (Dai et al., 2004). Typically, equilibrium exists between the bound and free forms of OTA in blood as well as in tissue (Castegnaro et al., 2006). In a healthy person, an increase in OTA in blood is usually rapidly compensated by an increase in urinary OTA excretion that brings the OTA concentration in blood back to the original steady-state level (Castegnaro et al., 2006; Pfohl-Leszkowicz et al., 2006). For this reason,

Table 4
OTA metabolite detected in urine of patients suffering from RCC.

N°	OTHQ-GSH 14.8 min[a]	OTB-GSH 18 min	OTHQ-NAC 23.2 min	OTB-NAC 24.4 min	DC-OTHQ 25.5 min	OP-OA 27 min	4S-OH-OTA 31.5 min	4R–OH–OTA 32.5 min	OTHQs 34 min/36 min[d]	OTBs 33.4 min/38 min[d]	Other metabolites
1	+[b]	+	+	+	+	nd[c]	+	+	+	+	OP-OTB (20.8 min); At 52 min
2	++	+++	nd	+++	+	nd	++	++	++	++	OP-OTB (20.8 min)
3	nd	nd	++	nd	nd	nd	+/−	+/−	nd	+/−	At 17 min; At 52 min; + OTC (53.6 min)
4	+	+	+	+	+	nd	+	+	nd	nd	At 46 min + OTC
5	nd	+	nd	+	nd	nd	+/−	+/−	nd	nd	At 50 min
6	+	nd	nd	nd	+	+	+	+	+	+	At 17 min ++
7	+	+	+	nd	+	+	+	+	+	+	OTα (16 min); At 49 min; At 50 min; OTC (53.6 min); 55 min
8	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
9	nd	+	nd	nd	nd	nd	nd	+	nd	nd	nd
10	+	++	+	nd	+	+	+	nd	+	+	OP- OTB (20.8 min); At 17 min; + OTC
11	nd	nd	+/−	+/−	+	+	+/−	+/−	+	+	At 17 min; 27.9 min; 28.7 min; + OTC
12	+	++	nd	+/−	+	++	+/−	nd	+	+	At 50 min; 52 min
13	+	+	+/−	+/−	nd	+	nd	+	nd	+	nd
14	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
15	+	+/−	+	+	++	+	nd	+	nd	+	nd
16	nd	nd	nd	nd	nd	nd	nd	+	nd	nd	OTα 19 min; 28 min
17	nd	nd	++	++	+	+	+	+	nd	+	OP-OTB++; 52 min ++
18	+	+	+	+	++	+	+	+	nd	nd	At 19 min; 52 min
19	+	nd	+	nd	nd	nd	+	+	nd	++	At 17; 19 min; 50 min
20	+	nd	nd	+	++	+	+	+	nd	+	At 17; 19 min; OTC

Abbr.: OTHQ-GSH, ochratoxin A hydroquinone-glutathione conjugate; OTB-GSH, ochratoxin B-glutathione conjugate; OTHQ-NAC, ochratoxin A hydroquinone-N-acetylcysteine; OTB-NAC, ochratoxin B hydroquinone-N-acetylcysteine; DC-OTHQ, decarboxylated ochratoxin A hydroquinone; OP-OA, ochratoxin A open lactone; 4S-OH-OTA, 4S-hydroxy ochratoxin A; 4R–OH–OTA, 4R-hydroxy ochratoxin A; OTHQ, ochratoxin A hydroquinone; OTB, ochratoxin B; OP-OTB, ochratoxin B open lactone.

[a] Retention time of OTA metabolites.

[b] Semi-quantitative analysis (nd - +++); on the basis of fluorescence intensity: inconclusive +/−, weak +, medium ++, intense +++.

[c] nd, not detectable.

[d] Two retention times for OTHQs and OTBs exist with a different elution time.

OTA in urine in general reflects short-term variations in OTA exposure (Castegnaro et al., 2006; Pfohl-Leszkowicz et al., 2006; Gilbert et al., 2001). In patients with impaired renal function and decreased filtration capacity, we could expect an increase in OTA concentration in blood and a decrease in OTA excretion compared to healthy persons with a similar OTA intake (Duarte et al., 2011). The following individuals (No. 12, 16, 17, 18, 19, 21, 23, 27, 28, 33) featured accumulation of OTA in the blood that was accompanied by a reduced elimination. These individuals also have positive CIT in plasma samples and limited excretion of this toxin confirming impaired renal kidney function (Malir et al., 2019). Co-exposure with CIT can explain the reduced excretion, as the presence of CIT modifies the transport of OTA (Pfohl-Leszkowicz et al., 2008).

The biotransformation of OTA in several metabolites is important as not only the half-lives and the route of elimination of them but also the toxicity varies for each individual (Tozlovanu et al., 2006; Li et al., 1997; Hadjeba-Medjdoub et al., 2012). For example, ochratoxin A open lactone (OP-OA) (Li et al., 2000) and ochratoxin C, which is ethyl ester ochratoxin A (OTC), have similar toxicity as OTA (Wu et al., 2011), while OTHQ is more toxic (Faucet-Marquis et al., 2006; Tozlovanu et al., 2006).

Several quinones metabolites including ochratoxin hydroquinone (OTHQ), decarboxylated OTHQ (DC-OTHQ), OTHQ conjugated to glutathione (OTHQ-GSH), OTHQ conjugated to N-acetylcystein (OTHQ-NAC) in addition to 4-S and 4-R–OH–OTA and dechlorinated OTA (OTB), were found in the urine of patients. Interestingly, these metabolites were found even in the urine of patients for which no OTA was detected in blood and urine (No. 5, 6, 9, 10, 11, 14). Thus OTB-GSH and OTHQ-GSH along with the corresponding NAC-conjugates can serve as appropriate biomarkers of OTA exposure as predicted by Tozlovanu et al. (2012). OTC has been found in the urine of several patients (No. 3, 4, 7, 10, 11, 20).

The biotransformation of OTA, which is a chlorinated compound, is complex and involves several biotransforming enzymes such as cytochrome P450s, glutathione S-transferases (GSTs), lipoxygenase, and cyclooxygenase (COX2) present in large quantity in the kidney. The metabolites conjugated to GSH and/or UDP are excreted in bile and kidney (Pfohl-Leszkowicz and Manderville, 2012; Pfohl-Leszkowicz and Castegnaro, 2005; Manderville and Pfohl-Leszkowicz, 2008; Tozlovanu et al., 2006; Pfohl-Leszkowicz et al., 2007b; Hadjeba-Medjdoub et al., 2012). OTQ can either undergo a two-electron reduction by the action of the NAD(P)H:quinone reductase to form OTHQ, or one-electron reduction occurs to yield a semi-quinone (Fig. 5.).

In general, GSTs are involved in detoxifying pathways. However, they contribute in some cases to the reactivity and toxicity of xenobiotics notably by the formation of thiyl radicals that react with macromolecules and yield peroxyl radicals. OTHQ could be formed directly from

Table 5
DNA adducts in the kidney samples of patients.

N°	C-C8dG-OTA (#1)	O6-OTA dG (#2)	OTHQ related adduct (#3) (dG-benzenoadduct)	OTHQ related adduct (#4) (dA-quinone adduct)
1	+[a]	nd[b]	nd	nd
2	+	+	+	+
3	+	nd	nd	nd
4	+	nd	nd	nd
5	nd	nd	nd	nd
6	+	+	+	+
7	+	+	+	+
8	nd	nd	nd	nd
9	nd	nd	nd	nd
10	+	+	+	+
11	+	+	+	+
12	+	+	+	+
13	+	+	+	+
14	nd	nd	nd	nd
15	+	nd	nd	nd
16	nd	nd	nd	nd
17	+	nd	nd	nd
18	+	nd	nd	nd
19	+	nd	nd	nd
20	+	nd	nd	nd

[a] +, detected.
[b] nd, not detected; Abbr.: C-C8dG-OTA, Carbon bound DNA adduct ochratoxin A – deoxyguanosine; O6-OTA dG, DNA adduct ochratoxin A – deoxyguanosine; OTHQ, ochratoxin A hydroquinone.

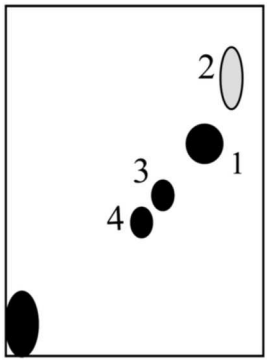

Fig. 4. Scheme of DNA adduct pattern monitored via TLC.
1 = C-C8dG-OTA adduct (OTB-dG); 2 = O-C8dG-OTA adduct; 3 = dG-Quinone adduct = benzenoadduct; 4 = dA-Quinone adduct.

OTA by GST and that can be oxidized in OTQ (Fig. 6.). Indeed, GSTs are involved in dehalogenation: The first step is the formation of an epoxide, while the epoxide is converted in phenol in the second step. This process can lead to OTHQ and/or OTB (Manderville and Pfohl-Leszkowicz, 2008; Faucet-Marquis et al., 2006; Tozlovanu et al., 2012; Manderville et al., 2006).

Chemically, CIT is a quinone derivative that is a pro-oxidant agent susceptible to transform OTA in OTHQ and OTB. In addition, a part of OTA is converted in OP-OA (ochratoxin A open lactone = open ring OA) (Manderville and Pfohl-Leszkowicz, 2008; Pfohl-Leszkowicz et al., 2008). This process represents a non-enzymatic pathway for OTA bioactivation that could play a key role in the synergistic effect observed in presence of both mycotoxins (Manderville and Pfohl-Leszkowicz, 2008; Pfohl-Leszkowicz et al., 2008).

Biological studies have predicted the role for ochratoxin quinone derivatives in OTA-DNA adduct formation (Faucet-Marquis et al., 2006; Tozlovanu et al., 2006; Pfohl-Leszkowicz et al., 2002b; Dai et al., 2002, 2004). OTA induced a different type of DNA adduct as a result of its metabolic transformation. The electrophile OTA metabolites reacted preferentially with deoxyguanine to form benzethenoadduct and C8dG-OTA (Fig. 5.) (Jennings-Gee et al., 2010). One deoxyadenine adduct could also be formed after biotransformation in quinone (Pfohl-Leszkowicz and Castegnaro, 2005). C-C8dG-OTA was found in 15/20 tissues samples we analysed (No. 1, 2, 3, 4, 6, 7, 10, 11, 12, 13, 15, 17, 18, 19, 20). Three other OTA-DNA adducts were observed in 7/20 patients (No. 2, 6, 7, 10, 11, 12, 13). These adducts corresponded to DNA adducts formed in the kidney of rat developing tumours (Faucet-Marquis et al., 2004) as well as in the kidney of pig that has developed OTA related nephropathy (Petkova-Bocharova et al., 2003). It also was previously observed in renal tumours in Bulgarian patients suffering from BEN/UTT (Pfohl-Leszkowicz et al., 1993), in renal tumours of Croatian patients (Pfohl-Leszkowicz, 2009), and in French patients with kidney tumours (Pfohl-Leszkowicz, 2009; Azemar et al., 1998). It has been demonstrated that the preferential formation of either C-C8dG-OTA or OTHQ-DNA adduct depended on the expression of some biotransforming enzymes. Indeed, OTHQ-related DNA adduct was formed after *in vitro* incubation in the presence of kidney microsomes of untreated pig and in healthy human expressing mainly cyclooxygenase COX1 and CYP 2C9, whereas C-C8dG-OTA was formed mainly after incubation in the presence of kidney microsomes from a pig fed with OTA and from a human tumour expressing mainly COX2 and lipooxygenase (Tozlovanu et al., 2006). It is noteworthy that induction of COX2 often occurred during the cancer process, notably in the kidney. Incubation in presence of microsome from the peri-tumoural part of the human kidney has led to the formation of two OTHQ adducts in addition to C-C8dG-OTA (Pfohl-Leszkowicz, 2009; Pfohl-Leszkowicz et al., 2007a; Manderville and Pfohl-Leszkowicz, 2008). The presence of OTB-GSH in samples was correlated to C-C8dG-OTA (OTB-dG), whereas OTHQ-GSH was correlated to dG-OTHQ and O6-C8dG-OTA. This can be explained through the fact that the conjugates (OTB-GSH/OTHQ-GSH) stem from the same electrophiles that were deemed important for OTA-mediated DNA adduction (Pfohl-Leszkowicz and Manderville, 2012; Tozlovanu et al., 2006; Hadjeba-Medjdoub et al., 2012).

DNA-xenobiotic binding is considered a critical step in the initiation of mutagenesis and carcinogenesis. The process of chemical carcinogenesis is initiated by the covalent binding of carcinogens or their reactive metabolites to DNA, thus forming DNA-adducts (Miller and Miller, 1981). A good correlation between DNA-adducts formation and the frequency of mutations (Lutz and Gaylor, 1996) and with the incidence of tumours was observed in animals (Poirier and Beland, 1992).

Akman et al. demonstrated using human mutation reporter plasmid pSP189 that OTA in presence of microsomal enzymes or by transition metal ions induced mutation after conversion into a genotoxic compound. Synthetic ochratoxin hydroquinone (OTHQ) was also mutagenic (Akman et al., 2012).

An increase in mutant frequency, as well as induction of double-strand breaks and deletion (frameshift) mutations at the red/gam gene at the carcinogenic target site of gpt delta transgenic rats strongly suggested the involvement of a genotoxic mechanism(s) in OTA-mediated carcinogenesis (Hibi et al., 2013a, 2013b).

OTB-dG has a tendency to adopt a mixture of major groove, wedge, and base displaced intercalated conformations when paired against complementary cytosine in the NarI sequence. The induction of double-strand breaks indicated that OTB-dG was not easily bypassed during replication by alternate enzymes (Kathuria et al., 2017; Sharma et al., 2014). The degree of misincorporation induced by the C-linked C8-dG adducts correlated with an ability to adopt the promutagenic syn-conformation within the NarI duplex as predicted by molecular dynamics simulations (Manderville and Wetmore, 2017). The dominant mutations induced by OTA were G → C transversions (Kathuria et al., 2018). Marin et al. recently observed that OTA altered miRNAs that were strongly connected to the engine of cancer, disturbing nodal points in different pathways such as TP53 signaling (Marin et al., 2019).

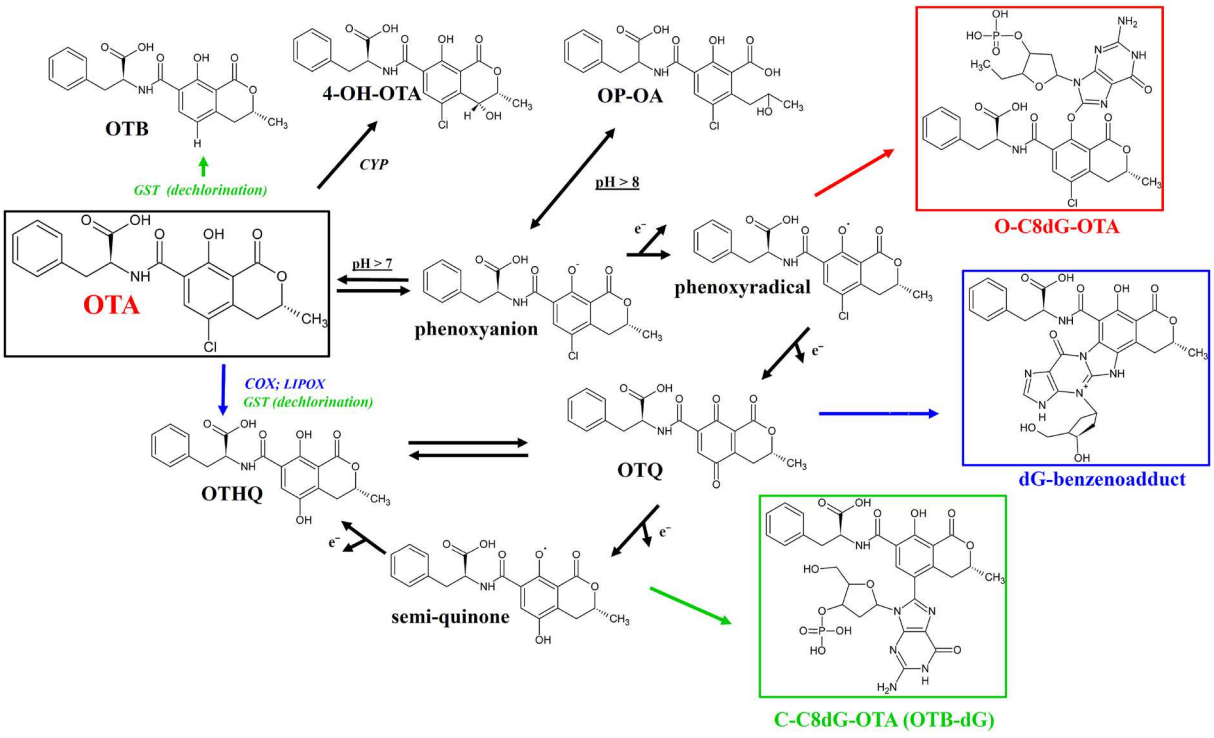

Fig. 5. OTA metabolites and DNA adduct formation
Abbr.:OTA, ochratoxin A; OTB, ochratoxin B; 4-OH-OTA, 4-hydroxy ochratoxin A; OP-OA, ochratoxin A open lactone; OTHQ, ochratoxin A hydroquinone; OTQ, ochratoxin A quinone; C-C8dG-OTA, DNA adduct ochratoxin A-deoxyguanosine; O-C8dG-OTA, DNA adduct ochratoxin A-deoxyguanosine.

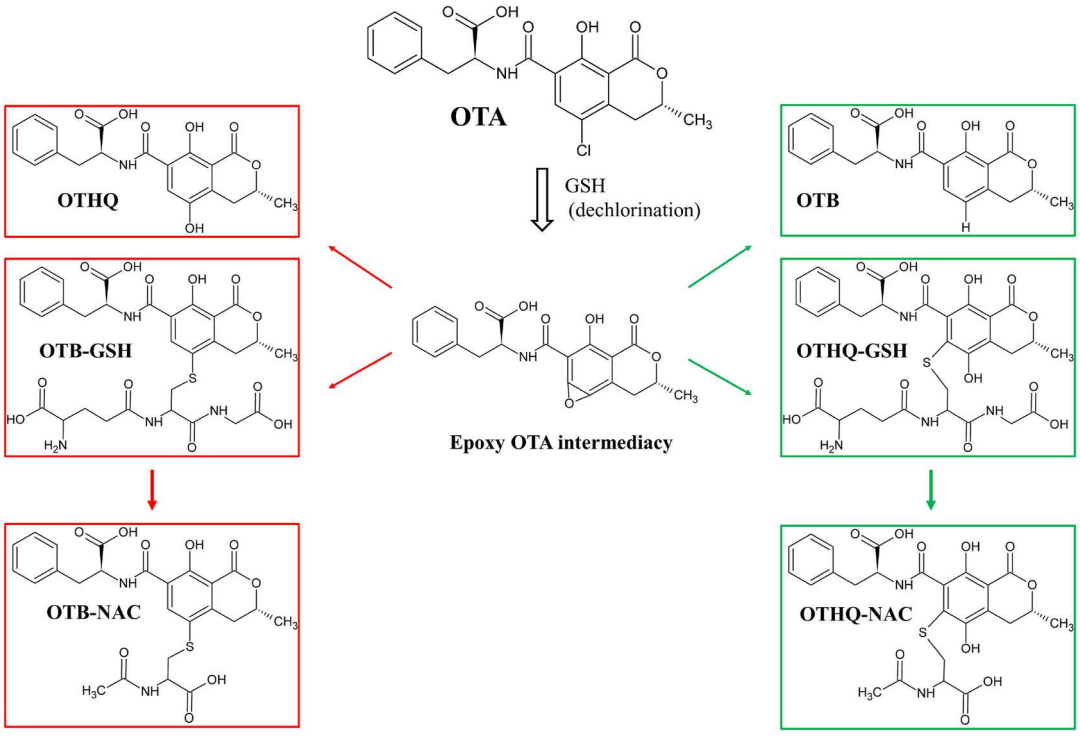

Fig. 6. The metabolites conjugated to GSH (glutathione) and NAC (N-acetylcystein)
Abbr.:OTA, ochratoxin A; OTHQ, ochratoxin A hydroquinone; OTB-GSH, ochratoxin B-glutathione conjugate; OTB-NAC, ochratoxin B hydroquinone-N-acetylcysteine; OTB, ochratoxin B; OTHQ-GSH, ochratoxin A hydroquinone-glutathione conjugate; OTHQ-NAC, ochratoxin A hydroquinone-N-acetylcysteine.

6. Conclusions

The presence of a chemical-specific DNA adduct in human DNA is a good indication of exposure to OTA (Swenberg, 2004). Carcinogenic DNA adducts in target tissues are more relevant markers than internal dose because the former reflects not only individual differences in

absorption and distribution but also differences in the metabolism (activation *versus* detoxification) and the extent of repair of DNA damage (Pfohl-Leszkowicz, 2008). Unlike the DNA adduct, OTB-dG, OTB-GSH, and OTHQ-GSH are stable to acid treatment and can be extracted from biological samples using methodology established for extraction of OTA. Thus, OTB-GSH and OTHQ-GSH along with the corresponding NAC-conjugates can serve as biomarkers of OTA exposure as predicted elsewhere (Tozlovanu et al., 2012). Our study confirmed the link between the presence of these metabolites in urine and the presence of OTA-DNA adduct in the patients suffering from RCC.

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Ethics approval

Approval for the biomonitoring study protocol was obtained from the Ethics Committee of the Charles University Medical School and Teaching Hospital, Sokolska 581, CZ – 500 05 Hradec Kralove (Reg. No. 201406 S02P). All participants responsible were informed about the scope and aim of this study. Written informed consent was obtained from all patients included in the study.

The study design was carried out in line with applicable ethical standards derived from the 1964 World Medical Association Declaration of Helsinki (ethical principles for medical research involving human subjects) and its nine amendments from years 1975–2013.

CRediT authorship contribution statement

Frantisek Malir: Conceptualization, Methodology, Project administration, Supervision, Writing – original draft, Writing – review & editing. **Miroslav Louda:** Methodology, Resources, Visualization, Writing – review & editing. **Jakub Toman:** Data curation, Formal analysis, Funding acquisition, Investigation, Validation, Visualization, Writing – review & editing. **Vladimir Ostry:** Conceptualization, Funding acquisition, Methodology, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Darina Pickova:** Formal analysis, Funding acquisition, Visualization, Writing – review & editing. **Jaroslav Pacovsky:** Methodology, Resources, Visualization, Writing – review & editing. **Milos Brodak:** Methodology, Resources, Visualization, Writing – review & editing. **Annie Pfohl-Leszkowicz:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

Akman, S., Adams, M., Case, D., Park, G., Manderville, R., 2012. Mutagenicity of ochratoxin A and its hydroquinone metabolite in the SupF gene of the mutation reporter plasmid Ps189. Toxins 4 (4), 267–280. https://doi.org/10.3390/toxins4040267.

Aurilio, G., Piva, F., Santoni, M., Cimadamore, A., Sorgentoni, G., Lopez-Beltran, A., et al., 2019. The role of obesity in renal cell carcinoma patients: clinical-pathological implications. Int. J. Mol. Sci. Multidiscip. Digit. Publ. Inst. 20 (22), 5683. https://doi.org/10.3390/ijms20225683.

Azemar, B., Pinelli, E., Plante, P., Escourrou, G., Petkova Bocharova, T., Pfohl-Leszkowicz, A., 1998. Some human kidney tumours in France exhibited a specific ochratoxin A-DNA adduct pattern. Revue de Medecine Veterinaire (France) 149 (6), 653.

Boorman, G., 1989. Toxicology and Carcinogenesis Studies of Ochratoxin A (CAS No. 303-47-9) in F344/N Rats (Gavage Studies). U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, Research Triangle Park NC, USA.

Bosetti, C., Bertuccio, P., Chatenoud, L., Negri, E., Vecchia, C.L., Levi, F., 1970–2008. Trends in mortality from urologic cancers in Europe. Eur Urol. Elsevier 60 (1), 1–15. https://doi.org/10.1016/j.eururo.2011.03.047, 2011.

TCGA., Cancer Genome Atlas Research Network, 2013. Comprehensive molecular characterization of clear cell renal cell carcinoma. Nature 499 (7456), 43–49. https://doi.org/10.1038/nature12222.

CanCon, 2014. (Cancer Control joint action) Czech national cancer Control programme [internet]. Cancer Burden in the Czech Republic: European and Worldwide Comparison [cited 2020 Oct 2]. Available from. https://www.onconet.cz/index-en.php?pg=news&aid=979.

Castegnaro, M., Mohr, U., Pfohl-Leszkowicz, A., Estève, J., Steinmann, J., Tillmann, T., et al., 1998. Sex-and strain-specific induction of renal tumours by ochratoxin A in rats correlates with DNA adduction. In: Int J Cancer, 77. Wiley Online Library, pp. 70–75, 1.

Castegnaro, M., Canadas, D., Vrabcheva, T., Petkova-Bocharova, T., Chernozemsky, I.N., Pfohl-Leszkowicz, A., 2006. Balkan endemic nephropathy: role of ochratoxins A through biomarkers. Mol. Nutr. Food Res. 50 (6), 519–529. https://doi.org/10.1002/mnfr.200500182.

Ceci, E., Bozzo, G., Bonerba, E., Di Pinto, A., Tantillo, M.G., 2007. Ochratoxin A detection by HPLC in target tissues of swine and cytological and histological analysis. Food Chem. 105 (1), 364–368. https://doi.org/10.1016/j.foodchem.2006.12.019.

Chan, C-K., Liu, Y., Pavlović, N.M., Chan, W., 2018. Etiology of balkan endemic nephropathy: an update on aristolochic acids exposure mechanisms. Chem. Res. Toxicol. Am. Chem. Soc. 31 (11), 1109–1110. https://doi.org/10.1021/acs.chemrestox.8b00291.

Cohen, S.M., Arnold, L.L., 2011. Chemical carcinogenesis. Toxicol. Sci. Oxford Acad. 120 (Suppl 1), S76–S92. https://doi.org/10.1093/toxsci/kfq365.

Coppa, C.F.S.C., Khaneghah, A.M., Alvito, P., Assunção, R., Martins, C., Eş, I., et al., 2019. The occurrence of mycotoxins in breast milk, fruit products and cereal-based infant formula: a review. Trends Food Sci. Technol. 92, 81–93. https://doi.org/10.1016/j.tifs.2019.08.014.

Dai, J., Park, G., Wright, M., Adams, M., Akman, S., Manderville, R., 2002. Detection and characterization of a glutathione conjugate of ochratoxin A. Chem. Res. Toxicol. 15 (12), 1581–2158. https://doi.org/10.1021/tx0255929.

Dai, J., Park, G., Perry, J.L., Il'ichev, Y.V., Bow, D.A., Pritchard, J.B., et al., 2004. Molecular aspects of the transport and toxicity of ochratoxin A. Acc. Chem. Res. ACS Publ. 37 (11), 874–881. https://doi.org/10.1021/ar0302134.

De Ruyck, K., De Boevre, M., Huybrechts, I., De Saeger, S., 2015. Dietary mycotoxins, co-exposure, and carcinogenesis in humans: short review. Mutat. Res. Rev. Mutat. Res. 766, 32–41. https://doi.org/10.1016/j.mrrev.2015.07.003.

Dohnal, V., Dvořák, V., Malíř, F., Ostrý, V., Roubal, T., 2013. A comparison of ELISA and HPLC methods for determination of ochratoxin A in human blood serum in the Czech Republic. Food Chem. Toxicol. 62, 427–431. https://doi.org/10.1016/j.fct.2013.09.010.

Duarte, S.C., Pena, A., Lino, C.M., 2009. Ochratoxin A non-conventional exposure sources — a review. Microchem. J. 93 (2), 115–120. https://doi.org/10.1016/j.microc.2009.06.002.

Duarte, S.C., Pena, A., Lino, C.M., 2011. Human ochratoxin A biomarkers-From exposure to effect. Crit. Rev. Toxicol. 41 (3), 187–2012. https://doi.org/10.3109/10408444.2010.529103.

Dusek, L., Muzik, J., Kubasek, M., Kopitkova, J., Zaloudik, J., Vyzula, R., 2012. Multimedia support in the education of clinical and health care disciplines. Portal of MU's Faculty of Medicine [Internet]. Epidemiology of Malignant Tumours in the Czech Republic. Masaryk University [cited 2020 Apr 18]. Available from: https://portal.med.muni.cz/article-584-epidemiology-of-malignant-tumours-in-the-czech-republic.html.

European Food Safety Authority, 2020. Risk assessment of ochratoxin A in food, 5. In: EFSA J, 18. John Wiley & Sons, pp. 1–150. https://doi.org/10.2903/j.efsa.2020.6113.

Faucet-Marquis, V., Pfohl-Leszkowicz, A., Dai, J., Castegnaro, M., Manderville, R., 2004. Evidence for covalent DNA adduction by ochratoxin A following chronic exposure to rat and subacute exposure to pig. Chem. Res. Toxicol. 17 (9), 1289–1296. https://doi.org/10.1021/tx049877s.

Faucet-Marquis, V., Pont, F., Størmer, F., Rizk, T., Castegnaro, M., Pfohl-Leszkowicz, A., 2006. Evidence of a new dechlorinated Ochratoxin A derivative formed in opossum kidney cell cultures after pretreatment by modulators of glutathione pathways: correlation with DNA-adduct formation. Mol. Nutr. Food Res. 50 (6), 530–542. https://doi.org/10.1002/mnfr.200500219.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., et al., 2015. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. In: Int J Cancer, 136. Wiley Online Library, pp. E359–E386. https://doi.org/10.1002/ijc.29210, 5.

Frenette, C., Paugh, R., Tozlovanu, M., Juzio, M., Pfohl-Leszkowicz, A., Manderville, R., 2008. Structure-activity relationships for the fluorescence of ochratoxin A: insight for detection of ochratoxin A metabolites. Anal. Chim. Acta 617 (1–2), 153–161. https://doi.org/10.1016/j.aca.2007.12.030.

Gilbert, J., Brereton, P., MacDonald, S., 2001. Assessment of dietary exposure to ochratoxin A in the UK using a duplicate diet approach and analysis of urine and plasma samples, 12. In: Food Addit Contam, 18. Taylor & Francis, pp. 1088–1093. https://doi.org/10.1080/02652030110070030.

Grajewski, J., Jarzemski, P., Twaruzek, M., Kuzminska, K., Trepala, M., 2007. The level of ochratoxin A in patients after nephrectomy. Mycotoxin Res. 23 (1), 22–26. https://doi.org/10.1007/BF02946020.

Hadjeba-Medjdoub, K., Tozlovanu, M., Pfohl-Leszkowicz, A., Frenette, C., Paugh, R., Manderville, R., 2012. Structure-activity relationships imply different mechanisms of action for ochratoxin A-mediated cytotoxicity and genotoxicity. Chem. Res. Toxicol. 25 (1), 181–190. https://doi.org/10.1021/tx200406c.

Hibi, D., Kijima, A., Kuroda, K., Suzuki, Y., Ishii, Y., Jin, M., et al., 2013a. Molecular mechanisms underlying ochratoxin A-induced genotoxicity: global gene expression analysis suggests induction of DNA double-strand breaks and cell cycle progression. J. Toxicol. Sci. 38 (1), 57–69. https://doi.org/10.2131/jts.38.57.

Hibi, D., Kijima, A., Suzuki, Y., Ishii, Y., Jin, M., Sugita-Konishi, Y., et al., 2013b. Effects of p53 knockout on ochratoxin A-induced genotoxicity in p53-deficient gpt delta mice. Toxicology 304, 92–99. https://doi.org/10.1016/j.tox.2012.12.005.

Hoang, M.L., Chen, C.-H., Chen, P.-C., Roberts, N.J., Dickman, K.G., Yun, B.H., et al., 2016. Aristolochic acid in the etiology of renal cell carcinoma, 12. In: Cancer Epidemiol. Biomark. Prev., 25 American Association for Cancer Research, pp. 1600–1608. https://doi.org/10.1158/1055-9965.EPI-16-0219.

Hornung, R.W., Reed, L.D., 1990. Estimation of average concentration in the presence of nondetectable values, 1. In: Appl Occup Environ Hyg, 5. Taylor & Francis, pp. 46–51. https://doi.org/10.1080/1047322X.1990.10389587.

Horwitz, W., 1995. Protocol for the design, conduct and interpretation of method-performance studies: revised 1994 (Technical Report), 2. In: Pure & Appl Chem, 67. Walter de Gruyter GmbH, pp. 331–343. https://doi.org/10.1351/pac199567020331.

Hsieh, J.J., Purdue, M.P., Signoretti, S., Swanton, C., Albiges, L., Schmidinger, M., et al., 2017. Renal cell carcinoma, 1. In: Nat. Rev. Dis. Prim., 3 Nature Publishing Group, pp. 1–19. https://doi.org/10.1038/nrdp.2017.9.

Hsu, C.C., Chow, W.-H., Boffetta, P., Moore, L., Zaridze, D., Moukeria, A., et al., 2007. Dietary Risk Factors for Kidney Cancer in Eastern and Central Europe. In: Am J Epidemiol, vol. 166. Oxford Academic, pp. 62–70. https://doi.org/10.1093/aje/kwm043, 1.

Hunt, J.D., Hel van der, O.L., McMillan, G.P., Boffetta, P., Brennan, P., 2005. Renal cell carcinoma in relation to cigarette smoking: meta-analysis of 24 studies. Int. J. Cancer 114 (1), 101–108. https://doi.org/10.1002/ijc.20618.

IARC., International Agency for Research on Cancer, 1993. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans: Some Naturally Occurring Substances: Food Items and Costituents, Heterocyclic Aromatic Amines and Mycotoxins [Internet]. IARC Press, Lyon, France [cited 2020 Oct 11]. Available from: http://monographs.iarc.fr/ENG/Monographs/vol56/mono56.pdf.

JECFA FAO/WHO, 2007. Evaluation of Certain Food Additives and Contaminants. Sixty-eight report of the Joint FAO/WHO Expert Committee on Food Additives. [Internet]. WHO Technical Report Series 947. World Health Organization, Geneva, Switzerland [cited 2020 Apr 18]. Available from: https://apps.who.int/iris/bitstream/handle/10665/43870/9789241209472_eng.pdf?sequence=1&isAllowed=y.

Jennings-Gee, J., Tozlovanu, M., Manderville, R., Miller, M., Pfohl-Leszkowicz, A., Schwartz, G., Ochratoxin, A., 2010. In Utero exposure in mice induces adducts in testicular DNA. Toxins 2 (6), 1428–1444. https://doi.org/10.3390/toxins2061428.

Kathuria, P., Sharma, P., Manderville, R., Wetmore, S., 2017. Molecular modeling of the major DNA adduct formed from food mutagen ochratoxin A in nar I two-base deletion duplexes: impact of sequence context and adduct ionization on conformational preference and mutagenicity. Chem. Res. Toxicol. 30 (8), 1582–1591. https://doi.org/10.1021/acs.chemrestox.7b00103.

Kathuria, P., Sharma, P., Manderville, R.A., Wetmore, S.D., 2018. Molecular dynamics simulations of mismatched DNA duplexes associated with the major C8-linked 2′-deoxyguanosine adduct of the food mutagen ochratoxin A: influence of opposing base, adduct ionization state, and sequence on the structure of damaged DNA. Chem. Res. Toxicol. 31 (8), 712–720. https://doi.org/10.1021/acs.chemrestox.8b00064.

Klaassen, C.D., 1986. Distribution, excretion, and absorption of toxicants. In: Klassen, C. D., Amdur, M.O., Doull, J. (Eds.), Casarett and Doull's Toxicology the Basic Science of Poisons, third ed. Macmillan, New York, pp. 33–63.

Kuiper-Goodman, T., 1996. Risk assessment of ochratoxin A: an update. Food Addit. Contam. 13 (Suppl. l), 53–57.

Kuiper-Goodman, T., Hilts, C., Billiard, S.M., Kiparissis, Y., Richard, I.D.K., Hayward, S., 2010. Health risk assessment of ochratoxin A for all age-sex strata in a market economy. Food Addit. Contam. 27 (2), 212–240. https://doi.org/10.1080/02652030903013278.

Kuroda, K., Hibi, D., Ishii, Y., Takasu, S., Kijima, A., Matsushita, K., et al., 2013. Ochratoxin A induces DNA double-strand breaks and large deletion mutations in the carcinogenic target site of gpt delta rats. Mutagenesis 29, 27–36. https://doi.org/10.1093/mutage/get054.

Kuroda, K., Hibi, D., Ishii, Y., Yokoo, Y., Takasu, S., Kijima, A., et al., 2015. Role of p53 in the progression from ochratoxin A-induced DNA damage to gene mutations in the kidneys of mice. Toxicol. Sci. Oxford Acad. 144 (1), 65–76. https://doi.org/10.1093/toxsci/kfu267.

Li, S., Marquardt, R.R., Frohlich, A.A., Vitti, T.G., Crow, G., 1997. Pharmacokinetics of ochratoxin A and its metabolites in rats. Toxicol. Appl. Pharmacol. 145 (1), 82–90. https://doi.org/10.1006/taap.1997.8155.

Li, S., Marquardt, R., Frohlich, A.A., 2000. Identification of Ochratoxins and some of their metabolites in bile and urine of rats. Food Chem. Toxicol. 38 (2–3), 141–152. https://doi.org/10.1016/S0278-6915(99)00153-2.

Lock, E.A., Hard, G.C., 2004. Chemically induced renal tubule tumors in the laboratory rat and mouse: review of the NCI/NTP database and categorization of renal carcinogens based on mechanistic information. In: Crit Rev Toxicol, 34. Taylor & Francis, pp. 211–299. https://doi.org/10.1080/10408440490265210, 3.

Lutz, W., Gaylor, D., 1996. Significance of DNA adducts at low dose: shortening the time to spontaneous tumor occurrence. Regul. Toxicol. Pharmacol. 23 (1), 29–34. https://doi.org/10.1006/rtph.1996.0005.

Malir, F., Roubal, T., Brndiar, M., Osterreicher, J., Severa, J., Knizek, J., et al., 2001. Ochratoxin a in the Czech republic. In: J Toxicol Toxin Rev, 20. Taylor & Francis, pp. 261–274. https://doi.org/10.1081/TXR-100108560, 3–4.

Malir, F., Ostry, V., Pfohl-Leszkowicz, A., Roubal, T., 2012. Ochratoxin A exposure biomarkers comparison with foreign countries. Biomarkers 17 (7), 577–589. https://doi.org/10.3109/1354750X.2012.692392.

Malir, F., Ostry, V., Dofkova, M., Roubal, T., Dvorak, V., Dohnal, V., 2013a. Ochratoxin A levels in blood serum of Czech women in the first trimester of pregnancy and its correspondence with dietary intake of the mycotoxin contaminant. Biomarkers 18 (8), 673–678. https://doi.org/10.3109/1354750X.2013.845609.

Malir, F., Ostry, V., Novotna, E., 2013b. Toxicity of the mycotoxin ochratoxin A in the light of recent data. Toxin Rev. 32 (2), 19–33. https://doi.org/10.3109/15569543.2013.782504.

Malir, F., Louda, M., Ostry, V., Toman, J., Ali, N., Grosse, Y., et al., 2019. Analyses of biomarkers of exposure to nephrotoxic mycotoxins in a cohort of patients with renal tumours. Mycotoxin Res. 35 (4), 391–403. https://doi.org/10.1007/s12550-019-00365-9.

Mally, A., 2012. Ochratoxin A and mitotic disruption: mode of action analysis of renal tumor formation by ochratoxin A, 2. In: Toxicol sci., 127 Oxford Academic, pp. 315–330. https://doi.org/10.1093/toxsci/kfs105.

Manderville, R., Pfohl-Leszkowicz, A., 2008. Bioactivation and DNA adduction as a rationale for ochratoxin A carcinogenesis. World Mycotoxin J. 1 (3), 357–367. https://doi.org/10.3920/WMJ2008.x039.

Manderville, R.A., Wetmore, S.D., 2017. Mutagenicity of ochratoxin A: role for a carbon-linked C8-deoxyguanosine adduct? https://doi.org/10.1021/acs.jafc.6b03897, 65,33,7097,7105.

Manderville, R.A., Pfohl-Leszkowicz, A., 2006. Genotoxicity of chlorophenols and ochratoxin A. In: Fishbein, J.C. (Ed.), Fishbein, JC Advances in Molecular Toxicology [Internet], first ed. Elsevier [cited 2021 May 14]. p. 85–138. Available from: https://www.sciencedirect.com/science/article/pii/S1872085406010046.

Mantle, P.G., 2009. Minimum tolerable exposure period and maximum threshold dietary intake of ochratoxin A for causing renal cancer in male Dark Agouti rats. Food Chem. Toxicol. 47 (10), 2419–2424. https://doi.org/10.1016/j.fct.2009.05.043.

Mantle, P.G., Faucet-Marquis, V., Manderville, R.A., Squillaci, B., Pfohl-Leszkowicz, A., 2010. Structures of covalent adducts between DNA and ochratoxin A: a new factor in debate about genotoxicity and human risk assessment. Chem. Res. Toxicol. Am. Chem. Soc. 23 (1), 89–98. https://doi.org/10.1021/tx900295a.

Marin, D., Braicu, C., Dumitrescu, G., Pistol, G., Cojocneanu, R., Berindan - Neagoe, I., et al., 2019. MicroRNA profiling in kidney in pigs fed ochratoxin A contaminated diet. Ecotoxicol. Environ. Saf. 184, 1–9. https://doi.org/10.1016/j.ecoenv.2019.109637.

Milićević, D., Jurić, V., Stefanović, S., Jovanović, M., Janković, S., 2008. Survey of slaughtered pigs for occurrence of ochratoxin A and porcine nephropathy in Serbia. Int. J. Mol. Sci. Mol. Divers. Preserv. Int. 9 (11), 2169–2183. https://doi.org/10.3390/ijms9112169.

Miller, E., Miller, J., 1981. Searches for ultimate chemical carcinogens and their reactions with cellular macromolecules. Cancer 47 (10), 2327–2345. https://doi.org/10.1002/1097-0142(19810515)47:10<2327::AID-CNCR2820471003>3.0.CO;2-Z.

WHO. International Agency for Research on Cancer [Internet]. WHO Classification of Tumours Online. 2020 [cited 2020 Mar 23]. Available from: https://tumourclassification.iarc.who.int.

Ojuri, O.T., Ezekiel, C.N., Sulyok, M., Ezeokoli, O.T., Oyedele, O.A., Ayeni, K.I., et al., 2018. Assessing the mycotoxicological risk from consumption of complementary foods by infants and young children in Nigeria. Food Chem. Toxicol. 121, 37–50. https://doi.org/10.1016/j.fct.2018.08.025.

Ostry, V., Malir, F., Roubal, T., Skarkova, J., Ruprich, J., Cerna, M., et al., 2005. Monitoring of mycotoxin biomarkers in the Czech Republic. Mycotoxin Res. 21 (1), 49. https://doi.org/10.1007/BF02954817.

Ostry, V., Malir, F., Ruprich, J., 2013. Producers and important dietary sources of ochratoxin A and citrinin. Toxins 5 (9), 1574–1586. https://doi.org/10.3390/toxins5091574.

Ostry, V., Malir, F., Toman, J., Grosse, Y., 2017. Mycotoxins as human carcinogens - the IARC Monographs classification. Mycotoxin Res. 33 (1), 65–73. https://doi.org/10.1007/s12550-016-0265-7.

Petkova-Bocharova, T., Adlouni, C.E., Faucet, V., Pfohl-Leszkowicz, A., Mantle, P.G., 2003. Analysis for DNA adducts, ochratoxin a content and enzyme expression in kidneys of pigs exposed to mild experimental chronic ochratoxicosis. FU Med. Biol. 10 (3), 111–115.

Pfohl-Leszkowicz, A., 2008. Formation, persistence and significance of DNA adduct formation in relation to some pollutants from a broad perspective. Adv. Mol. Toxicol. 2, 183–239. https://doi.org/10.1016/S1872-0854(07)02007-3.

Pfohl-Leszkowicz, A., 2009. Ochratoxin A and aristolochic acid involvement in nephropathies and associated urothelial tract tumours. Arh. Hig. Rada. Toksikol. 60 (4), 465–483. https://doi.org/10.2478/10004-1254-60-2009-2000.

Pfohl-Leszkowicz, A., Castegnaro, M., 2005. Further arguments in favour of direct covalent binding of Ochratoxin A (OTA) after metabolic biotransformation. Food Addit. Contam. 22 (Suppl 1), 75–87. https://doi.org/10.1080/02652030500309400. Suppl 1.

Pfohl-Leszkowicz, A., Manderville, R.A., 2012. An update on direct genotoxicity as a molecular mechanism of ochratoxin A carcinogenicity. Chem. Res. Toxicol. Am. Chem. Soc. 25 (2), 252–262. https://doi.org/10.1021/tx200430f.

Pfohl-Leszkowicz, A., Grosse, Y., Castegnaro, M., Nicolov, I.G., Chernozemsky, I.N., Bartsch, H., et al., 1993. Ochratoxin A-related DNA adducts in urinary tract tumours of Bulgarian subjects. IARC Sci. Publ. 124 (124), 141–148.

Pfohl-Leszkowicz, A., Petkova-Bocharova, T., Chernozemsky, I.N., Castegnaro, M., 2002a. Balkan endemic nephropathy and associated urinary tract tumours: a review on aetiological causes and the potential role of mycotoxins. Food Addit. Contam. 19 (3), 282–302. https://doi.org/10.1080/02652030110079815.

Pfohl-Leszkowicz, A., Bartsch, H., Azémar, B., Mohr, U., Estève, J., Castegnaro, M., 2002b. MESNA protects rats against nephrotoxicity but not carcinogenicity induced by ochratoxin A, implicating two separate pathways. Facta Univ. Ser. Med. Biol. Citeseer 9 (1), 57–63.

Pfohl-Leszkowicz, A., Vrabcheva, T., Petkova-Bocharova, T., Garren, L., Grosso, F., Nikolov, I., et al., 2006. Analysis of Ochratoxin a in Serum, Urine and Food Consumed by Inhabitants from an Area with Balkan Endemic Nephropathy: a One Month Follow up Study. In: Njapau, H., Trujillio, S., Van Egmond, H.P., Park, D.L. (Eds.), Mycotoxins and Phycotoxins Advances in Determination, Toxicology and Exposure Management. Wageningen Academic Publishers, The Netherlands. https://doi.org/10.3920/978-90-8686-585-7 cited 2020 Dec 8]. p. 217–24. Available from:

Pfohl-Leszkowicz, A., Tozlovanu, M., Manderville, R., Peraica, M., Castegnaro, M., Stefanovic, M., 2007a. New molecular and field evidences for the implication of mycotoxins but not aristolochic acid in human nephropathy and urinary tract tumor. Mol. Nutr. Food Res. 51 (9), 1131–1146. https://doi.org/10.1002/mnfr.200700045.

Pfohl-Leszkowicz, A., Manderville, R., Ochratoxin, A., 2007b. An overview on toxicity and carcinogenicity in animals and humans. Mol. Nutr. Food Res. 51 (1), 61–99. https://doi.org/10.1002/mnfr.200600137.

Pfohl-Leszkowicz, A., Molinié, A., Tozlovanu, M., Manderville, R.A., Siantar, D.P., Trucksess, M.W., Scott, P.M., Herman, E.M., 2008. Combined toxic effects of ochratoxin A and citrinin, In Vivo and in Vitro. In: Food Contaminants [Internet]. American Chemical Society. https://doi.org/10.1021/bk-2008-1001.ch003 cited 2020 Apr 18]. p. 56–79. Available from:

Poirier, M., Beland, F., 1992. DNA adduct measurements and tumor incidence during chronic carcinogen exposure in animal models: implications for DNA adduct-based human cancer risk assessment. Chem. Res. Toxicol. 5 (6), 749–755. https://doi.org/10.1021/tx00030a003.

R-Biopharm - Rhône Ltd, 2014. OCHRAPREP®. Immunoaffinity columns for use in conjunction with HPLC or LC MS/MS. For in vitro use only. Version P14/V16/22.09.14. Darmstadt, Germany.

Ringot, D., Chango, A., Schneider, Y.-J., Larondelle, Y., 2006. Toxicokinetics and toxicodynamics of ochratoxin A, an update. Chem. Biol. Interact. 159 (1), 18–46. https://doi.org/10.1016/j.cbi.2005.10.106.

Robert Koch Institut R., 2019. Krebs in Deutschland für 2015/2016 [Internet]. Berlin, Germany [cited 2020 Apr 18]. Available from: https://doi.org/10.25646/5977.

Sanfilippo, K.M., McTigue, K.M., Fidler, C.J., James, D.N., Chang, Y., Fried, L.F., et al., 2014. Hypertension and obesity and the risk of kidney cancer in 2 large cohorts of US men and women, 5. In: Hypertension, 63. American Heart Association, pp. 934–941. https://doi.org/10.1161/HYPERTENSIONAHA.113.02953.

Schwartz, G.G., 2002. Hypothesis: does ochratoxin A cause testicular cancer?, 1. In: Cancer Causes & Control, 13. Springer, pp. 91–100.

Schwartz, G.G., Manderville, R.A., Pfohl-Leszkowicz, A., 2010. Response to comments of peter G. Mantle, 10. In: Toxins, 2. Molecular Diversity Preservation International, pp. 2337–2339. https://doi.org/10.3390/toxins2102337.

Selvaraj, J.N., Wang, Y., Zhou, L., Zhao, Y., Xing, F., Dai, X., et al., 2015. Recent mycotoxin survey data and advanced mycotoxin detection techniques reported from China: a review, 4. In: Food Addit Contam Part A, 32. Taylor & Francis, pp. 440–452. https://doi.org/10.1080/19440049.2015.1010185.

Sharma, P., Manderville, R., Wetmore, S., 2014. Structural and energetic characterization of the major DNA adduct formed from the food mutagen ochratoxin A in the NarI hotspot sequence: influence of adduct ionization on the conformational preferences and implications for the NER propensity. Nucleic Acids Res. 42 (18), 11831–11845. https://doi.org/10.1093/nar/gku821.

Slack, R., Cherrie, J., Tongeren, M.V., Fortunato, L., Hutchings, S., Rushton, L., 2012. The Burden of Occupational Cancer in Great Britain: Kidney Cancer [Internet]. London, UK, p. 1–45. Report No.: RR854 [cited 2020 Apr 18]. Available from: https://www.hse.gov.uk/research/rrpdf/rr854.pdf.

Stoev, S., 2017. Balkan Endemic Nephropathy – still continuing enigma, risk assessment and underestimated hazard of joint mycotoxin exposure of animals or humans. Chem. Biol. Interact. 261 (5), 63–79. https://doi.org/10.1016/j.cbi.2016.11.018.

Studer-Rohr, I., Schlatter, J., Dietrich, D.R., 2000. Kinetic parameters and intraindividual fluctuations of ochratoxin A plasma levels in humans. Arch. Toxicol. 74 (9), 499–510. https://doi.org/10.1007/s002040000157.

Swenberg, J.A., 2004. Toxicological considerations in the application and interpretation of DNA adducts in epidemiological studies. IARC Sci. Publ. 157, 237–246.

Tesar, V., Viklicky, O., Bartonickova, K., Boucek, P., Bürgelova, M., Certikova-Chabova, V., et al., 2015. Klinická Nefrologie, 2., Zcela Přepracované Vydání (Clinical Nephrology, 2nd completely revised edition second ed. Grada Publishing, a. s., Prague.

Thompson, M., Ellison, S.L., Wood, R., 2002. Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report), 5. In: Pure Appl Chem, 74. De Gruyter, pp. 835–855. https://doi.org/10.1351/pac200274050835.

Timbrell, J.A., 1998. Biomarkers in toxicology. Toxicology 129 (1), 1–12. https://doi.org/10.1016/S0300-483X(98)00058-4.

Tozlovanu, M., Pfohl-Leszkowicz, A., 2010. Ochratoxin A in roasted coffee from French supermarkets and transfer in coffee beverages: comparison of analysis methods, 8. In: Toxins, 2. Molecular Diversity Preservation International, pp. 1928–1942. https://doi.org/10.3390/toxins2081928.

Tozlovanu, M., Faucet-Marquis, V., Pfohl-Leszkowicz, A., Manderville, R.A., 2006. Genotoxicity of the hydroquinone metabolite of ochratoxin A: structure-activity relationships for covalent DNA adduction. Chem. Res. Toxicol. 19 (9), 1241–1247. https://doi.org/10.1021/tx060138g.

Tozlovanu, M., Canadas, D., Pfohl-Leszkowicz, A., Frenette, C., Paugh, R.J., Manderville, R.A., 2012. Glutathione conjugates of ochratoxin A as biomarkers of exposure, 4. In: Arh Hig Rada Toksikol, 63. Institut za medicinska istraživanja i medicinu rada, pp. 417–426. https://doi.org/10.2478/10004-1254-63-2012-2202.

Wu, Q., Dohnal, V., Huang, L., Kuca, K., Wang, X., Chen, G., et al., 2011. Metabolic pathways of ochratoxin A. Curr. Drug Metabol. 12 (1), 1–10. https://doi.org/10.2174/138920011794520026.

Zepnik, H., Pähler, A., Schauer, U., Dekant, W., 2001. Ochratoxin A-induced tumor formation: is there a role of reactive ochratoxin A metabolites? Toxicol. Sci. Oxford Acad. 59 (1), 59–67. https://doi.org/10.1093/toxsci/59.1.59.

Zepnik, H., Völkel, W., Dekant, W., 2003. Toxicokinetics of the mycotoxin ochratoxin A in F 344 rats after oral administration. Toxicol. Appl. Pharmacol. 192 (1), 36–44. https://doi.org/10.1016/s0041-008x(03)00261-8.

Zimmerli, B., Dick, R., 1995. Determination of ochratoxin A at the ppt level in human blood, serum, milk and some foodstuffs by high-performance liquid chromatography with enhanced fluorescence detection and immunoaffinity column cleanup: methodology and Swiss data. J. Chromatogr. B Biomed. Sci. Appl. 666 (1), 85–99. https://doi.org/10.1016/0378-4347(94)00569-Q.